Novel Approaches of Generating and Selecting High Genetic Merit *In Vitro* Produced Pig and Cattle Embryos

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

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Dedicated to my lovely wife

Chiara.

Table of Contents

List of Figures

Fig. 1.9 – Ovum pick-up (OPU). Following ovarian stimulation, the mature oocytes are retrieved from their follicles shortly before ovulation. An ultrasound guided needle is inserted through the cervix and used to aspirate the contents of the antral follicles. (Image adapted from http://thamaraihealthcare.com).

Fig. 1.10 – Immature porcine cumulus-oocyte complexes (COC) of different morphology. A) Good quality oocyte surrounded by a compact and dense cumulus. B) An oocyte surrounded only by a single layer of cumulus cells is expected to have a low developmental competence. C) Denuded oocyte, no cumulus cells are present leading to the expectation of very low developmental competence. Scale bar represents 50 µm. (Image adapted from Alvarez *et al.* 2009).

Fig. 1.11 – Key steps of *in vitro* fertilisation (IVF). Mature oocytes from a superovulated donor or oocytes matured *in vitro* are co-cultured with prepared semen to achieve fertilisation outside of the body (1), resulting in the formation of a zygote (2) and finally of an embryo (3) which can then be transferred *in utero* (4). (Image adapted from http://www.cpma.ch).

Fig. 1.12 – The reproductive tract of the cow. Notably, the cranial part of the uterus is divided in two separate chambers known as the uterine horns. During embryo transfer, a catheter is used to deliver an embryo through the cervix and up to the middle point of one of the uterine horns. (Image from www.ansci.wisc.edu).

Fig. 1.13 – Comparative morphology of human and bovine cleavage stage embryos. A) Human 8-cell stage embryo of excellent morphology presenting eight even and translucent blastomeres. B) Bovine 8-cell stage embryo of excellent morphology. The specimen is opaque due to the presence of lipid droplets, which make the morphological assessment more challenging. (Image sources: part A: atlas.eshre.eu; part B: adapted from Lechniak *et al.* 2008).

Fig. 1.14 – Embryo classification in cattle at day 7 of culture. Embryos of grade 1 are considered excellent or good, with minor irregularities and less than 15% fragmentation and are suitable for transfer and cryopreservation. Embryos of grade 2 are considered fair and display moderate irregularities; their use for transfer is acceptable but they are expected to display reduced cryotolerance. Embryos of grade 3 are considered poor with major irregularities and obvious fragmentation; they are not expected to survive freeze/thawing and provide very modest results upon transfer. Embryos of grade 4 are dead or degenerating and should be immediately discarded, as they are non-viable. (Image adapted from Bo & Mapletoft 2013).

Fig. 1.15 – Well of the well (WOW) culture dish. In the WOW culture system, embryos are cultured in the same dish (left) and can therefore take advantage of the benefits of co-culture. Additionally, the embryos can be individually tracked thanks to their positioning within micro-wells (zoom, right). (Image source: http://www.vitrolife.com).

Fig. 1.16 – Application of optical coherence tomography (OCT) to the imaging of early stage murine embryos. The images on the top row were captured by standard bright field microscopy and are compared to images of the same specimen captured through the use of OCT. With OCT, nuclei are clearly visible in zygotes and cleavage stage embryos. At the same time, all the characteristic features of a blastocyst (trophectoderm ring, inner cell mass, blastocoel) can be easily observed (Image adapted from Karnowski *et al.* 2017).

Fig. 1.17 – Cleavage stage embryo splitting. Two 4-cell stage murine embryos (1) are subjected to enzymatic digestion of their zona pellucida (2). Once freed from the zona pellucida, the

blastomeres are separated (3) and transferred either singly or in groups into an empty zona pellucida (4). The embryos are then cultured until the blastocyst stage (5-6). (Image adapted from Tang *et al.* 2012).

Fig. 1.18 – Blastocyst stage embryo splitting. In this form of embryo splitting a blastocyst is divided mechanically in two equal halves. To remain viable, the resulting demi-embryos should receive similar proportions of both the trophectoderm (TE) and, more importantly, of the inner cell mass (ICM). With reference to the above, the dashed line indicates the optimal position for the cut. (Image adapted from Escriba *et al.* 2002).

Fig. 1.19 – Somatic cell nuclear transfer (SCNT). A schematic representation of the SCNT method is presented. An enucleated oocyte is fused with the nucleus of an adult somatic cell. The activated embryo is transferred to a foster mother leading to the birth of an animal genetically identical to the somatic cell nuclear donor (Image from Gifford & Gifford 2013).

Fig. 1.20 – Examples of segregation patterns arising from normal disjunction, non-disjunction and premature predivision of chromatids. Maternal chromosomes in red, paternal chromosome in blue. PB: polar body. The net result of each segregation pattern is described on the right of each panel as gain (G), loss (L) or normal (N) copy number for PB1, PB2 and Zygote, respectively. Note that not all possible segregation patterns are represented and for premature predivision reciprocal patterns are also possible leading to a balanced outcome. (Image from Handyside *et al.* 2012).

Fig. 1.21 – Application of karyomapping in a case of preimplantation genetic diagnosis (PGD) in human embryos. The genotype of an affected sibling (reference) at specific single nucleotide polymorphism (SNP) loci was compared to 7 embryo biopsies. Two pairs of columns are given for each chromosome examined. P: paternal chromosome, M: maternal chromosome. Blue: paternal informative SNP concordant with reference; Red: paternal informative SNP different from reference; Yellow: maternal informative SNP shared with reference; Green: maternal informative SNP different from reference; Grey: absence of information. In embryos 3 and 6, the paternal chromosome is missing (paternal monosomy). (Image from Thornhill *et al.* 2015)

Fig. 1.22 – Biopsy stages. A) Polar body biopsy. B) Cleavage stage biopsy, a single blastomere is being removed from an 8-cell stage embryo. C) Trophectoderm biopsy, a group of cells are removed from a blastocyst. (Image sources: A: www.cambridge.org; B: www.mitosis.gr; C: nordicalagos.org).

Fig. 2.1 – Grading of porcine cumulus oocyte complexes (COCs). Grades were assigned based on the extension of the cumulus complex. A) Three or more intact cumulus cell layers; B) two intact cumulus cell layers; C) one incomplete cumulus layer; D) denuded oocyte. Images captured with a Hoffman inverted microscope at x200 total magnification.

Fig. 2.2 – Principle of the Ellman's reaction. Glutathione disulphide (GSSG) is reduced by the enzyme glutathione reductase (GR) to glutathione (GSH). The Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with GSH to form a new disulphide bond (GSTNB), releasing 2-nitro-5-thiobenzoate (TNB). Then, the GR reduces GSTNB releasing a second TNB molecule, thus allowing GSH to re-enter the cycle. The accumulation of TNB can be tracked by a spectrophotometer set at 412 nm. Assuming the reagents are provided in excess, the reaction rate is only limited by the starting concentration of GSH. (Image adapted from https://www.funakoshi.co.jp/data/datasheet/NWS/NWK-GSH01.pdf).

Fig. 2.3 – Embryo splitting strategies applied in this study. Several alternative protocols are shown. A 2-cell stage embryo is disaggregated into two 1/2 type splits. Alternatively, an 8-cell stage embryo can be split in several symmetric ways leading to the formation of either two 4/8 type splits, four 2/8 type splits or eight 1/8 type splits or even to a combination of these. Finally, blastocysts can be bisected leading to the formation of two blastocyst splits.

Fig. 2.4 – Bisectioning setup and demonstration. A) Assembly of a disposable P-730 microsurgical blade on an Integra TI micromanipulation rig. B-E) The image sequence (from left to right) demonstrates a blade bisectioning test performed on an arrested oocyte, which resulted in a neat cut. The microscopical images were captured with a Hoffman inverted microscope at x200 total magnification.

Fig. 2.5 – Schematic of serial embryo splitting by disaggregation and bisectioning. A potential way of performing serial splitting could be to bisect blastocysts derived from a previous blastomere separation experiment. In the diagram, an 8-cell stage embryo is divided in two 4/8 splits by blastomere separation. The resulting blastocysts are then bisected to produce up to four twins.

Fig. 2.6 – Total cell count estimation with SmartCapture 3 on Hoechst 33342 stained bovine blastocysts. To improve the accuracy of the cell count, the embryo was sequentially divided into smaller sections (red rectangles, panels 1 to 4) which could be individually brought into focus. Cell nuclei from the different focal planes were then counted in each section. The total magnification used was x200.

Fig. 2.7 - SS-OCT microscope experimental set-up. The dish containing the embryo was positioned on the microscope's translation stage for lateral scanning. C1-C2: optical couplers, PC: polarisation controllers, L1,2 – lenses, Is: isolator. This image was kindly provided by Miss Sophie Caujolle (School of Physical Sciences, University of Kent, Canterbury, UK).

Fig. 3.1 – Meiotic stage of porcine oocytes visualized by α-tubulin immunostaining. Tubulin in green, DNA counterstained with Hoechst 33342 (blue). A) Prophase I, no spindle is detected. B) Metaphase I, a clear tubulin spindle has formed around the chromosomal compartment. C) Anaphase I, two sets of chromosomes are separated by a tubulin bridge. D) Metaphase II, two independent spindles can be detected belonging to either the oocyte or to polar body I (arrow). Images captured by fluorescence microscopy at x200 total magnification.

Fig. 3.2 – Meiotic stage of porcine oocytes of different grades after IVM. Oocytes clad by more cumulus layers were more likely to achieve full nuclear maturation (metaphase II). However, the number of oocytes in metaphase I appeared consistent across grades A-C. Grade D oocytes were found to be unable to complete nuclear maturation.

Fig. 3.3 – Cortical granules (CG) stain with PNA-lectin in porcine oocytes. CGs in green, DNA counterstained with Hoechst 33342 (blue). In the images, N indicates the oocyte's nucleus, PB indicates polar body I. A) A cytoplasmically immature oocyte, no clear CG distribution is present. The presence of a polar body indicates asynchrony between nuclear and cytoplasmic maturation in this cell. B) A cytoplasmically mature oocyte, a clear ring of CGs can be detected (arrow). Images captured by fluorescence microscopy at x200 total magnification.

Fig. 3.4 – Rates of cytoplasmic maturation in gilt oocytes after IVM. Oocytes clad by either 2 or 3+ layers of cumulus cells (grades A and B) performed similarly in this test and complete cytoplasmic maturation more often than oocytes of other grades. Data given as mean ± S.E.M.

Fig. 3.5 - Calibration curve for the measurement of glutathione (GSH). Known amounts of GSH (0.01 nmol, 0.1 nmol and 1 nmol) were used to calculate the average increase in A_{412} for each 20 second interval. A calibration curve was then produced by linear regression.

Fig. 3.6 - Reaction rate curves for the measurement of glutathione (GSH) levels in oocytes of different grades. The reaction rate was measured by tracking the increase in the A_{412} of each sample over time, a metric directly proportional to its intrinsic GSH content. Each reaction curve presented is the average obtained from triplicate experiments. Grade A and grade B oocytes showed very similar kinetics, whilst grade C and grade D oocytes displayed much slower reaction rates, indicating a reduced GSH content. Error bars given as S.E.M.

Fig. 3.7 - Total glutathione (GSH) content per oocyte according to grade. Oocytes clad by more layers of cumulus cells (grades A and B) were richer in GSH in absolute terms. However, the GSH content of grade B oocytes was found to be highly variable. Data given as mean ± S.E.M.

Fig. 3.8 - Polyspermic zygote. Three pronuclei can be distinguished (arrows), indicating this zygote has been penetrated by two separate sperm cells. DNA stained with Hoechst 33342, total magnification x200.

Fig. 3.9 - Penetration rate (A) and polyspermy rate (B) in zygotes derived from oocytes of different grades. A) The cumulus investment appeared to have only a moderate effect on sperm penetrability with only denuded oocytes (grade D) showing a reduction. B) Moreover, no difference was found in the polyspermy rates across the four experimental groups. Data given as mean ± S.E.M, statistical analysis performed with chi-square using α=0.05.

Fig. 3.10 - Cleavage stage porcine embryos. Image captured with a Hoffman microscope at x200 total magnification.

Fig. 3.11 - Cleavage rates achieved by fertilized oocytes of different grades. The original cumulus investment appeared to correlate with embryo developmental potentials. Oocytes clad by two or more layers of cumulus cells (grades A and B) produced cleavage stage embryos significantly more often, while denuded oocytes (grade D) only rarely produced embryos. Data given as mean ± S.E.M.

Fig. 3.12 - Cleavage pattern in embryos derived from oocytes of different grades. The lines portray the proportion of embryos reaching each successive cleavage stage. A pattern can be seen with embryos derived from grade A and B oocytes being both more abundant and tending to reach more advanced stages.

Fig. 4.1 – Embryo splits produced following different strategies. A) A 4/8 type split produced by blastomere separation from an 8-cell stage embryo. B) An embryo split derived from the bisectioning of a blastocyst, the embryo appears collapsed, a normal occurrence immediately after the cut. Images captured by phase contrast microscopy at x200 total magnification in a well-of-the-well culture system.

Fig. 4.2 – Variation in the blastulation rate of 8-cell stage splits in accordance with the splitting ratio. A significant correlation was detected by logistic regression between the two variables. For display purposes, in this graph grouping of data was operated and a trendline was given to guide the reader. However, logistic regression was performed on the raw, ungrouped data.

Fig. 4.3 – Variation in the cell count of blastocysts derived from 8-cell stage splits in accordance with splitting ratio. A direct correlation appears to be present between the two variables in study.

Fig. 4.4 – Blastulation and recovery rates achieved by embryos split following different strategies. While 1/8 type splits consistently underperformed, the other splitting strategies appeared to produce blastocyst stage embryos at similar rates. Averages with different superscripts differ significantly (Chi-square, $P < 0.05$). Data given as mean ± S.E.M.

Fig. 4.5 – Projected blastocyst yield for different embryo splitting strategies. When the potentials of each different strategy and its efficiency were taken into account, a 2/8 type split was the strategy that yielded the greatest number of blastocysts per starting zygote. Data given as mean ± S.E.M.

Fig. 4.6 – Blastocyst staining for cell count estimation. Blastocysts were stained using two alternative strategies. A) Immunostaining for CDX-2. Cells expressing CDX-2 were stained in red, and Hoechst 33342 (blue) was used a nuclear counterstain. CDX-2 appeared to co-localise with Hoechst 33342 in all cells indicating that the immunostaining had no ability to discriminate between trophectoderm and inner cell mass cells. B) Simple nuclear staining by Hoechst 33342, all nuclei stained in blue. This approach consistently allowed total cell counts to be estimated. Images captured by fluorescence microscopy at x200 total magnification.

Fig. 4.7 – Average cell counts in day 7 post-IVF blastocysts produced according to different embryo splitting strategies as compared to unsplit controls. Averages with different superscripts differ significantly (Tukey-Kramer *post-hoc* test, $P < 0.05$). Data given as mean ± S.E.M.

Fig. 4.8 – Example blastocysts derived from different splitting strategies. A) Blastocyst derived from a 1/8 type split. B) Blastocyst derived from a 2/8 type split. C) Blastocyst derived from a 4/8 type split. All the pictures were captured 168 h after IVF using a PrimoVision EVO microscope. The splitting ratio appears to have a clear effect on blastocyst size.

Fig. 4.9 – Series of ideal comparisons between cell counts in blastocysts derived from embryos split according to different strategies and control blastocysts the counts of which were reduced by an appropriate factor. Cell counts form intact control embryos were divided by 2 for comparison against 4/8, 1/2 and blastocyst type splits; by 4 for comparison against 2/8 type splits and by 8 for comparison against 1/8 type splits. Embryo splits had statistically more cells than these ideal controls in all cases but for blastocyst stage splits. Data given as mean ± S.E.M.

Fig. 4.10 – Blastulation rates for embryos produced by serial splitting as compared to embryos produced by single splitting strategies. Embryos split according to serial splitting strategy B and C blastulated to statistically similar rates to embryos produced by comparable single splitting strategies. Data given as mean ± S.E.M.

Fig. 4.11 – Average cell counts in blastocysts produced by serial splitting as compared to embryos produced by single splitting strategies. Blastocysts derived from single splitting strategies consistently showed higher cell counts when compared to embryos produced by serial splitting. Data given as mean ± S.E.M.

Fig. 4.12 – Timing of developmental landmarks in control and cleavage stage split embryos. For each event, columns with different superscripts differ significantly (Tukey-Kramer *post-hoc* test $P<0.05$). Data given as mean ± S.E.M.

Fig. 5.1 – A 2-cell stage bovine embryo imaged by SS-OCT. While cell boundaries are not evident, the embryo clearly shows two dark areas (arrows) which are consistent with the presence of nuclei.

Fig. 5.2 – Bovine blastocysts imaged by SS-OCT. A) Axial view showing a section of the entire embryo. The white lines determine the boundaries used for the transversal analysis. B) Selection of transversal cross-sections at different depths, allowing distinguishing the size, shape, and distribution of the inner cell mass.

Fig. 5.3 – 3D reconstruction of a bovine blastocyst. A) A selection of *en-face* images captured at different depths in the embryo. B) 3D model of the embryo reconstructed on ImageJ from approximately 300 *en-face* images. For display purposes, images containing cumulus cells or reflections from the plastic dish were cropped, artificial colours were applied to the trophectoderm (blue) and the inner cell mass (purple) and a window was created to allow the visualisation of the inside of the embryo.

Fig. 5.4 – Average speckle variance measured on live bovine blastocysts at an arbitrary fixed depth over 10 minutes. Five live embryos were observed and one dead embryo was used as a control. Data given as mean ± S.E.M.

Fig. 5.5 – Total speckle variance (SV) for a single blastocyst at several depths over 26 hours. Movement was simultaneously detected by SV at multiple depths within the same embryo. In the graph, motion measurements at superficial (25 μm and 300 μm), intermediate (75 μm and 225 μm) and central layers (150 μm) are displayed.

Fig. 5.6 – Percentage of cross-sectional (*en-face*) images displaying speckle variance values above threshold at any given time for a single blastocyst. Information from all depths within the same embryo was considered to track the decreasing motion of the embryo over time.

Fig. 5.7 – Cross-sectional SS-OCT images of a blastocyst over long-term observation and corresponding motion maps. A) *En-face* SS-OCT images of the observed embryo over 26 h (actively monitored over the first 18 h) at a fixed depth (150 μm from the top embryo surface). B) Corresponding motion maps, black represents absence of movement while movement intensity is given on a Red/Green scale (red higher). Scale bar = 50 μm. The images were kindly provided by Sophie Cajoule (Applied Optics, University of Kent).

Fig. 5.8 - 3D reconstruction of a day 8 bovine blastocyst for automated cell count. A) 3D model of the embyo. TE: trophectoderm, IMC: inner cell mass. B) By using ImageJ, the 3D model was analysed and the position of each putative cell was highlighted with a dot of a different colour, then a count was produced.

Fig. 6.1 – Call rates obtained from blade assisted or laser assisted biopsies. After whole genome amplification, the embryo biopsy samples obtained with either method were submitted for Single Nucleotide Polymorphism (SNP) typing and the call rates achieved by each group were recorded. No statistical differences were evident between the two methods ($P>0.05$).

Fig. 6.2 – Birth of the first karyomapped calf in the world. Cookie Four is the daughter of the sire Cinderdoor (Semex) and the dam Crossfell Uno Cookie (Paragon Veterinary Group) and was born in 2017 in Cumbria following the transfer of a karyomapped embryo.

Fig. 6.3 – Number of embryos displaying zero, one or multiple chromosomal abnormalities. Interestingly, the affected embryos had either few or many chromosomal errors with no in-between clusters.

Fig. 6.4 – Example karyomaps. A series of karyomaps are presented from both normal and abnormal cases. Gray bands imply absence of information. Euploid chromosomes are characterised by few, large alternating blocks of blue/red (for paternal) or green/yellow (for maternal) bands, representing alternating haplotypes. Monosomies appear as complete or almost complete lack of information for a full chromosome, while trisomies appear as frequent and short blocks of alternating haplotypes. Uniparental disomies appear as a monosomy for one chromosome and a trisomy for its homologue, and, finally, parthenogenetic activation events appear similar to a monosomy of paternal origin at all loci. Chr: Chromosome; Ref.: Reference sibling; MS: Monosomy; TS: Trisomy; UPD: Uniparental Disomy. These karyomaps were produced by BoVision (version 3). Additionally, karyomapping was employed in order to characterise the frequency and type of numerical abnormalities on a chromosome by chromosome basis. Parthenogenetically activated embryos were excluded from this analysis to avoid overrepresenting paternal monosomy events. Moreover, triploidy and uniparental disomy were only detected once and therefore could not be investigated further from a statistical point of view.

Fig. 6.5 – Number of monosomies and trisomies detected per chromosome. The results were collected from n=56 embryo karyomaps and a total of 3248 chromosomes. No information on the paternal X chromosome was available.

Fig. 6.6 – Number of monosomies per chromosome by parent of origin. No obvious pattern was detected from the analysis of 56 embryos.

Fig. 6.7 – Number of trisomies per chromosome by parent of origin. Overall, trisomies appeared more common in the maternal chromosome population (chi-square, $P<0.05$) as detected from the analysis of 56 embryos.

Fig. 6.8 – Average number of crossovers per chromosome and parent of origin. The analysis was completed on a total of 56 embryos. For each chromosome pair, a star (*) indicates that a significant difference was found between the number of events per parent (paired student t-test, $P<0.05$). Data given as mean ± S.E.M.

Fig. 6.9 – Average number of crossovers as correlated with chromosome size measured in mega base pairs (Mb). A moderate but significant association was found suggesting that smaller chromosomes allow for less space between crossover events. Chromosome X appeared as an outlier due to its reduced recombination frequency.

Fig. 6.10 – Non-recombination rate per chromosome and parent of origin. The analysis was completed on a total of 61 embryos and error bars are given as confidence intervals for proportions. Smaller chromosomes appeared to be more often affected by non-recombination. Data given as mean ± C.I. 95% for proportions.

List of Tables

List of Abbreviations

aCGH array comparative genomic hybridisation

ADO allele drop out

AI artificial insemination

ART assisted reproductive technology

CI confidence interval

cAMP cyclic adenosine monophosphate

CG cortical granule

CGH comparative genomic hybridisation

cGMP cyclic guanosine monophosphate

COC cumulus-oocyte complex

DAPI 4'6-diamidino-2-phenylindole

db-cAMP dibutyryl cyclic-adenosine monophosphate

DF degree of freedom

DTNB 55'-Dithiobis2-nitrobenzoic acid

DTT dithiothreitol

EDTA ethylenediaminetetraacetic acid

EGTA ethylene-bisoxyethylenenitrilotetraacetic acid

FBS foetal bovine serum

FF-OCT full field optical coherence tomography

FISH fluorescence *in situ* hybridisation

FITC fluorescein isothiocyanate

FSH follicle stimulating hormone

GEBV genomic estimated breeding values

GR glutathione reductase

GSH glutathione

GSSG Glutathione disulphide

hCG human chorionic gonadotrophin

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HLA human leukocyte antigen

HTF human tubal fluid

ICM inner cell mass

ICSI intracytoplasmic sperm injection

IVC *in vitro* culture

IVF *in vitro* fertilisation

IVM *in vitro* maturation

IVP *in vitro* production

LH luteinising hormone

LOS large offspring syndrome

M Morgan

Mb mega base pairs

MOET multiple ovulation and embryo transfer

MOPS 3-(N-morpholino)propanesulfonic acid

mTRIS modified trishydroxymethylaminomethane medium

MZ monozygotic

NCSU-23 North Carolina state university 23

NGS next generation sequencing

OCT optical coherence tomography

OCTA optical coherence tomography angiography

OPU ovum pick-up

PBS phosphate buffered saline

PCR polymerase chain reaction

PFA paraformaldehyde

pFF porcine follicular fluid

PGD preimplantation genetic diagnosis

PGS preimplantation genetic screening

PHE penicillamine hypotaurine and epinephrine

PIPES 14-piperazinediethanesulfonic acid

PMSG pregnant mare serum gonadotrophin

PNA peanut agglutinin

PN pronucleus

PVA poly vinyl alcohol

QTL quantitative trait locus

ROS reactive oxygen species

RT room temperature

RT-qPCR quantitative real-time polymerase chain reaction

SCNT somatic cell nuclear transfer

SDS sodium dodecyl sulphate

SEM standard error of the mean

SNP single nucleotide polymorphisms

SOF synthetic ovarian fluid

SOFaaci synthetic ovarian fluid amino acids citrate inositol

SS-OCT swept source optical coherence tomography

STR short tandem repeat

SV speckle variance

TALP Tyrode's albumin lactate pyruvate

TCM-199 tissue culture medium 199

TE trophectoderm

TNB 2-nitro-5-thiobenzoate

TR Texas red

TRIS trishydroxymethylaminomethane

UHR-OCT ultra high resolution optical coherence tomography

UPD uniparental disomy

WGA whole gnome amplification

WOW well-of-the-well

ZP zona pellucida

Abstract

In vitro production (IVP) in agricultural species such as pigs and cattle is performed in a similar manner to human IVF, and involves ovum pick up or oocyte maturation, fertilization outside the body and subsequent transfer of viable embryos. Its application in agricultural breeding can significantly speed up genetic gain rates by reducing generational intervals, increasing selection intensity, and facilitating the dissemination of valuable genetics. IVP is an increasingly important tool for breeding companies given the ongoing emphasis on feeding a growing population with fewer resources. With the above in mind, the aim of this work was to improve the yield of competent, euploid, and high genetic merit IVP embryos available for transfer.

Oocytes destined for IVP are commonly assessed for developmental competence by morphological screening. To determine whether it would be possible to improve the utilisation of oocytes per donor, the developmental competence of porcine oocytes with decreasing cumulus oocyte complex (COC) investment was examined. It was found that current IVP practice is wasteful, through the elimination of oocytes with slightly impaired morphology, which still have remarkable developmental potentials.

Moreover, whilst it is accepted that embryo splitting (generating more than one embryo from a single fertilized zygote) could benefit the breeding industry by increasing the offspring of the most desirable parents, a comparative analysis of the different splitting methodologies available, including stage of the split and single versus serial splitting strategies, is currently missing in the literature. Here, the splitting of an 8-cell stage embryo into four identical twins was identified as the strategy producing the greatest output of good quality embryos. Additionally, time-lapse investigation of the embryo splits found evidence of the existence of a developmental clock that tightly regulates early cleavage events.

Normally, only embryos that display satisfactory morphology are selected for transfer. However, in cattle and pigs, this assessment is complicated by the accumulation of lipid droplets within the embryo, which renders it opaque. Consequently, there is scope for

the application of new imaging modalities, such as optical coherence tomography (OCT), which are able to image an embryo in full depth and non-invasively. In this work, swept source OCT was successfully tested for use in early stage bovine embryos to obtain both structural and functional imaging. Moreover, micron-scale movements were measured within blastocysts by OCT as a way to rapidly discriminate between living and deceased embryos, representing a novel application of this methodology.

Embryo biopsies can be used to establish the genetic merit of an embryo through single nucleotide polymorphism (SNP) analysis, allowing the application of genomic selection soon after fertilisation rather than at birth. Moreover, SNP information can be analysed by karyomapping to select the most chromosomally normal embryos for transfer. Here, the birth of the first five karyomapped calves in the world is reported. Additionally, karyomapping was used to measure the incidence of aneuploidy in bovine blastocysts by parent of origin and to determine the recombination frequency for each chromosome, demonstrating the applicability of this methodology to basic research.

1. Introduction

The current trend in world population growth suggests that the human population might reach 9 billion individuals by 2050, which in return will cause an increase in the food demand of between 70 to 100% (Godfray *et al.* 2010). Already today, demand for meat is rising globally due to an increased consumption in the developing countries (Godfray *et al.* 2010). Because of the environmental costs of animal production and concerns about animal welfare (Vries & Boer 2010), it is important that this demand is met by way of greater production efficiency rather than just by a crude increase in the number of animals bred (Godfray *et al.* 2010). A possible way for the breeding industry to cope with current and future challenges could be the application of new breeding and selection methodologies like *in vitro* production (IVP) and genomic selection by means of pre-implantation genetic screening (PGS) which have the potential to rapidly improve the genetic merit of the breeding population. Whilst these principles can be applied to several farm animal species, the work of this thesis will focus in particular on cattle and pigs, whose meat is the most consumed worldwide (Alexandratos & Bruinsma 2012).

1.1 Natural reproduction

1.1.1 The cell division cycle

The cell division cycle is a series of events that characterise dividing eukaryotic cells (Bell & Dutta 2002; Bertoli *et al.* 2013). The cycle features a number of distinct phases as presented in figure 1.1. A cell that has just originated will enter the gap phase one (G1) during which it will display a high metabolism (Pardee 1989, Bell & Dutta 2002). During G1, the cell might interrupt the cell cycle by entering the rest phase G0 (Nurse & Bissett 1981; Freytag 1988) or it might enter the synthesis phase (S phase) during which its genetic material is duplicated (Nurse & Bissett 1981; Aparicio *et al.* 1997). After the S phase, the cell enters a new gap phase (G2) which allows the recruitment of the resources necessary for duplication (Zhai *et al.* 1996). Importantly, the G2 phase acts as a checkpoint before a division (O'Connor 1997; O'Connell *et al.* 2000); errors that cannot

be repaired will conduct the cell to apoptosis (Li *et al.* 1998), otherwise the cell will proceed towards mitosis.

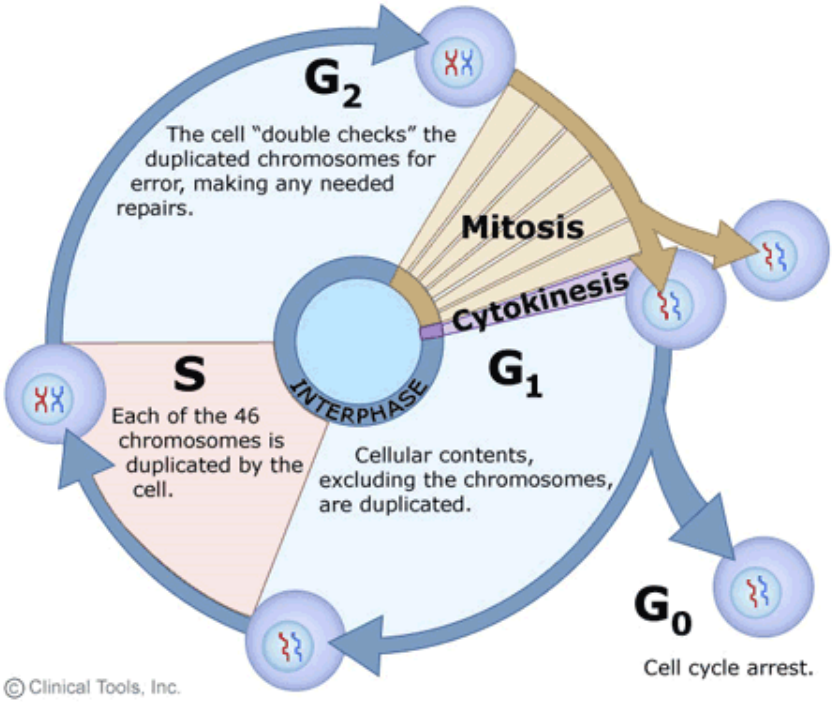


Fig. 1.1 - The cell division cycle. Several distinct phases characterise the cycle. Two gap phases (G1 and G2); an S phase (synthesis) during which DNA replication occurs; and an M phase (mitosis) during which the cell divides to form two copies of itself. After the M phase, the cell cycle begins again. However, a cell might also exit the cycle during G1 by entering a rest phase, which is quiescent from a replication standpoint and is known as the G0 phase. (Image source: www2.le.ac.uk).

1.1.2 Mitosis

During mitosis, two daughter cells with an identical genome are produced by the balanced segregation of the chromosomes replicated during the S phase (Minton 2014). The process of mitosis is divided in separate stages, which are depicted in figure 1.2. At the beginning of mitosis, chromosomes are formed of two sister chromatids held together by the centromere (Michaelis *et al.* 1997). During prophase, the nuclear membrane disintegrates, the chromosomes condense and their centrosomes become connected by a network of microtubules: the mitotic spindle (Zhu *et al.* 2005; Foley *et al.* 2013; Mitchinson 2014; Wieser & Pines 2015). At prometaphase, the mitotic spindle binds to the centromeres through the kinetochore (Fugakawa *et al.* 2014; Godek *et al.* 2015), then at metaphase, the chromosomes align (Chan & Yen 2003) and the sister

chromatids are pulled to opposing poles during anaphase (Civelekoglu-Scholey & Cimini 2014). Finally, the cell enters telophase, a nuclear membrane reforms (Sansregret & Petronczki 2013) and the cell divides by cytokinesis (Skop *et al.* 2004).

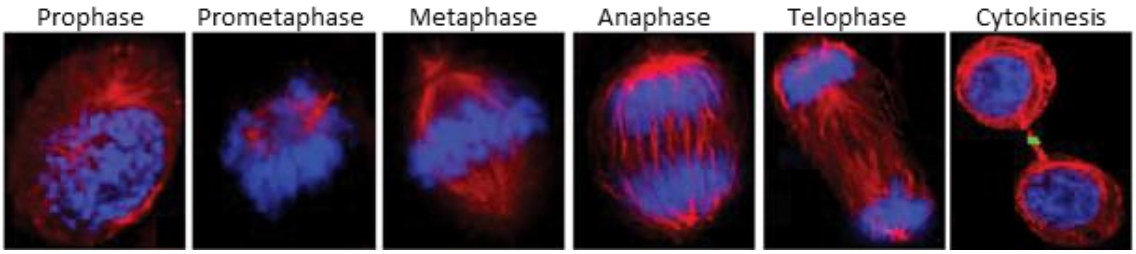


Fig. 1.2 – Mitotic phases. The images illustrate the behaviour of a human cell during the different stages of mitosis, from prophase to cytokinesis. Microtubules are coloured in red, while DNA in blue. The images were captured by fluorescence microscopy. (Adapted from Zhu *et al.* 2005).

1.1.3 Meiosis

Meiosis is a specialised form of cell division, which is characterised by two successive phases (meiosis I and meiosis II), and the formation of four unique haploid cells (Kleckner 1996; Teresawa *et al.* 2007). The steps necessary for the completion of meiosis are summarised in figure 1.3.

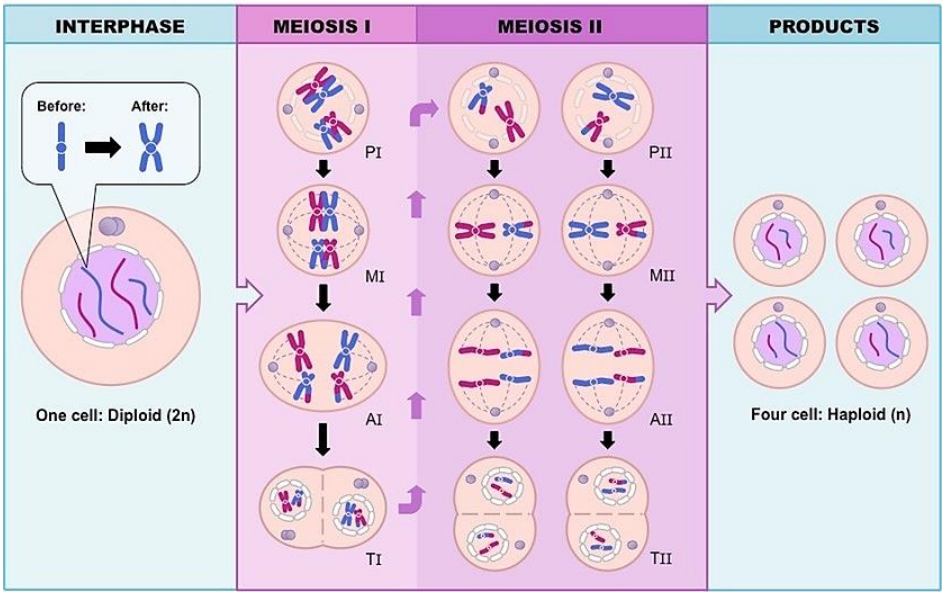


Fig. 1.3 – Meiosis. The diagram illustrates the successive phases that characterise meiosis I and II which lead to the formation of four haploid cells from a common precursor. P: prophase; M: metaphase; A: anaphase; T: telophase. (Image source: http://ib.bioninja.com.au).

The prophase of meiosis I (prophase I) is further divided in: leptotene, zygotene, pachytene, diplotene and diakinesis (Page & Hawley 2003). During leptotene, chromosomes condense and the homologous chromosomes connect forming the synaptonemal complex during zygotene (Sym *et al.* 1993). In pachytene, homologous chromosomes form chiasmata and non-sister chromatids exchange genetic material through crossing over (Jones & Franklin 2006), see figure 1.4.

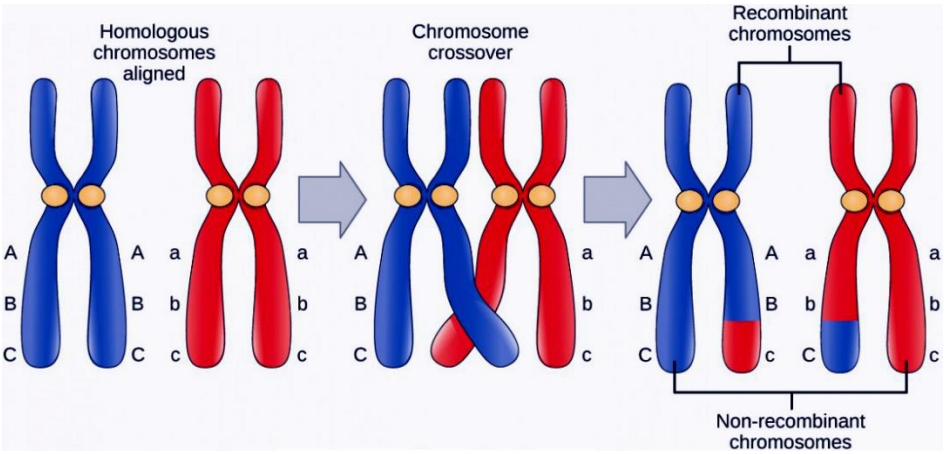


Fig. 1.4 – Crossing over. During meiosis, each chromosome is formed of two sister chromatids and is paired to its homologue. The exchange of genetic material between non-sister chromatids can results in new combinations of alleles (letters a-c) to be inherited by the offspring. Chromatids carrying combinations of alleles not present in the parental genotypes are called recombinants. (Image adapted from opentextbc.ca).

Interestingly, the number of crossovers per meiotic event differs significantly between the two sexes in several mammalian species (Barton & Charlesworth 1998; Otto & Lenormand 2002; Lynn et al 2005). For example, female mice have higher recombination rates as compared to males (Otto & Lenormand 2002) but crossovers are more frequent in rams rather than in ewes (Maddox *et al.* 2001). The formation of chiasmata is considered essential for the successive correct segregation of chromosomes (Fledel-Alon *et al.* 2009) as these structures are responsible for maintaining the correct positioning of the homologous chromosome pairs (Fedotova *et al.* 1989).

By the time the cell reaches diakinesis, chromosomes are fully aligned at the equator and the nuclear envelope fragments. The remaining phases of meiosis I (metaphase, anaphase and telophase) mimic the homonym mitotic phases (Fedotova *et al.* 1989).

Meiosis II is normally initiated immediately after meiosis I. During this phase, sister chromatids separate and segregate to different daughter cells, producing four non-identical haploid cells named gametes (Lamb *et al.* 2005). In mammals and other organisms, specialised forms of meiosis occur in the female and in the male leading to the formation of oocytes and sperm cells, respectively (Hunt & Hassold 2002).

1.1.4 Oogenesis, ovulation and the menstrual/oestrus cycle

In the mammalian female, the oocyte pool (ovarian reserve) is established during gestation (Gondos *et al.* 1986). The primordial germ cells migrate to the ovary where they form oogonia and then primary oocytes by differentiation. The primary oocytes initiate meiosis but become arrested at diplotene and enter a prolonged quiescent phase known as dictyate while encapsulated in the primordial follicles and surrounded by a monolayer of granulosa cells (Czolowska & Tarkowski 1996). In humans, this dormant state can last for up to approximatively 50 years (Wallace & Kelsey 2010).

During the menstrual cycle in primates, or the oestrus cycle in other species (Martin 2007), follicle activation leads primordial follicles to give rise to primary follicles, and then to secondary follicles while the oocyte grows and is surrounded by the zona pellucida (ZP) and several layers of cumulus cells forming the cumulus-oocyte complex (COC) (Gilula *et al.* 1978; Malgorzata *et al.* 2016). The follicle stimulating hormone (FSH) then rescues follicles of the appropriate stage from apoptosis and allows for the development of tertiary follicles. As the amount of FSH decreases over the cycle, only a few (in multiparous species) or one (in uniparous species) dominant follicle(s) develop(s) further. Following the luteinising hormone (LH) surge, the oocyte resumes meiosis, extrudes the first polar body, becomes arrested at metaphase II (Mueller *et al.* 2015), and is then expelled from the follicle (ovulation). If the oocyte is fertilised, it will then complete meiosis II and extrude its second polar body (Austin & Braden 1954). The process of ovulation is schematically represented in figure 1.5.

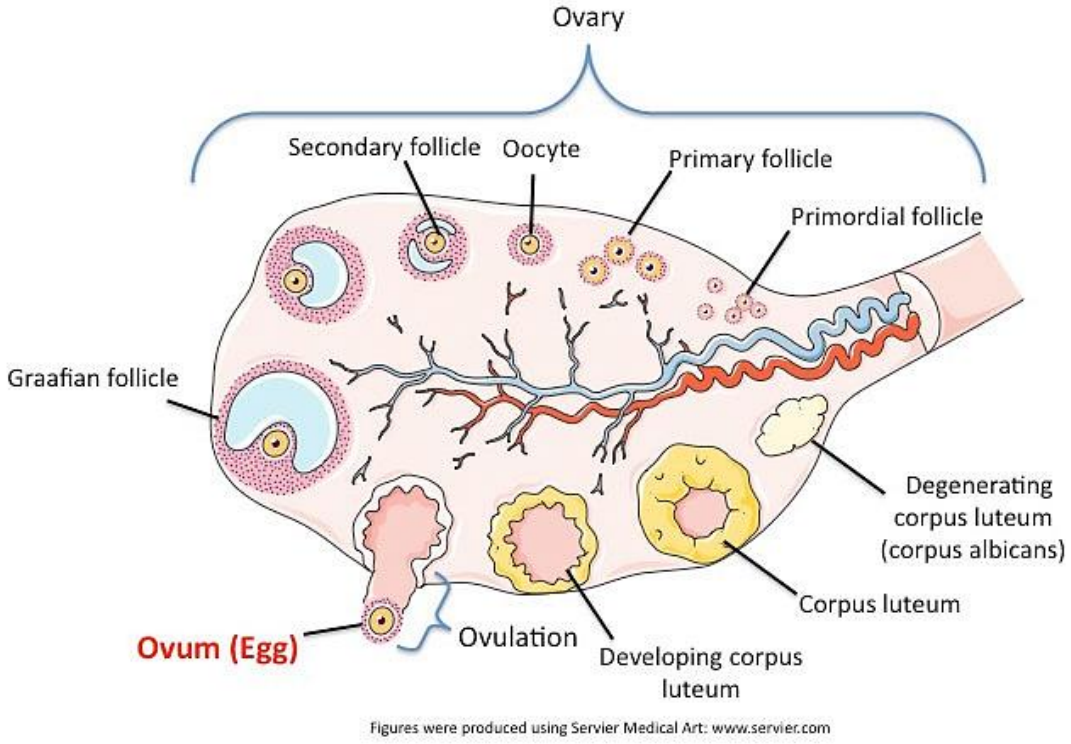


Fig. 1.5 - The process of ovulation. In the ovary, primary oocytes reside within the primordial follicle. A fully mature follicle is called the antral (or Graafian) follicle; inside it, the oocyte resumes meiosis to produce a secondary oocyte, which arrests again at metaphase II. The mature oocyte, also called an ovum, is expelled from the follicle during the process of ovulation. The follicle develops in the corpus luteum and then degenerates. (Image source: https://www.repropedia.org).

1.1.5 Spermatogenesis

The formation of spermatozoa (spermatogenesis) begins immediately prior to puberty and proceeds continuously over the entire reproductive life of the male (Hilscher 1974). This process is divided in three main stages: the proliferative, the meiotic and, finally, the differentiation phase. During the proliferative phase, part of the spermatogonial stem cell population undergoes differentiation and forms primary spermatocytes. During the meiotic phase, a primary spermatocyte undergoes meiosis I to form two secondary spermatocytes that, in turn, will complete meiosis II to produce a total of four haploid spermatids (Bellevé *et al.* 1977). The spermatids enter the differentiation phase which is characterised by tight packaging of the genomic content, substitution of histones with protamines, the formation of the acrosome, the exocytosis of excess cytoplasm, and the formation of the flagellum (Gliki *et al.* 2004; Miller *et al.* 2010). The

fully differentiated cells are called spermatozoa and are released in the lumen of the seminiferous tubule (Russell *et al.* 1993) and then transported to the epididymis where they are stored in readiness for ejaculation (Hoskins *et al.* 1978). The process of spermatogenesis is further illustrated in figure 1.6.

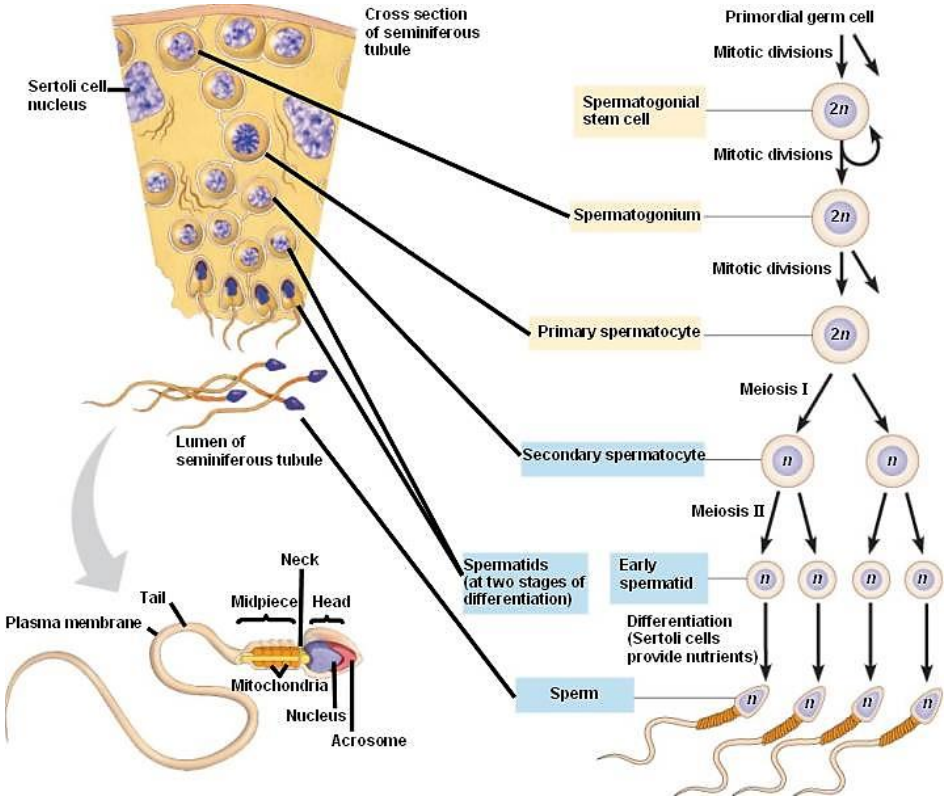


Fig. 1.6 - The process of spermatogenesis. In the testis, spermatogonia form primary spermatocytes, which in turn form spermatocytes by meiotic division. The round, early spermatids differentiate into elongated spermatids and are released into the seminiferous tubule lumen as mature spermatozoa. Sertoli cells support the process of male gametogenesis. (Image taken from: human-fertility.com).

1.1.6 Fertilisation

Spermatozoa undergo their final maturation steps within the female reproductive tract and acquire the ability to fertilise during the process of capacitation (Banerjee & Chowdhury 1995; Austin 2012; Ma *et al.* 2012). The phenomenon of capacitation was firstly observed in the early 1950s (Austin 1951), and since then much effort has been dedicated to optimising the conditions for capacitation *in vitro* in view of the application of several reproductive technologies (reviewed in Bailey 2010). Importantly, during capacitation an influx of calcium results in increased cell motility, a phenomenon called

hyperactivation (White & Aitken 1989). At the same time, chemoattractant molecules released from the COC impart a preferential movement direction to spermatozoa (Sun *et al.* 2005).

To complete fertilisation, the sperm cell needs to breach through the cumulus cells and the ZP, which surround the oocyte. This is achieved by both the swimming motion and the release of lytic enzymes such as hyaluronidase and acrosin as part of the acrosome reaction (Lin *et al.* 1994). The presence of extracellular calcium is fundamental for the acrosome reaction to take place, as is the binding of the sperm cell to the ZP3 protein of the ZP (Rossato *et al.* 2001; Sánchez-Cárdenas *et al.* 2014). The spermatozoon can then enter the oocyte by fusing its equatorial segment to the oolemma (Langlais & Kenneth 1985; De Jonge *et al.* 2013).

As an immediate response to a sperm cell entry, the oocyte initiates the release of the cortical granules (CG), in what is described as the cortical reaction. The content of the granules causes the proteolytic removal of sperm specific binding sites from the ZP (Dandekar & Talbot 1992) and is one of the primary mechanisms employed by the oocyte to prevent polyspermy (penetration of multiples sperm cells into a single oocyte) (Wang *et al.* 1997). At the same time, the oocyte establishes a membrane block to prevent fusion of additional sperm cells (Gardner & Evans 2005; Gardner *et al.* 2007). A schematic of fertilisation is presented in figure 1.7.

Finally, the tightly packed male genome decondenses and forms the male pronucleus while the sperm tail degenerates (Perreault *et al.* 1984). During syngamy, the male and female pronuclei fuse to reconstitute a diploid organism with the formation of the zygote (Sathananthan & Trounson 1985).

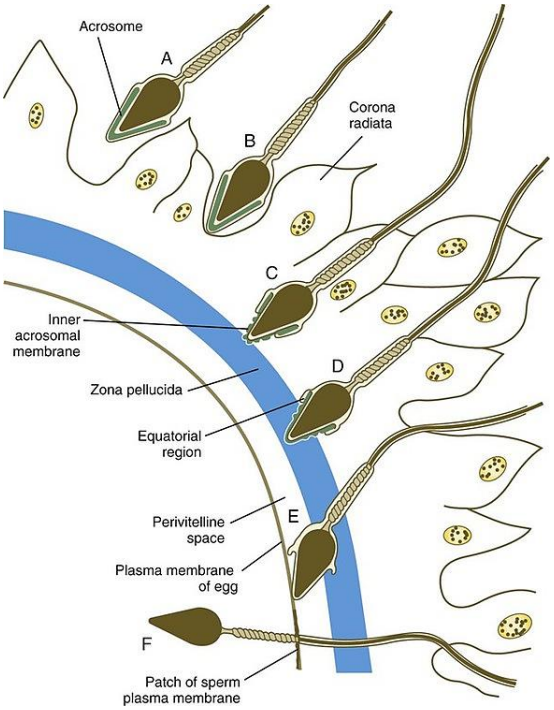


Fig. 1.7 – Key events in fertilisation. A-B) The sperm cell makes contact with the cumulus cells. C) The acrosome reaction is initiated with release of lytic enzymes. D) The spermatozoon breaches through the zona pellucida. E) The equatorial segment of the sperm head fuses to the oocyte's membrane. F) The sperm enters the oocyte. (Image source: clinicalgate.com).

1.1.7 Early embryonic development

After fertilisation, the zygote initiates a series of rapid mitotic division known as cleavage events, which are characterised by absence of cellular growth. As a result, the originating cells, which are known as the blastomeres, decrease in size at each division. During the cleavage stage, the embryo has a distinctive appearance and the number of blastomeres can often easily be counted by standard microscopy (Boiso *et al.* 2002). The duration of the cleavage stage is different across different species; in cattle, for example, it has an approximate duration of four days (Holm *et al.* 1998). After several cleavage divisions the blastomeres undergo the process of compaction and form a morula as the cells become tightly bound to each other (Ducibella & Anderson 1975; Iwata *et al.* 2014). Compaction is made possible by calcium-dependent adhesion molecules like E-caderin, which accumulate at the sites of cell-to-cell contact (Winkel *et al.* 1990; Adams *et al.* 1998). Following compaction, an increased activity of ionic pumps causes an influx of water and sodium, which accumulate within the spaces between the internal blastomeres, ultimately causing the formation of a liquid filled cavity. This process is

called cavitation while the cavity itself is referred to as the blastocoel (Manejwala *et al.* 1989). After the formation of the blastocoel, the embryo is identified as a blastocyst. Importantly, by this time two separate cell populations have become established in the embryo: the inner cell mass (ICM) lineage and the trophectoderm (TE) lineage. The ICM will eventually differentiate in two cell layers known as the hypoblast and the epiblast; this latter layer will then proceed to form the entireity of the foetus, while the hypoblast and the TE will form the extraembryonic tissues (Gardner & Papaioannou 1975). During early development, the embryo travels through the fallopian tube and reaches the uterus where implantation can occur after the embryo has hatched out of the ZP (Aplin & Kimber 2004). Figure 1.8 provides a visual summary of some key developmental events in a mammalian embryo.

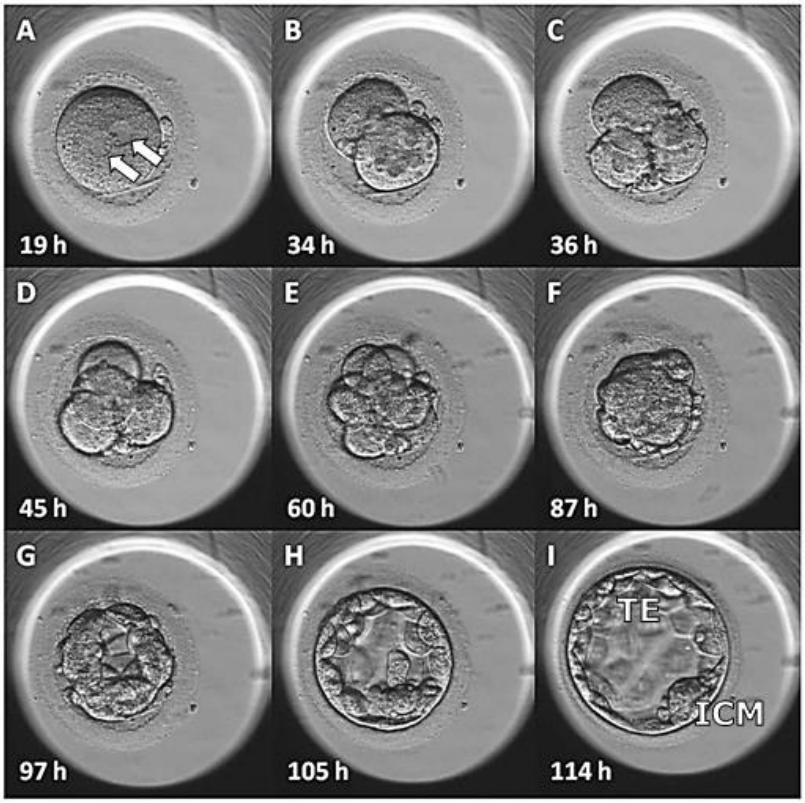


Fig. 1.8 – Early development of a human embryo. A) A zygote is forming as the pronuclei (arrow) are about to merge. B) 2-cell stage embryo after the first cleavage division. C) 3-cell stage embryo. D) 4-cell stage embryo. E) 8-cell stage embryo. F) Compaction: the blastomeres become tightly adherent and it is no longer possible to obtain a cell count by simple observation. G) Cavitation, the blastocoel begins to form. H) The embryo has developed into a blastocyst. I) The blastocyst has fully expanded; both the trophectoderm (TE) and the inner cell mass (ICM) are well evident. (Image adapted from Montag *et al.* 2014).

1.1.8 Developmental clock and cell fate specification models in early development

Interestingly, work in the murine model has demonstrated that twin embryos derived from the application of embryo splitting (an artificial twinning method further discussed in section 1.4.2) maintain very similar developmental kinetics (Morris *et al.* 2012). Indeed, several studies have reported the existence of a clock that paces early developmental events and ensures that the whole embryo is synchronised. This mechanism has been named the "developmental clock" and is thought to have a role in the timing of cell fate specification in early embryos (Satoh 1982; Poueymirou & Schultz 1989; Rougvie 2001; Morris *et al.* 2012; Noli *et al.* 2015). However, the molecular mechanism underlying the operation of this clock are yet to be elucidated (Noli *et al.* 2015).

The first cell fate decision appears evident at the morula stage, when asymmetric cell divisions cause one daughter cell to orientate towards the periphery of the embryo and the other daughter cell to be pushed towards its centre (Bruce & Zernicka-Goetz 2010; Lorthongpanich *et al.* 2012). Currently, at least three models have been proposed to explain how fate specification occurs. The inside-out model (Tarkowski & Wroblewska 1967), postulates that the asymmetric cell divisions ultimately cause differentiation by exposing the inner and the outer cell populations to different microenvironments. In similarity to this model, the cell polarity model (Johnson & Ziomek 1981) also assumes that a position dependent mechanism is responsible for fate specification, by suggesting that the asymmetrical cell division observed after the 8-cell stage are by themselves the cause of lineage specification by enforcing an asymmetrical distribution of certain cellular structures between daughter cells. In contrast, a third model, the pre-patterning model (Piotrowska & Zernicka-Goetz 2001), suggests that ICM and TE fate specifications are predetermined at fertilisation due to the asymmetrical distribution of determinants in the oocyte, which are then inherited by the blastomeres.

While no clear consensus exists in the literature about which model is the most correct, from a molecular standpoint, it is known that immediately after asymmetric division,

the outer cells still exhibit a degree of plasticity as they express both pluripotency markers like SOX2, NANOG and OCT4 which are typically found in ICM cells, and TE specific markers like HLA-G and KRT18 (Cauffman *et al.* 2009; Chen *et al.* 2009; Verloes *et al.* 2011). However, as development proceeds, the appearance of other transcription factors such as CDX2 is known to reduce or remove this plasticity, finally committing the outer cells towards TE fate specification (Niwa *et al.* 2005; Strumpf *et al.* 2005).

1.2 Assisted reproductive technology (ART)

In humans, reproduction is a rather inefficient process. It has been estimated that 10-15% of couples have difficulties conceiving (Evans 2002); moreover, around 30% of pregnancies result in spontaneous loss (Dey 2010). The term assisted reproductive technology (ART) indicates an array of methodologies used to enable subfertile couples to conceive (Kupka *et al.* 2014). However, ART has also been used successfully in farm animal breeding where it can provide important benefits including: a rapid distribution of a genetics of interest, enhanced selection intensity, reduced generational intervals, and better disease control (Mapletoft & Hasler 2005).

The first studies describing the manipulation of mammalian embryos outside of the body and their transfer to recipient animals were published in 1890 and these methodologies were further developed during the first half of the 20th century (Betteridge 1981). Following these preliminary studies, improvements in superovulation protocols (Rowson 1951), culture media (Foote & Onuma 1970), and recovery and transfer of embryos (Rowson & Dowling 1949) led to the establishment of a viable embryo transfer industry for animal breeding (Betteridge 1981; Hasler 2014). Today, embryo transfer is a massive commercial undertaking. For example, in 2015 in North America alone, over 360,000 good quality *in vivo* derived bovine embryos were recovered from donors and more than 200,000 bovine embryos were produced by fertilisation of oocytes *in vitro* (Perry 2016).

The following sections will focus on describing some of the ARTs that have found wider scope in farm animal breeding: artificial insemination (AI), IVP, embryo transfer (ET) and

cryopreservation. The redistribution across several recipient animals of embryos produced *in vivo* following superovulation and insemination of high merit dams (multiple ovulation and embryo transfer, MOET) has also been widely applied to animal breeding programmes (Hasler *et al.* 2014); however, for reasons of brevity, this topic will not be discussed in detail.

1.2.1 Artificial insemination (AI)

The practice of AI might be the less sophisticated of the ARTs. The procedure simply consists in delivering through a catheter a sufficient dose of semen into the female reproductive tract at an appropriate time during the oestrus cycle (Diskin & Sreenan 1980). AI has been used in animal breeding since the 1900s and became routine practice in the 1980s (Knox 2016).

The semen used for AI may be fresh, but in commercial settings semen is almost always collected at a dedicated facility, then diluted and preserved in an appropriate medium known as an extender which will keep it viable for 3 to 7 days whilst the doses are distributed to farms (Waterhouse *et al.* 2004). The use of an extender not only allows the wide geographical distribution of the genetics carried by a particularly desirable sire, but it also increases the number of dams that can be serviced by each single ejaculate (Roca *et al.* 2006). The use of AI is wide-spread in farm animal breeding; for example, by the year 2000 several countries were reported to rely almost exclusively on AI for commercial pig breeding (Weitze 2000) where AI has been shown to be highly dependable with farrowing rates above 85% and litter sizes of 14 now common (Yeste *et al.* 2014). Overall, the spreading of AI practices reduced the need to breed male animals at farms, reduced venereal diseases and improved the accuracy of breeding records (Funk 2006). In cattle breeding, the ability to use top sires to inseminate a large pool of dams resulted in a steady rate of genetic gain over the end of the last century (Cunningham 1999) thanks to an increased selection intensity on the male line (Robinson & Buhr 2005; Safranski 2008).

More recently, the development of suitable protocols for the cryopreservation of semen has eliminated transportation problems (Yeste *et al.* 2014). Additionally, the development of technologies for sperm sexing (Johnson *et al.* 1999) allowed performing AI with sex-sorted spermatozoa reducing the wastage and animal welfare issues associated with the birth of animals of unwanted sex (Garner 2006). Although sex-sorted sperm doses for AI are more expensive, more fragile and more diluted than regular extended doses, their use has been exploited in a number of domesticated animals including sheep (De Graaf *et al.* 2009), pig (Guthrie *et al.* 2002) and is especially common in cattle (Trigal *et al.* 2012; Seidel *et al.* 2014).

1.2.2 *In vitro* production (IVP)

The most obvious limitation of AI is that it only allows the distribution of male genetics. IVP is a more sophisticated technique, which involves the manipulation of the female gametes outside of the body and the production of embryos *in vitro*, which can then be transferred to synchronised recipients. IVP involves several steps. Firstly, mature oocytes are obtained either by ovum pick-up (OPU), or by *in vitro* maturation (IVM) of abattoir-derived material. Secondly, the oocytes are fertilised by *in vitro* fertilisation (IVF). Thirdly, the resulting embryos are maintained in an incubator during *in vitro* culture (IVC) untill good quality embryos can be transferred to synchronised recipients by embryo transfer. Not only can IVP be used to benefit the breeding industry directly, it also paves the way for the application of more refined breeding strategies which might include reproductive cloning (discussed under section 1.4) and preimplantation genetic screening (discussed under section 1.6).

1.2.2.1 Ovum pick-up (OPU)

Before mature oocytes can be harvested by OPU, donors are normally subjected to ovarian stimulation therapy (da Silva *et al.* 2017). As discussed under section 1.1.4, during a normal cycle, only a limited number of follicles (usually one in uniparous animals) will develop enough to contain a mature oocyte, limiting the possible OPU yield. Appropriate drugs, notably analogues of FSH and LH, are administered to favour

the maturation of multiple antral follicles (Revelli *et al.* 2015; Mikkola & Taponen 2017). The timing and nature of the pharmacological regimen is pivotal to successful superovulation and has been reviewed elsewhere (Bó & Mapletoft 2014; Landry *et al.* 2016). The *in vivo* matured oocytes can then be recovered surgically (Metz & Mastroianni 1979), although minimally invasive methods are generally preferred (Van Wagtendonk-de Leeuw 2006). In the latter approach, shortly before ovulation an ultrasound guided needle is inserted through the vagina and is used to aspirate the follicles and recover the oocytes (Galli *et al.* 2001), as illustrated in figure 1.9.

The advantage of OPU is that it allows the collection of oocytes which are already mature and therefore ready to be further employed for IVP. Moreover, OPU enhances selection intensity among females by increasing the oocyte availability from elite dams, and can be used for the reduction of generation intervals thanks to the ability to harvest oocytes from appropriately managed peripubertal animals (Callesen *et al.* 1996).

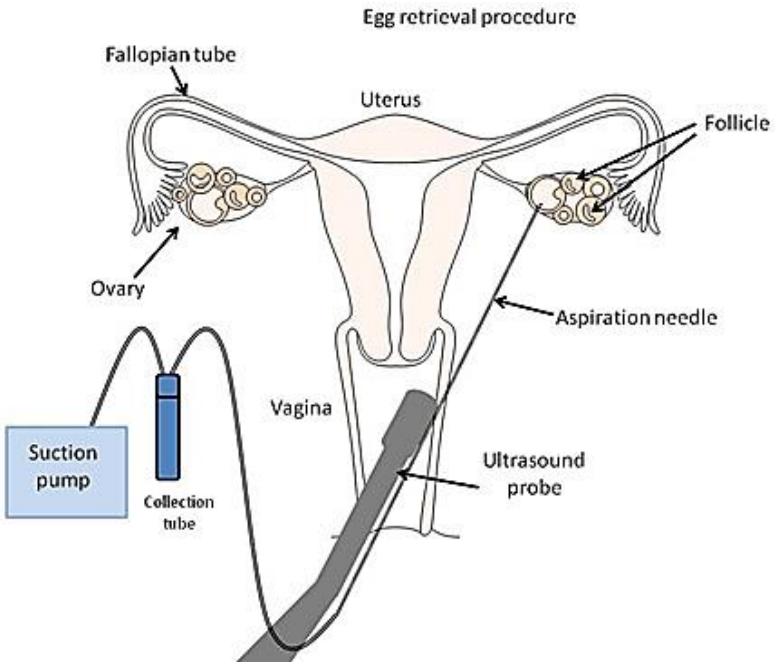


Fig. 1.9 – Ovum pick-up (OPU). Following ovarian stimulation, the mature oocytes are retrieved from their follicles shortly before ovulation. An ultrasound guided needle is inserted through the cervix and used to aspirate the contents of the antral follicles. (Image adapted from http://thamaraihealthcare.com).

1.2.2.2 *In vitro* maturation (IVM)

As an alternative to OPU, oocytes can also be harvested directly from abattoir material, shortly after the culling of the donor (Neglia *et al.* 2003). Commonly, ovaries are recovered from an abattoir and transported to a laboratory in a warmed saline solution within a few hours after collection (Wongsrikeao *et al.* 2005). The oocytes contained in superficial follicles are then aspirated through a needle of appropriate size either manually or with the aid of an aspirator pump (Jain *et al.* 1995). Alternative methods for the collection exist such as slicing of the follicles with a blade or complete dissection of the ovary (Lonergan *et al.* 1991).

The oocytes retrieved by any of these methods, however, are likely to be immature due to the animal being culled at a random point during its oestrus cycle. This is not necessarily a shortcoming since immature follicles are more common than mature follicles (Bagg *et al.* 2007) and, therefore, it is possible to harvest large numbers of oocytes in this way without need for ovarian stimulation. However, the immature oocytes will need to be cultured *in vitro* under appropriate conditions in order for them to reach the MII stage and be ready for fertilisation, a process known as IVM (Lonergan & Fair 2016).

Successful methods for the IVM of both bovine (Gandolfi *et al.* 1998; Gilchrist & Thompson 2007; Bernal *et al.* 2015) and porcine oocytes (Nagai 1996; Jeon *et al.* 2014; Appeltant *et al.* 2015) have been described in the literature. In cattle, IVM oocytes displayed blastulation rates of 40 to 50% following fertilisation and embryo culture (Bavister *et al.* 1992; Watson 2007). However, success rates appear reduced in pigs where blastulation rates of about 20% are common (Gil *et al.* 2010). Therefore, IVM is still a very active field of research, especially in the porcine model.

Unfortunately, the oocytes aspirated from follicles of different stages form a heterogeneous population adding variability to this setup (Van Blerkom *et al.* 1990). Therefore, methods for the selection of just the competent oocytes before IVM appear fundamental to avoid a waste of resources. Oocyte quality is known to be linked to

follicle size. Indeed, only oocytes derived from follicles larger than 3 mm are known to be developmentally competent in the pig (Bagg *et al.* 2007) and similar observations were reported for cattle (Lonergan *et al.* 1994; Carolan *et al.* 1996). Additionally, the morphology of the COC has been linked to oocyte developmental competence and used as a screening parameter. Although the cumulus is not essential for fertilisation (Schroeder & Eppig 1984), it is known to support the oocyte's ability to complete meiosis and its ability to develop into an embryo (Nagai *et al.* 1993; Dang-Nguyen *et al.* 2011; Lin *et al.* 2016). Cumulus cells directly control meiosis resumption in the oocyte (Mattioli & Barboni 2000; Norris *et al.* 2008), support the migration of CG to the oocyte's periphery (Galeati *et al.* 1991), and protect the oocyte from reactive oxygen species (ROS) damage through the supply of glutathione (GSH) (Tatemoto *et al.* 2000). Several studies have recommended selecting for IVM only COCs formed of multiple (three or more) compact layers of cumulus cells (Rath *et al.* 1995; Long *et al.* 1999; Esaki *et al.* 2004; Sherrer *et al.* 2004; Bagg *et al.* 2007; Alvarez *et al.* 2009; Lee *et al.* 2012, Lin *et al.* 2015). However, a comprehensive investigation of the developmental potentials of oocytes with reduced cumulus investment appears missing in the literature. Figure 1.10 presents a selection of porcine COCs with different expected developmental potentials.

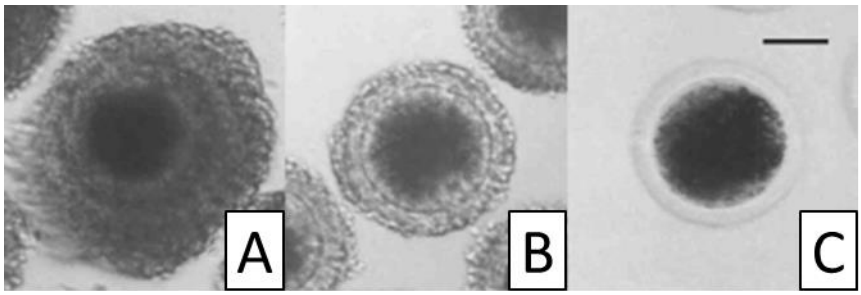


Fig. 1.10 – Immature porcine cumulus-oocyte complexes (COC) of different morphology. A) Good quality oocyte surrounded by a compact and dense cumulus. B) An oocyte surrounded only by a single layer of cumulus cells is expected to have a low developmental competence. C) Denuded oocyte, no cumulus cells are present leading to the expectation of very low developmental competence. Scale bar represents 50 µm. (Image adapted from Alvarez *et al.* 2009).

After selection, the most suitable oocytes are matured for 18-22 h in cattle (Rizos *et al.* 2008) or for 44 h in pigs (Abeydeera & Day 1997; Appeltant *et al.* 2015) under an appropriate hormonal stimulation based on FSH and LH analogues, in similarity to

ovarian stimulation regimens (Knitlova *et al.* 2017). During this time, the oocytes have the opportunity to progress to the MII stage of meiosis (nuclear maturation) and to complete cytoplasmic maturation (Dang-Nguyen *et al.* 2011).

The cytoplasmic maturation encompasses a number of metabolic and structural changes that allow an oocyte to be fertilised and develop (Ferreira *et al.* 2009). The redistribution of CG to the periphery of the cell is one of the most notable examples of a cytoplasmic maturation event; other examples include mitochondria redistribution and accumulation of ribosomes and are reviewed elsewhere (Ferreira *et al.* 2009). While both nuclear and cytoplasmic maturation are essential for development, the two processes are not necessarily synchronised in IVM oocytes leading to a possible reduction in their developmental competence (Eppig 1996; Grupen *et al.* 1997). As a result, methods have been put forward to favour the synchronisation between nucleus and cytoplasm. In the oocyte, cyclic adenosine monophosphate (cAMP) is known to inhibit nuclear maturation (Norris *et al.* 2009). Therefore, during IVM, cAMP modulating agents can be supplied to the oocytes to postpone meiosis and to improve synchronisation between nucleus and cytoplasm (Appeltant *et al.* 2015).

In order to achieve shorter generational intervals, IVM has also been used for the production of embryos from peripubertal animals. Indeed, the vast majority of the abattoir population of pigs is constituted of peripubertal gilts, which in turn become the principal donors of oocytes for IVM. However, it is well established that oocytes derived from gilts display reduced developmental potentials when compared to oocytes derived from sows (Marchal *et al.* 2001; Bagg *et al.* 2004; Bagg *et al.* 2006; Lechniak *et al.* 2007). Moreover, IVM oocytes generally show reduced ability to produce blastocyst stage embryos as compared to *in vivo* derived oocytes (Rizos *et al.* 2002) and polyspermy is known to be more common in IVM rather than in *in vivo* derived oocytes (Wang *et al.* 1998).

1.2.2.3 *In vitro* fertilisation (IVF)

The technique of IVF was firstly developed to treat human infertility and involves the co-culture of both mature oocytes and sperm cells under controlled conditions (Steptoe & Edwards 1978). If successful, IVF will lead to the formation of a zygote, which can then be further cultured *in vitro* prior to transfer to a recipient in an attempt to establish a pregnancy (Hasler *et al.* 1995). The general principle of IVF is illustrated in figure 1.11.

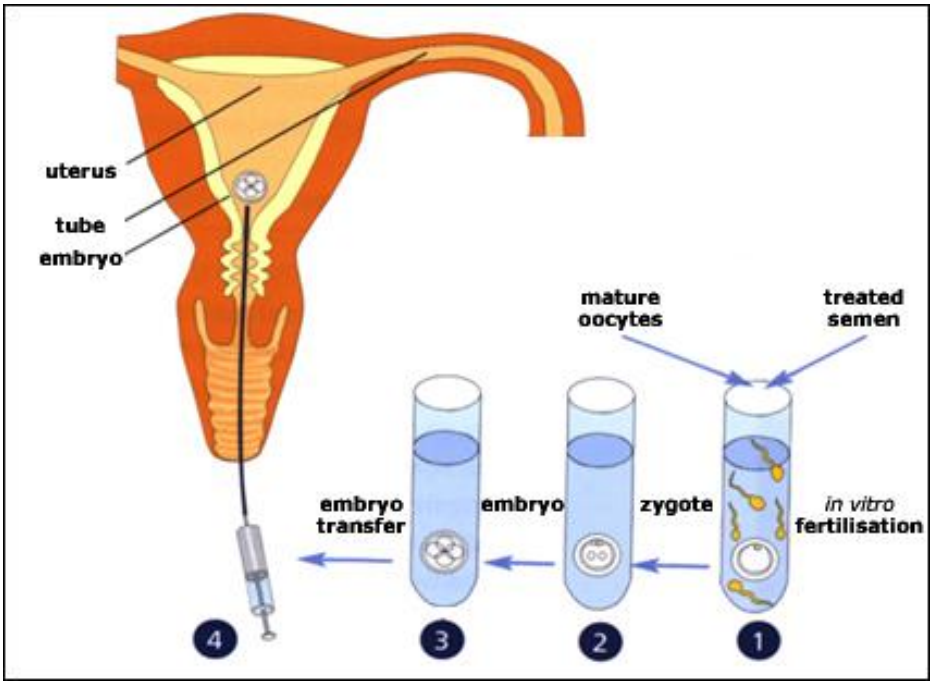


Fig. 1.11 – Key steps of *in vitro* fertilisation (IVF). Mature oocytes from a superovulated donor or oocytes matured *in vitro* are co-cultured with prepared semen to achieve fertilisation outside of the body (1), resulting in the formation of a zygote (2) and finally of an embryo (3) which can then be transferred *in utero* (4). (Image adapted from http://www.cpma.ch).

As discussed above, IVF can also be applied to farm animal breeding where it forms part of an IVP system. Both *in vivo* derived oocytes retrieved by OPU and IVM oocytes can be fertilised by IVF (Wang *et al.* 1999). IVF is normally carried out in specialised media designed to assist the capacitation of the sperm (Izquierdo *et al.* 1998). As described in section 1.1.6, sperm cells would normally acquire the ability to fertilise an oocyte (capacitation) during their voyage through the female reproductive tract. Therefore, during IVF appropriate conditions must be created to induce sperm capacitation. Most media for mammalian IVF contain high concentrations of calcium ions, which are known to induce capacitation *in vivo* (Parrish *et al.* 1999). Other important factors are

bicarbonate and magnesium (Cran *et al.* 1986). Moreover, most IVF media described in the literature make use of additional compounds to stimulate capacitation: these are mainly caffeine for porcine IVF (Yamaguchi *et al.* 2012; Nabavi *et al.* 2013) and heparin for bovine IVF (Parrish *et al.* 1988). Additional components that have been shown to assist the capacitation of bull semen are epinephrine, penicillamine and hypotaurine (Miller *et al.* 1994).

However, one of the key issues with IVF is the incidence of polyspermy (Tanihara *et al.* 2013; Kosman *et al.* 2014). The problem of polyspermy is especially relevant for pig IVP where rates of up to 65% have been detected (Wang *et al.* 1998). While the causes of this unusually high rate of polyspermy in pig IVP are still uncertain, some risk factors have been identified in the literature (Coy & Avilés 2010), such as the high concentration of sperm cells surrounding the oocyte *in vitro* as compared to natural fertilisation (Nagai *et al.* 1990) and the specific formulation of the capacitating agents in the culture medium (Coy *et al.* 1993; Abeydeera *et al.* 1997; Abeydeera 2001; Abeydeera 2002; Gil *et al.* 2010). Caffeine has been shown to induce spontaneous acrosome reactions in boar spermatozoa in the absence of contact with an oocyte, which is thought to cause actively polyspermy (Gil *et al.* 2008; Yamaguchi *et al.* 2012; Nabavi *et al.* 2013). As a result, several alternatives to caffeine have been described including the use of adenosine, theophylline, cysteine and fertilisation promoting peptide (Funahashi *et al.* 2000; Yoshioka *et al.* 2003). In cattle IVF, heparin and sperm concentration have been shown to have a direct effect on polyspermy, so that both these parameters should be carefully calibrated based on the IVF system used and the specific bull employed (Parrish 2014). Additionally, it has been noted that alterations in the culturing method can also reduce polyspermy. For example, accurate washing of the zygotes after co-culture, and optimised co-incubation timing can positively reduce polyspermy (Matas *et al.* 2003).

1.2.2.3.1 Sperm preparation for IVF

Successful IVF has been described with sperm cells derived from a variety of sources including fresh semen (Parrish *et al.* 1985), extended semen (Xu *et al.* 1996), cryopreserved semen (Parrish *et al.* 1986) and sex sorted semen (Xu *et al.* 2006),

allowing great flexibility to this tool. The use of cryopreserved semen is particularly common in bovine IVF due to its wide commercial availability. The use of cryopreserved semen is advantageous because it allows high reproducibility over time (Parrish 2014). However, sperm samples of any kind are formed of both viable and non-viable sperm cells, and while motile sperm cells are prevalent in fresh ejaculates, they may represent as little as 30% of the population in cryopreserved samples, making it difficult to measure the effective dose used for IVF and potentially impairing culture (Parrish *et al.* 1995).

To circumvent this issue, methods for the selection of the motile fraction of an ejaculate have been developed, most notably the swim up method and the Percoll gradient method (Van der Zwalmen *et al.* 1991). During swim up, a sperm sample is deposited at the bottom of a column of medium, and then incubated for a set time. The top fraction of the column is then collected and is expected to contain only sperm cells that were able to swim actively towards the top and are therefore motile (Younglai *et al.* 2001). The Percoll gradient method instead relies on the different density exhibited by viable and non-viable sperm cells. By centrifugation of a sperm sample through an appropriate density gradient, is possible to separate the heavier viable sperm cells from the lighter non-viable ones (McClure *et al.* 1989). Of the two methodologies, the Percoll gradient method has found greater scope in animal IVF due to its rapidity and ability to recover greater amounts of viable cells (Parrish *et al.* 1995).

1.2.2.4 *In vitro* culture (IVC)

Following successful fertilisation, embryos must be cultured under appropriate conditions to be able to develop further, a phase known as IVC. In particular, the ability to culture embryos until the blastocyst stage is thought to be pivotal to selecting the best quality embryos for transfer thus ensuring high implantation rates (Gardner *et al.* 1998).

1.2.2.4.1 Development of an adequate IVC system

Early attempts in the field failed to maintain the embryos in culture for prolonged periods. Research into methods for extended IVC was intense in the 1950s, leading to the development of media that could fully support the growth of *in vivo* derived embryos to the blastocyst stage (Whitten 1956; Whitten 1957; McLaren & Biggers 1958) and to the development of the M16 medium that could support blastulation of early embryos reliably (Whittingham 1971). However, initial media could not support the complete development of zygotes, as these arrested at specific stages depending on the model organism employed: 2-cell in mice, 8-cell in cattle, 4-cell in pigs and humans; at a time seemingly correlated with the activation of the embryonic genome (Rieger 1992).

Later studies demonstrated that this cell block could be overcome by the supplementation of adequate energy sources (like glutamine, pyruvate and lactate) and by the addition to the culture medium of chelating agents like ethylenediaminetetraacetic acid (EDTA) (Chatot *et al.* 1989). Furthermore, the analysis of the composition of tubal fluids led to the development of several efficient IVC media still in use today, like the human tubal fluid (HTF) medium (Quinn *et al.* 1984) and the synthetic ovarian fluid (SOF) medium, which is commonly employed in cattle IVP (Tervit *et al.* 1972). Following the initial success, IVC stayed an active field of research. For example, modern media for cattle IVC based on SOF generally carry other improvements like the inclusion of essential amino acids, citrate and myo-inositol (Holm *et al.* 1999). However, a medium which could support the growth of porcine embryos was not developed until 1992 after much optimisation, this medium was called North Carolina State university 23 (NCSU-23) (Petters & Wells 1993). The rather tardive development of an IVC system for the porcine model somewhat explains the reduced impact IVP has made in this species to date as compared to cattle.

At the same time, efforts were dedicated towards the optimisation of culture conditions other than the medium itself. In particular, it was found that the use of adequate buffers is important for embryo development (reviewed in Pool 2004; Swain 2010). Bicarbonate is the main buffering system employed in embryo culture as the pH of the culture

medium can be conveniently controlled by adjusting the CO_2 pressure in the incubator. However, several other buffers were described for the manipulation of embryos outside an incubator, which included among the others trishydroxymethylaminomethane (TRIS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 3-(N-morpholino)-propanesulfonic acid (MOPS) (Will *et al.* 2011). The osmolarity of the medium was also investigated and it was found that embryos tend to develop better when cultured in slightly hypoosmolar media, which are close to 280 mOsm (Baltz & Tartia 2009). Additionally, the presence of high levels of ROS is known to affect embryo development (Yang *et al.* 1998), and he culture of embryos under low O_2 pressure, typically 5%, was generally found to increase the quantity and quality of the blastocysts produced (Umaoka *et al.* 1992; Noda *et al.* 1994). It was also observed that embryos tend to develop better when cultured in groups because they can benefit from the reciprocal secretion of paracrine growth factors (Gandolfi 1994; Nagao *et al.* 2008). As a result, some efforts were dedicated towards determining the optimal number of embryos to be cultured in a specific volume to maintain a balance between nutrients, catabolites and growth factors. Although there is no clear consensus in the literature regarding the optimal embryo density, culture conditions featuring one embryo per 3 to 10 µl of medium are commonly employed (Choi *et al.* 2003; Fujita *et al.* 2006; Rebollar–Lazaro *et al.* 2010).

Finally, the addition of serum, often in the form of foetal bovine serum (FBS), to IVC media in the proportion of 5 to 10%, is commonplace, since serum is an abundant source of nutrients, growth factors and antioxidants (Burnouf *et al.* 2016). However, the current trend in the literature is to move away from serum-supplemented media towards the use of completely defined IVC media instead, which guarantee higher reproducibility and eliminate biosecurity concerns (Rizos *et al.* 2002). Defined media able to fully support the development of zygotes for the IVC of both cattle (Holm *et al.* 1999) and pig embryos (Yoshioka *et al.* 2008) have been published.

1.2.2.4.2 Effects of culture media composition on embryo development

The "quiet embryo hypothesis" put forward by Leese (2002) suggests that embryos with a moderately active metabolism have fewer chances of becoming arrested as compared to embryos with high metabolism. The rationale behind this hypothesis is that viable embryos are efficient in their utilisation of resources while stressed embryos will require a higher energy input to cope with the damage that might be present (Leese *et al.* 2007). The quiet embryo hypothesis is supported by evidence suggesting that embryos with a greater developmental competence display a reduced amino acid turnover (Stokes *et al.* 2007) as well as reduced protein and DNA synthesis (Baumann *et al.* 2007).

Even though, as described in the previous section, progress has been made in the development of culture media that can mimic the conditions encountered by embryos *in vivo*, IVP embryos generally exhibit a reduced developmental competence (Pomar *et al.* 2005). For example, it has been demonstrated that IVP embryos have a significantly higher glucose metabolism as compared to *in vivo* derived embryos of the same age (Khurana *et al.* 2000), which, according to the quiet embryo hypothesis, suggests they might not be as viable. Moreover, IVP embryos are known to accumulate lipids as a consequence of the media used for IVC (Abe *et al.* 2002; Sudano *et al.* 2012; Sanches *et al.* 2013), a behaviour that has been associated with decreased developmental potentials (Sturmey *et al.* 2009). As a result, methods to reduce lipid accumulation during IVP have been proposed, such as the addition to the medium of L-carnitine, a co-factor of lipid β-oxidation (Dunning *et al.* 2011; Sutton-McDowall *et al.* 2012).

These observations indicate that there is still scope for the development of optimal IVC media and indeed, research in this field is currently very active (Baldoceda-Baldeon *et al.* 2014; Wang *et al.* 2014; Mullaart *et al.* 2015; Kelly & Gardner 2017; Murillo-Ríos *et al.* 2017).

1.2.3 Embryo transfer

The final step of IVP is the transfer *in utero* of the embryos produced. Usually, the recipient animal is treated with oestrus controlling drugs to ensure that the embryos are

transferred into a uterus that is ready to accept implantation (Spell *et al.* 2001; Stroud & Hasler 2006).

Embryo transfer, often in the form of MOET, has been commercially exploited in cattle breeding over the last four decades to improve the genetic merit of livestock by enhancing selection for traits like milk production and disease resistance (Hasler 2014). Initially, the methods used for embryo transfer were surgical, and the transfer was operated with local anaesthesia on standing cows (Smith 1988; Hasler 2014). However, the development of catheter based non-surgical methodologies for embryo transfer has revolutionised the industry (Dawson 2000). Non-surgical embryo transfer simply involves the use of a catheter not too dissimilar from those used for AI, while the recipient animal is restrained in a standing position. The catheter is designed to pass through the cervix so that the embryos can be deposited approximatively in the middle of one uterine horn (Rowe *et al.* 1980; Wright 1981; Mapletoft 2006); a schematic representation of the cow's reproductive tract is presented in figure 1.12. Several studies have indicated that pregnancy rates between 41-56% can be achieved following non-surgical embryo transfer of IVP embryos in cattle (Hastler *et al.* 1995; Trigala *et al.* 2012; Merton *et al.* 2013). However, the management of the recipients at a farm level is known to play a key role for success (Stroud & Hasler 2006).

On the other hand, embryo transfer has not been applied as extensively in pigs. However, specialised catheters, which take into account the peculiar length of the uterine horns found in the porcine female reproductive tract, have been developed, allowing non-surgical embryo transfer in the pig (Martinez *et al.* 2004). By this method, the completion of an embryo transfer round in less than five minutes in around 95% of recipient sows has been made possible (Martinez *et al.* 2002) and a study by Martinez *et al.* (2013) achieved a farrowing rate of 80% and an average litter size of 9.5 following the transfer of 30 embryos per recipient, indicating the birth of one piglet per each 4 embryos transferred.

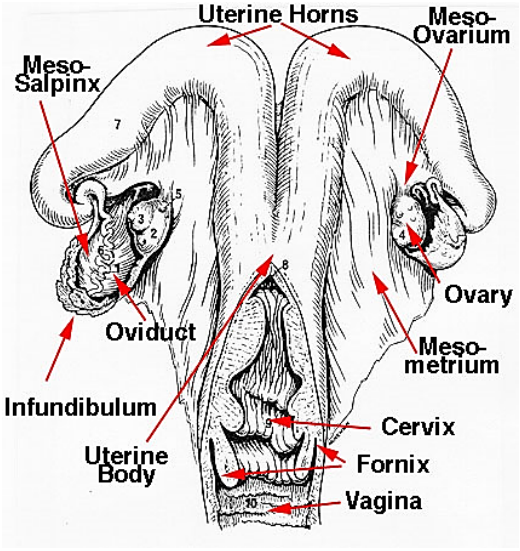


Fig. 1.12 – The reproductive tract of the cow. Notably, the cranial part of the uterus is divided in two separate chambers known as the uterine horns. During embryo transfer, a catheter is used to deliver an embryo through the cervix and up to the middle point of one of the uterine horns. (Image from www.ansci.wisc.edu).

1.2.4 Cryopreservation

Cryopreservation is a technology that allows the storage of sperm, oocytes and embryos at sub-zero temperatures and their successive recovery (Saragusty & Arav 2011; Medeiros *et al.* 2012). In IVP, cryopreservation allows the long-term storage of embryos and their commercialisation across long geographic distances (Sudano *et al.* 2012).

Two general methods are used for cryopreservation: slow freezing and vitrification. During slow freezing, the cooling rate of the sample is strictly controlled to prevent the formation of ice crystals inside cells (Van Wagtendonk-De Leeuw *et al.* 1995). The main advantage of slow freezing is the use of low concentrations of cryoprotecting agents (Vajta & Nagy 2006). Whilst cryoprotectants can be detrimental to cell survival due to their toxicity, their use in cryopreservation is essential due to their ability to reduce or eliminate the damages associated with ice crystal formation (cryodamage) (Bondioli 2015). Conversely, during vitrification high concentration of cryoprotectants are used to dehydrate the sample before direct immersion in liquid nitrogen to achieve a very steep cooling rate, which prevents the formation of ice crystals altogether (Vajta *et al.* 1998). Of the two techniques, vitrification has found a wider use in animal IVP because of its effectiveness and low cost (Van Wagtendonk-de Leeuw *et al.* 1997; Mucci *et al.* 2006).

Despite some technical advances in cryopreservation (Lane *et al.* 1999), such as the use of microdroplets (Papis *et al.* 2000) and cryotop techniques (Kuwayama *et al.* 2005; Dode *et al.* 2013), the freezing process can still impair the viability of embryos due to cryodamage (Overstrom 1996; Baguisi *et al.* 1999a). This is especially true for IVP embryos, where cryopreservation is complicated by their lower cryotolerance as compared to *in vivo* derived embryos (Pollard & Leibo 1994). This reduced tolerance to cryopreservation is thought to be caused, at least in part, by the greater amount of lipids found in IVP embryos (Abe *et al.* 2002; Sanches *et al.* 2013) and their reduced number of mature mitochondria (Crosier *et al.* 2001; Farin *et al.* 2004). In particular, lipid accumulation has been shown to be detrimental for the cryopreservation of porcine embryos (Larman *et al.* 2014) and, interestingly, the removal of lipids (delipation) has been shown to increase markedly their cryotolerance (Esaki *et al.* 2004). Furthermore, the use of serum-free media or the modulation of the lipid content of embryos through appropriate changes in the culture media have been shown to increase cryotolerance as well (Sudano *et al.* 2013).

1.3 Non-invasive methods for embryo selection

Embryos manipulated *in vitro* are normally screened to estimate their developmental potentials before being selected for embryo transfer. Several non-invasive selection methodologies have been described in the literature including morphology, morphokinetics and assessment of spent culture medium.

1.3.1 Morphology

The morphological evaluation of embryos has found wide use in human IVF (Boiso *et al.* 2002). However, the appearance of human embryos is somewhat different to the appearance of many ungulate embryos, such as porcine and bovine embryos, due to the distinctive accumulation of lipid droplets in the latter specimens, which renders the embryo opaque, and more challenging to assess though conventional methods (Van Soom *et al.* 2003; Sturmey *et al.* 2009). Figure 1.13 further illustrates this difference by comparing a human embryo to a bovine embryo of the same developmental stage.

Nevertheless, the cattle embryo transfer industry has long been able to exploit the principle that the transfer of embryos of better morphology leads to higher pregnancy rates (Lindner & Wright 1983).

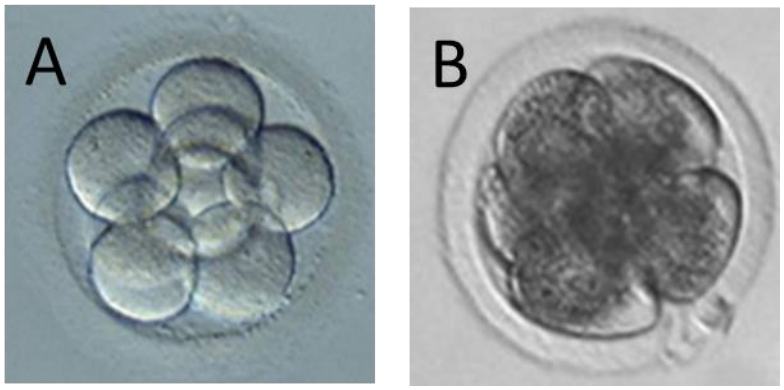


Fig. 1.13 – Comparative morphology of human and bovine cleavage stage embryos. A) Human 8-cell stage embryo of excellent morphology presenting eight even and translucent blastomeres. B) Bovine 8-cell stage embryo of excellent morphology. The specimen is opaque due to the presence of lipid droplets, which make the morphological assessment more challenging. (Image sources: part A: atlas.eshre.eu; part B: adapted from Lechniak *et al.* 2008).

A vast body of literature has been published regarding which embryo morphological parameters best correlate with developmental competence, the most noteworthy example being drafted after the Istanbul consensus workshop (Balaban *et al.* 2011). According to the developmental stage of an embryo, different parameters can be taken into account. Examples of the most common parameters considered are given below.

In zygotes, pronuclei should be of equal size, abutted, and close to the centre of the zygote (Rienzi *et al.* 2013). This configuration has been demonstrated to be correlated positively to developmental competence in humans (Nagy *et al.* 2003; Balaban *et al.* 2011). However, the morphology of the pronuclei is difficult to identify in ungulates due to the discussed opacity of their oocytes and embryos. Some studies have suggested the use of centrifugation to accumulate the lipid content of the zygote towards the periphery of the cell thereby allowing visualisation of the pronuclei (Tatham *et al.* 1995; Wall 2001), however this method is most commonly used to facilitate certain applications requiring nuclei micromanipulation rather than for embryo selection.

In cleavage stage embryos, the presence of fragmentation, intended as the accumulation of non-viable, sub-cellular material in the embryo, has been identified as having a negative impact on developmental competence (Alikani *et al.* 1999; Balaban *et al.* 2011). The degree and pattern of fragmentation is known to have clinical significance (Alikani *et al.* 1999), although embryos seem to be able to tolerate fragmentation <10% without a loss in developmental competence (Van Royen *et al.* 2001). The percentage of fragmentation within an embryo is often estimated subjectively by microscopy observation, presenting a challenge to reproducibility (Alikani *et al.* 1999; Ebner *et al.* 2001; Johansson *et al.* 2003). Uneven cleavage, resulting in the formation of blastomeres of unequal sizes has also been correlated with a reduced developmental competence (Hardarson *et al.* 2001; Racowsky *et al.* 2011).

The morphological assessment of blastocyst stage embryos is possibly the methodology that has found greater use in animal IVP due to this stage being favoured for embryo transfer (Rizos *et al.* 2002). The main parameters evaluated in blastocysts are the extension of the ICM, the extension of the TE and blastocoel expansion (Gardner & Schoolcraft 1999; Balaban *et al.* 2011). With particular reference to cattle IVP, a formal grading system has been developed to classify blastocysts in four grades from 1 to 4 (Bo & Mapletoft 2013) with only embryos belonging to grades 1 and 2 being of interest for embryo transfer, and embryos of grade 1 being generally expected to produce higher pregnancy rates than embryos of grade 2 (Hasler *et al.* 1987). Details of this classification are presented in figure 1.14.

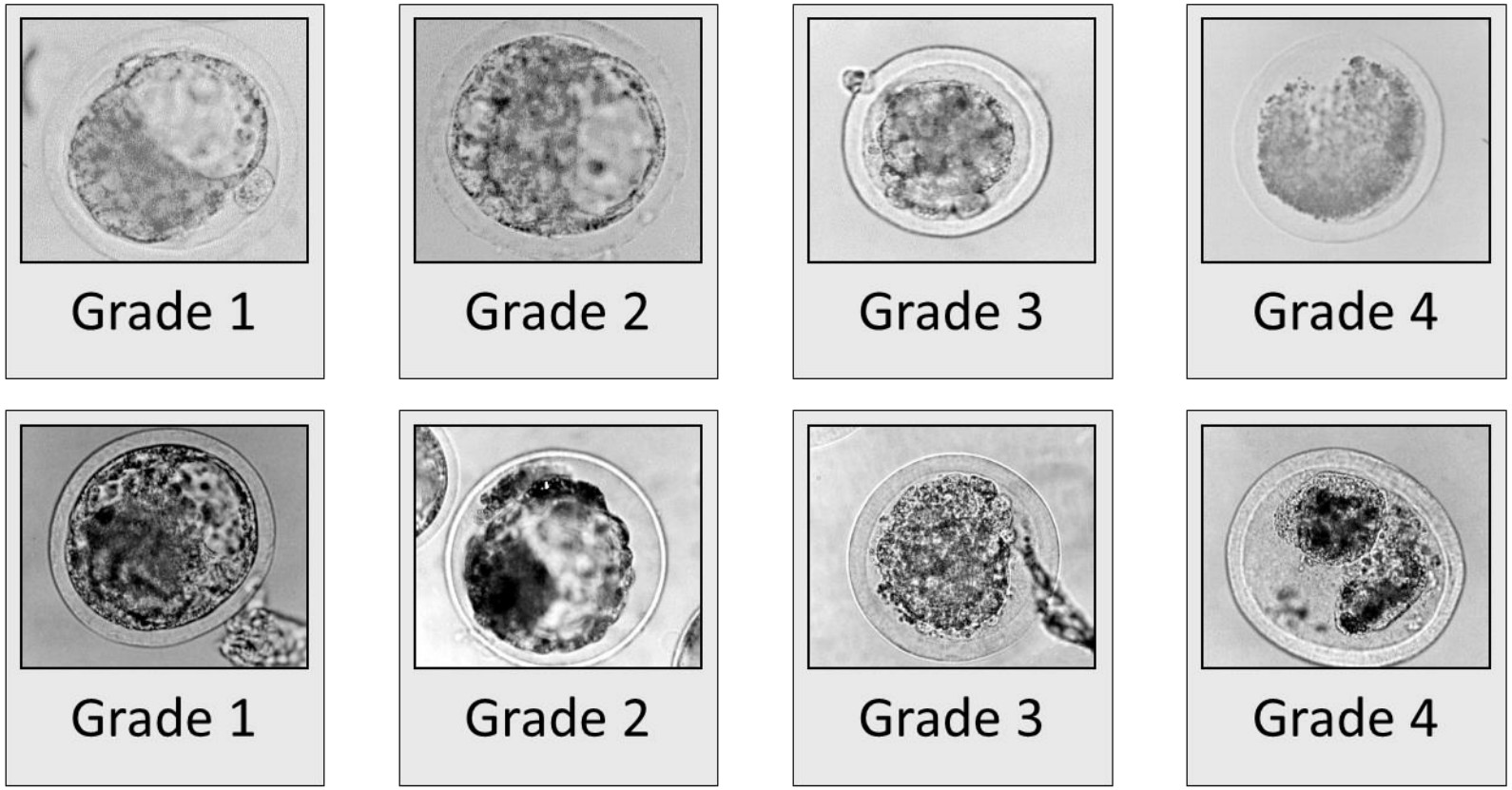


Fig. 1.14 – Embryo classification in cattle at day 7 of culture. Embryos of grade 1 are considered excellent or good, with minor irregularities and less than 15% fragmentation and are suitable for transfer and cryopreservation. Embryos of grade 2 are considered fair and display moderate irregularities; their use for transfer is acceptable but they are expected to display reduced cryotolerance. Embryos of grade 3 are considered poor with major irregularities and obvious fragmentation; they are not expected to survive freeze/thawing and provide very modest results upon transfer. Embryos of grade 4 are dead or degenerating and should be immediately discarded as they are non-viable. (Image adapted from Bo & Mapletoft 2013).

1.3.2 Time lapse

To support the morphological assessment of embryos, and in an attempt to provide a better prediction of viability, time-lapse systems have been introduced in clinical IVF settings. These systems require a microscope and a camera and are able to tack embryo development during the entire duration of IVC (Kaser & Racowsky 2014). Several systems are commercially available like Embryoscope and PrimoVision, both from Vitrolife, Sweden. Some systems, like Embryoscope, feature fully insulated culture chambers that function like incubators, while other systems, like PrimoVision, are designed to fit inside standard incubators.

The availability of time-lapse systems allows for continuous embryo monitoring providing embryologists with a greater amount of information as compared to static observation (Cruz *et al.* 2011; Chamayou *et al.* 2013). Moreover, observations can be carried out without removing the specimen from its incubator, allowing for an undisturbed culture. The use of time-lapse as a method to predict accurately the developmental competence of embryos has generated some controversy in the literature, with several studies finding a net positive impact (Hesters *et al.* 2008; Ciray *et al.* 2012; Meseguer *et al.* 2012; Gardner *et al.* 2015) and some authors failing to detect significant improvements (Racowski *et al.* 2015; Goodman *et al.* 2016). Moreover, it has been suggested that continuous monitoring could have a negative impact on embryo development due to frequent exposure of the embryos to light, leading to phototoxicity (Pribenszky *et al.* 2010; Wong *et al.* 2013). However, this effect, if present, appears to be minimal (Sugimura *et al.* 2010; Kirkegaard *et al.* 2012; Kaser & Racowsky 2014).

One interesting feature offered by some time-lapse platforms like PrimoVision, is the ability to culture embryos in individual micro-wells within the same culture dish, an approach referred to as the well-of-the-well (WOW) culture system (Wood *et al.* 1993). This application permits tracking of several embryos at the same time whilst also allowing the embryos to benefit from the effects of co-culture, which have been described under section 1.2.2.4. A commercial dish designed for the WOW culture of embryos is shown in figure 1.15. Interestingly, the WOW culture system has immediate

applications in research because it allows experimenters to track individual embryos under the same experimental conditions, an approach described by Vajita *et al.* (2000). Nonetheless, in IVP, time-lapse technology has found limited application from a commercial perspective with most users focusing on the research benefits offered (Holm *et al.* 1998; Somfai *et al.* 2010). Indeed, time-lapse is well suited to study the dynamics of early embryonic development (Wong *et al.* 2010; Kirkegaard *et al.* 2012; Herrero *et al.* 2013; Kellam 2015) which, as discussed under section 1.1.8, are not yet fully elucidated.

Fig. 1.15 – Well of the well (WOW) culture dish. In the WOW culture system, embryos are cultured in the same dish (left) and can therefore take advantage of the benefits of co-culture. Additionally, the embryos can be individually tracked thanks to their positioning within micro-wells (zoom, right). (Image source: http://www.vitrolife.com).

1.3.3 Novel imaging modalities for the study of embryonic development

Time-lapse systems used for research purposes are limited in that they offer a poor depth of view, and this problem is made worse in post-compaction embryos like blastocysts, and in ungulate embryos due to their unfavourable lipid distribution (Van Soom *et al.* 2003). Moreover, time-lapse analysis packages do not allow for an embryo to be easily be observed from different angles, and the observation becomes especially difficult when the embryo presents overlapping cells or is highly fragmented (Wong *et al.* 2010). Therefore, there is currently some interest in the literature for the development of new imaging modalities which can resolve the embryo structure in full depth, in real time, and non-invasively. A technique currently showing some promise in this context is optical coherence tomography (OCT) (Drexler & Fujimoto 2015).

1.3.3.1 Optical coherence tomography (OCT)

Due to its ability to non-invasively produce well defined images through living tissues up to a depth of several millimetres, OCT is currently considered one of the most innovative emerging optical methods (Huang *et al.* 1991; Fercher *et al.* 2003; Schuman *et al.* 2003; Drexler *et al.* 2014; Drexler & Fujimoto 2015), a fact well testified by the filing of over one thousand related patents since 1980 (Drexler *et al.* 2014).

From an intuitive point of view, OCT can be thought of as a being similar to ultrasonography, in that it is capable of producing cross-sectional images from multiple depth layers within the sample. However, OCT uses light instead of sound and displays a far greater resolution than ultrasound (Drexler *et al.* 1999). In OCT, an optical beam, often from a laser source, is directed at a sample. To ensure penetrability, low power laser sources are normally employed (infrared lasers) ensuring the safety of the technique (Choma *et al.* 2005). A small proportion of the illuminating light is reflected by the sample whilst the majority of the light is scattered in all possible directions. In standard microscopy, scattered light contributes to background noise; however, in OCT scattered light is filtered out of the image by interpherometry, a method to measure the optical path of photons and remove the information carried by those with a scattered pattern (Fercher *et al.* 1999).

Originally, OCT was developed for use in ophthalmology (Puliafito *et al.* 1995) where it has become a standard tool for the investigation of the posterior part of the eye (Hee *et al.* 1995; Budenz *et al.* 2007; Staurenghi *et al.* 2014). Resolutions of 1 µm have become achievable allowing detailed investigations at the cellular and subcellular level (Drexler *et al.* 2000). Other medical applications of OCT include diagnostics procedures in oncology (Assayag *et al.* 2014), cardiology (De Rosa *et al.* 2017), gastroenterology (Kirtane & Wagh 2014) and dermatology (Dalimier *et al.* 2014). Whilst OCT can certainly be used to obtain a structural characterisation of a sample and reconstruct 3D models of the specimen analysed, this method can also be used to acquire functional images from living tissues. One example is Optical Coherence Tomography Angiography (OCTA), a method used to detect flowing blood cells (Jia *et al.* 2014). This application is made

possible by algorithms such as speckle variance (SV) analysis, which can detect and quantify the changes in the optical pattern of a sample associated with movement (Federici *et al.* 2015; Ruminski *et al.* 2015; Gorczynska *et al.* 2016).

Today, OCT encompasses a number of different but related techniques, including ultra-high-resolution (UHR)-OCT (Herman *et al.* 2004), full field (FF)-OCT (Bonin *et al.* 2010), and swept source (SS)-OCT (Shimada *et al.* 2014). In particular, SS-OCT employs as a light source a tuneable laser that sequentially emits light at different frequencies over time, a method that can increase tissue penetration. Moreover, SS-OCT has the potential to offer higher scanning speeds as compared to other methods and might be better suited to analyse samples in motion (Drexler *et al.* 2014). Finally, recent advancements in the SS-OCT field have greatly simplified image acquisition as compared to earlier platforms, rendering the operation of SS-OCT microscopes more similar to standard microscopes from an operator's perspective (Podoleanu & Bradu 2013; Cernat *et al.* 2017).

1.3.3.1.1 Applications of OCT in embryology

OCT has already found some limited application in developmental biology. The first of such studies was completed by Drexler *et al.* (1999) who used UHR-OCT to produce detailed cross-sectional imaging of a living tadpole. A later paper by Larina *et al.* (2009a) applied the principles of SS-OCT to rat embryos, which were recovered surgically from donors 10 or 11 days after mating. In their work, the authors used SS-OCT and OCTA to produce 3D reconstructions of the beating heart of the embryos and to detect the blood flow within vessels. More recently, OCT has been applied to the study of early mammalian embryos as well. Zheng *et al.* (2012) applied FF-OCT to the imaging of cleavage stage and blastocyst stage murine embryos, and were able to reconstruct 3D models of the embryos examined which included an assessment of the cell numbers completed with no need for fluorescent labelling. Additionally, in a recent study, high-resolution intracellular imaging on live mouse and pig oocytes and embryos has been reported (Karnowski *et al.* 2017). In their work, Karnowski and colleagues were able to record fertilisation and early cleavage divisions, effectively representing the first application of OCT to time-lapse. In this context, the fundamental advantages of OCT

are its ability to image embryos in a complete non-invasive way thanks to the use of low power light sources, the absence of chemical labelling, and the ability to penetrate through the lipid content of ungulate embryos (Zheng *et al.* 2012; Karnowski *et al.* 2017). An example of the images produced by the application of OCT on early mouse embryos is given in figure 1.16.

Considering the limited number of publications in this field, it is safe to assume that the application of OCT to embryology is still a new area of research. While several authors have described the application of structural OCT imaging to the study of embryo development, just one paper has applied functional OCT imaging to early embryos (Larina *et al.* 2009a), while the applicability of SS-OCT and SV to the detection of the structure and the vital status of early ungulate embryos remains completely unexplored.

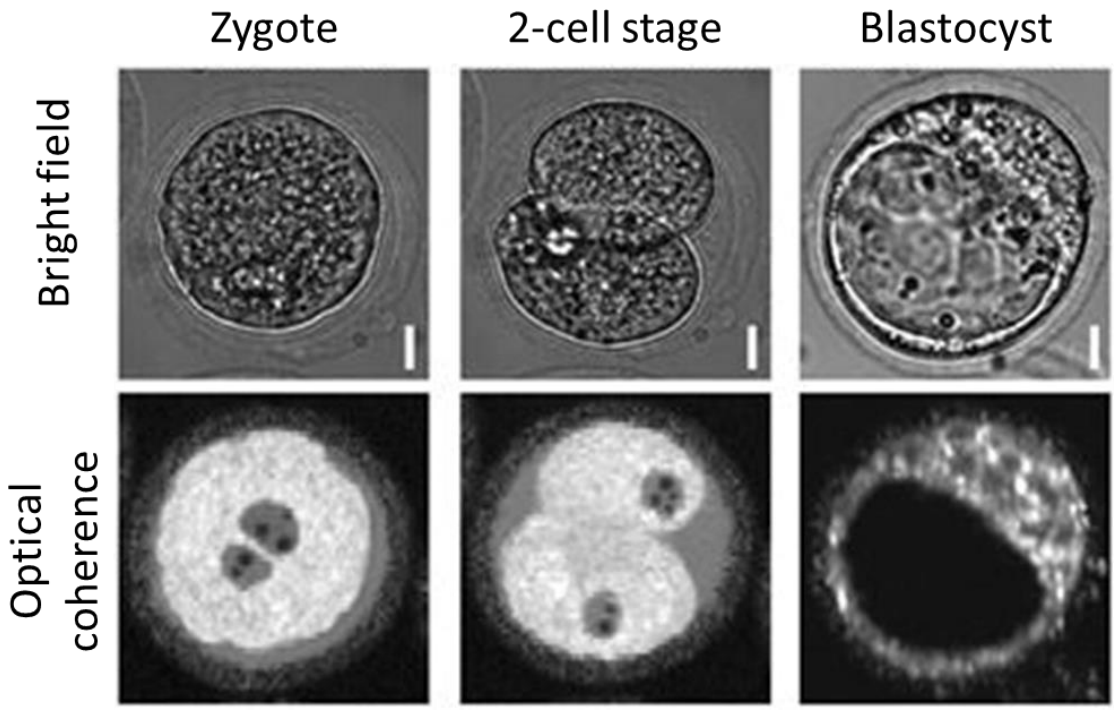


Fig. 1.16 – Application of optical coherence tomography (OCT) to the imaging of early stage murine embryos. The images on the top row were captured by standard bright field microscopy and are compared to images of the same specimen captured through the use of OCT. With OCT, nuclei are clearly visible in zygotes and cleavage stage embryos. At the same time, all the characteristic features of a blastocyst (trophectoderm ring, inner cell mass, blastocoel) can be easily observed (Image adapted from Karnowski *et al.* 2017).

1.3.4 Analysis of culture medium

The idea of analysing spent culture medium as a method to gain insight into an embryo's developmental competence non-invasively has also been discussed in the literature (Sturmey *et al.* 2008). A key breakthrough in this direction was described in 2002 when it was demonstrated that the pattern in which embryos modify the amino acid content of the culture medium is predictive of their ability to develop to the blastocyst stage (Houghton *et al.* 2002). Further investigation found that the pattern of amino acid consumption, and particularly of asparagine, glycine and leucine, was also related to the ability of the embryo to establish an ongoing pregnancy (Brison *et al.* 2004).

This methodology for the selection of highly competent embryos appears especially powerful when used in combination with other more traditional selection methods. For example, Booth *et al.* (2007) showed that the combination of amino acid profiling from spent culture medium with classic morphokinetic parameters resulted in the ability to predict which porcine zygotes would proceed to form a blastocyst with an accuracy of 80%. More recently, Guerif *et al.* (2013) proposed combining amino acid profiling with the measurement of the consumption rates of pyruvate and glucose in order to gauge the developmental competence of bovine embryos. The authors found that embryos with a high pyruvate uptake were less likely to develop to the blastocysts stage than embryos with a moderate uptake, providing further evidence for the quiet embryo hypothesis discussed in section 1.2.2.4.2.

Additionally, it is known that male and female embryos can exhibit different gene expression and metabolic profiles (Gutiérrez-Adán *et al.* 2000). This led to the development of criteria for the determination of embryo sex from culture media analysis through amino acid profiling, an application that could have immediate applications in farm animal IVP (Sturmey *et al.* 2010). A different but related approach was proposed by Muñoz *et al.* (2014) who applied infrared spectroscopy to obtain non-invasive sexing of cattle embryos with an accuracy of 74.4 to 86.0%.

Finally, recent studies have demonstrated the presence of cell-free RNA and DNA of embryonic origin in spent culture medium (Capalbo *et al.* 2016; Shamonki *et al.* 2016; Liu *et al.* 2017). The analysis of the microRNA profile recovered in this way has been proposed as a methodology to assess developmental competence, although this approach seems only suitable for blastocyst stage embryos (Capalbo *et al.* 2016). Moreover, several authors have attempted to use DNA samples extracted from spent culture medium to establish a diagnosis of aneuploidy through conventional PGS methods (Shamonki *et al.* 2016; Xu *et al.* 2016; Feichtinger *et al.* 2017; Liu *et al.* 2017). However, the concordance between these investigations and the diagnosis established by classic embryo biopsy ranged between 46.6 and 90.0%, suggesting that further optimisation will be required before introducing aneuploidy screening based on spent culture medium into clinical practice (Xu *et al.* 2016; Feichtinger *et al.* 2017; Liu *et al.* 2017).

1.4 Reproductive cloning

The term “clones” identifies separate organisms with essentially identical genomic constitution. In mammals, clones can appear spontaneously when monozygotic (MZ) twins are born, or their formation can be induced by the application of reproductive cloning techniques (Paterson *et al.* 2003).

1.4.1 Natural twinning

The mechanisms leading to the formation of MZ twins *in vivo* are not yet well understood, although it is known that they arise from the fragmentation of a single embryo (Knopman *et al.* 2014; Herranz 2015). The frequency of MZ twins in natural human reproduction has been estimated to be 1 in 250 conceptions (MacGillivray 1986). Interestingly, this figure becomes significantly higher for pregnancies established from IVF embryos, although, once again, the reasons for this are not understood (Knopman *et al.* 2014). In cattle, the MZ twinning rate appears to vary slightly according to the specific breed considered; however, from the information presented in table 1.1, it can be estimated to be close to 1 in 360 births.

Breed	Calvings (n)	MZ births (n)	MZ twinning (% of births)	Reference
Holstein Friesians	10,885	14	0.13	(1)
Holstein Friesians	7,387	22	0.30	(2)
Swedish Red & White	495,470	842	0.17	(3)
Swedish Friesians	169,144	237	0.14	(3)
German Schwarzbunt	734,297	2,056	0.28	(3)
Bavaria	140,054	252	0.18	(3)
German Fleckvieh	683,807	2,257	0.33	(3)
German Braunvieh	271,283	1,194	0.44	(3)
Holstein	23,978	55	0.23	(4)
Holstein Friesians	11,951	27	0.23	(5)
Holstein Friesians	24,843	184	0.74	(6)
Holstein	1,212	4	0.33	(7)
TOTAL	**2,574,311**	**7,140**	**0.27**	**-**

Table 1.1 – Frequency of monozygotic (MZ) twins across several cattle breeds. From the information presented, it can be estimated that, in the general cattle population, MZ twins appear with an average frequency of 0.27% equivalent to 1 in 360 calving events. References: (1) Meadows *et al.* 1957; (2) Erb & Morrison 1959; (3) Johansson *et al.* 1974; (4) Cady & Van Vleck 1978; (5) Nielen *et al.* 1989; (6) Ryan & Boland 1991; (7) Del Rio *et al.* 2006.

On the other hand, it is difficult to describe the frequency of MZ twinning in multiparous animals such as the pig, since only fingerprinting tests rather than standard observation can discriminate between littermates (dizygotic twins) and true MZ twins. While a few studies have reported the occurrence of MZ twins in the pig, their sample size was too small to draw any conclusions about the incidence of MZ twinning in the general porcine population (Ashworth & Barrett 1998; Bjerre *et al.* 2009).

The frequency of MZ twinning appears low in cattle and possibly in the pig (Del Rio *et al.* 2006; Bjerre *et al.* 2009); however, the ready availability of identical animals could be beneficial to the breeding industry as a means of breeding more efficiently from parents of high genetic merit or for use in research. The formation of identical animals can, however, be induced through the reproductive cloning techniques embryo splitting and nuclear transfer, which are discussed hereafter.

1.4.2 Embryo splitting

It is possible to take advantage of the totipotency of early stage embryos to create MZ twins purposely. The technique of embryo splitting is effectively a type of reproductive cloning: by artificially dividing embryos and transferring the resulting splits to surrogate mothers it is possible to obtain multiple, genetically homogenous offspring (Willadsen 1979; Willadsen & Polge 1981; Escriba *et al.* 2002).

Arguably, the first successful reproductive cloning experiment on a vertebrate was carried out by Spemann at the beginning of the 20th century when the embryologist used a baby-hair loop to split a salamander embryo in two halves, and discovered that both the halves had the ability to develop into fully formed twins if the embryo splitting had been performed at an early enough stage (Spemann 1901). One of the very first embryo splitting procedures completed on a mammal was performed in 1967 to investigate the developmental competence of 1/4, 1/8 and 2/8 mouse embryo splits produced from *in vivo* derived cleavage stage embryos (Tarkowski & Wroblewska 1967). Later studies in the field found that embryo splits derived from 2-cell stage embryos had better survivability than splits derived from 8-cell stage embryos when transferred to foster mothers (65% versus a maximum of 27%) (Tsunoda & McLaren 1983).

Two general methods have been described for the purpose of generating embryo splits: cleavage stage embryo splitting (or blastomere separation) (Willadsen *et al.* 1981), and blastocyst stage embryo splitting (or embryo bisectioning) (Williams *et al.* 1982). Interestingly, there are currently no reports in the literature directly comparing the efficiency of cleavage stage and blastocyst stage splitting strategies performed under the same IVP system.

1.4.2.1 Cleavage stage embryo splitting (blastomere separation)

During cleavage stage embryo splitting, intact blastomeres are removed from their ZP. A common approach is to remove the ZP completely via mechanical or chemical procedures. The zona-free embryos can then be fully disaggregated by culture in Ca2+ and Mg2+ free medium and gentle agitation. Therefore, the separation is achieved by weakening the intracellular junctions between blastomeres rather than just by mechanical action. After separation, single or multiple blastomeres can be placed into an evacuated ZP often derived from an oocyte (Escriba et al 2002). This methodology is further illustrated in figure 1.17.

One of the most interesting features of the cleavage stage embryo splitting is that it can potentially lead to the formation of a number of embryos equal to the number of blastomeres recovered. Unfortunately, no study has reported the birth of MZ octuplets as a result of cleavage stage embryo splitting. However, the technique has produced the birth of triplets and quadruplets in cattle (Johnson *et al.* 1995; Willadsen & Polge 1981) and pairs of twins in several other farm animal species including sheep (Willadsen 1979; Willadsen 1980), goat (Tsunoda *et al.* 1984), pig (Saito & Niemann 1991), and horse (Allen 1982; Allen & Pashen 1984).

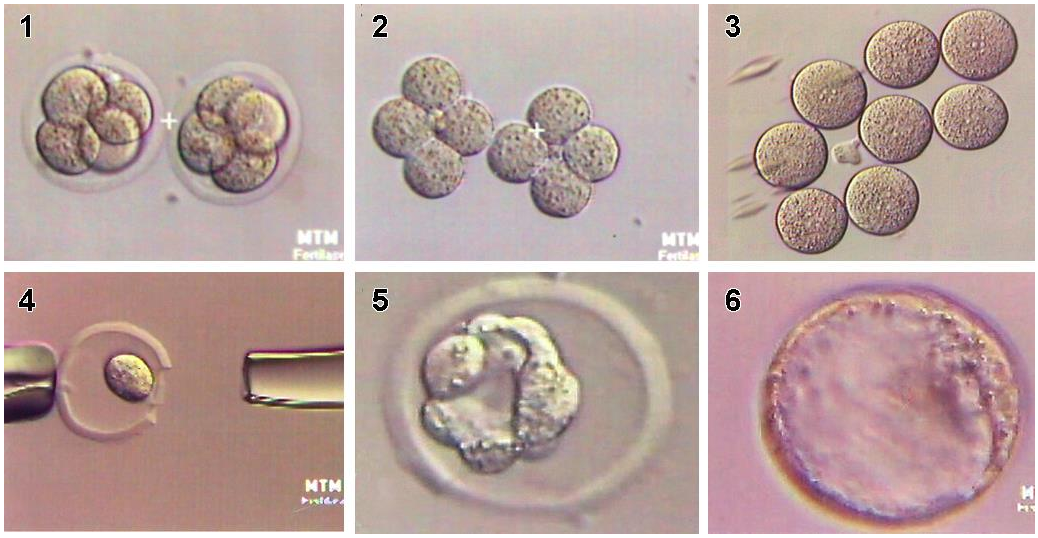


Fig. 1.17 – Cleavage stage embryo splitting. Two 4-cell stage murine embryos (1) are subjected to enzymatic digestion of their zona pellucida (2). Once freed from the zona pellucida, the blastomeres are separated (3) and transferred either singly or in groups into an empty zona pellucida (4). The embryos are then cultured until the blastocyst stage (5-6). (Image adapted from Tang *et al.* 2012).

1.4.2.2 Blastocyst stage embryo splitting (embryo bisectioning)

The splitting of a blastocyst is commonly performed by passing a fine bore pipette through the embryo so that the separation is achieved purely by mechanical means. This approach limits the number of splits producible to just two, with each identical half of the embryo known as “demi”. Only demi-embryos receiving a sufficient proportion of the ICM will be able to survive the splitting and continue development (Escriba *et al.* 2002). An example of this methodology is presented in figure 1.18.

Embryo bisectioning has led to the birth of MZ twins in several mammals (Leibo & Rall 1987; Williams *et al.* 1984; Tsunoda *et al.* 1984; Nagashima *et al.* 1989). The success of the technique, however, is limited to high quality blastocysts, as only good quality embryos appear able to withstand the stress caused by the splitting (Escriba *et al.* 2002).

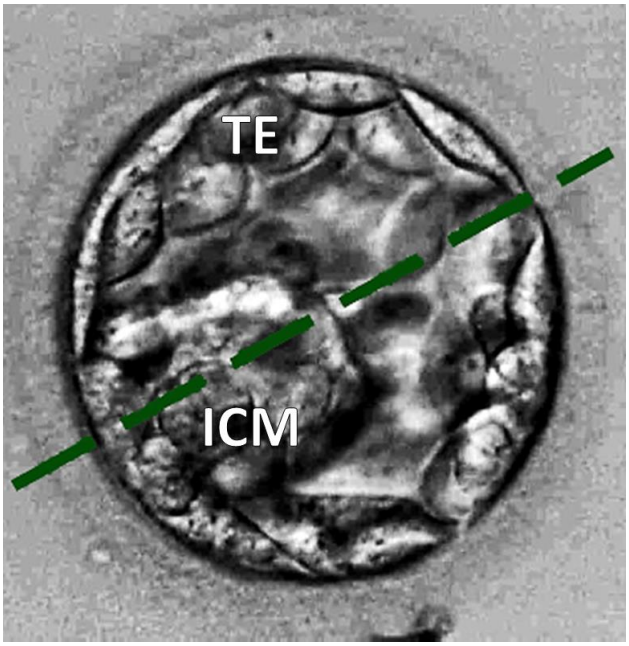


Fig. 1.18 – Blastocyst stage embryo splitting. In this form of embryo splitting, a blastocyst is divided mechanically in two equal halves. To remain viable, the resulting demi-embryos should receive similar proportions of both the trophectoderm (TE) and, more importantly, of the inner cell mass (ICM). With reference to the above, the dashed line indicates the optimal position for the cut. (Image adapted from Escriba *et al.* 2002).

1.4.2.3 Zona-free embryo splitting

The standard embryo splitting technique recommends culturing the splits into evacuated ZPs. This recommendation serves at least two purposes: it allows the experimenter to keep track of his work with more ease (for example blastomeres from different embryos might diffuse in the medium) and, more importantly, provides the embryo with a solid matrix within which it may correctly organise itself (Tagawa *et al.* 2008). However, a study in mouse demonstrated that the functions of the ZP might be completely replaced by the use of the WOW system (Wood *et al.* 1993), and a similar finding was confirmed in cattle (Vajta *et al.* 2000).

The application of the WOW culturing technique to embryo splitting provides for a quicker, more effective protocol and removes the need for the collection and preparation of additional ZPs, although this approach seems suited to cleavage stage embryo splitting only (Tagawa *et al.* 2008). Interestingly, post-compaction embryos that are denuded of their ZP, bisected and immediately transferred to their recipient show conception rates comparable to zona-clad controls (Seike *et al.* 1989). In similarity to what described for the WOW culture system, this approach provides for a quicker and less resource intensive protocol (Seike *et al.* 1989).

1.4.2.4 Applications of embryo splitting to livestock production

The application of embryo splitting might be beneficial to the breeding industry in those circumstances when an IVP system is in place. In this scenario, embryo splitting could be used to maximise the number of embryos available for transfer as well as to increase embryo transfer success rates (Gary *et al.* 1991). Therefore, embryo splitting could further amplify selection intensity on both female and male lines due to the ability to produce numerous animals from a few elite parents.

In cattle, pregnancy rates >50% per demi-embryo have been reported resulting on average in more than one pregnancy established per single embryo and therefore in a true multiplication of the offspring (Gary *et al.* 1991). Embryo splitting in cattle is a well-established technique with bisected embryos achieving similar pregnancy rates to intact

embryos (respectively 50-60% and 55-61%) (Lopes *et al.* 2001). As a result, embryo splitting has already been applied commercially in this species (Gary *et al.* 1991; Kippax *et al.* 1991).

Conversely, embryo splitting has been applied only sparingly in pigs. Nevertheless, splitting of cleavage stage porcine embryos has been reported and led to the formation of blastocysts *in vitro* (Menino & Wright 1983) and even to live births (Niemann & Reichelt 1992). Moreover, live births have been recorded after blastocyst stage embryo splitting (Rorie *et al.* 1985; Nagashima *et al.* 1988) with one study reporting pregnancy rates of 82% with healthy piglets but reduced litter sizes (Reichelt & Niemann 1994). However, in the pig, embryo bisectioning can lead to substantial disruption of the embryo's developmental ability, especially when blastocysts of a lesser grade are split (Tao *et al.* 1995). In general, the survivability of porcine embryo splits appears somewhat compromised, as they tend to perform poorly in comparison to intact control embryos with pregnancy rates upon transfer of 14.3% and 50%, respectively, reported in one study (Brüssow & Schwiderski 1990).

In the horse, *in vivo* derived cleavage stage embryos could be split, cultured into ligated ewe oviducts and then transferred to foster mares leading to normal term pregnancy (Allen & Pashen 1984). While the need for *in vivo* culture seems surpassed (Skidmore *et al.* 1989), horse embryo splitting has been reported only occasionally in the literature.

1.4.2.5 Embryo splitting and genomic screening

It is well accepted that the breeding industry would benefit from the ability to select the sex of the offspring before a pregnancy is established (Johnson *et al.* 2005). Reliable sex determination of IVP embryos before embryo transfer was first reported in the early 1990s (Herr & Reed 1991) and subjected to several improvements shortly after (Bredbacka *et al.* 1995). Currently, the procedure is used to some extent with 0.3% and 3.7% of biopsied cattle embryos being sexed in the USA and in Canada, respectively (Hasler 2014). Furthermore, at present there is a growing interest into applying genomic selection to farm animal IVP, which would result in an improved rate of genetic gain

thanks to the ability to perform a selection without the need to establish a pregnancy (Hall *et al.* 2013), as further discussed in section 1.8. In this context, the availability of twin embryos could simplify sexing and genomic selection by providing additional samples for the analysis. At the same time, it could reduce the cost of the screening since a single test would be informative for all twins simultaneously.

1.4.2.6 Embryo splitting in biomedical research

Animal embryo splitting might also be useful in biomedical research. Indeed, the availability of identical twins would provide for excellent control samples, allowing hypothesis testing against a fixed genetic background. For example, pharmacokinetic studies are known to benefit from the availability of MZ twins (Tang & Endrenyi 1998). Additionally, splits can be frozen and transferred at different times to test epigenetic effects such as the role of maternal hypothyroidism in reduced IQ (Haddow *et al.* 1999); or to allow the investigation of identical protocols in twins of different ages to address cellular senescence questions (Chan *et al.* 2000).

1.4.2.7 Embryo splitting applications in primates

The possibility of applying embryo splitting to non-human primates has received some interest. The production of clones of model organisms like the rhesus monkey could find immediate research applications in the fields of epigenetics, tissue transplant, and disease prevention among the others (Schramm & Paprocki 2004). A breakthrough in this direction was achieved in 2000 with the birth of Tetra, the first live rhesus monkey born from cleavage stage embryo splitting after numerous unfruitful attempts (Chan *et al.* 2000). Since then, both pre- and post-compaction embryo splitting techniques have been trialled in the rhesus monkey (Mitalipov *et al.* 2002). However, the complex requirements of the rhesus embryo led to sub-optimal birth rates and mostly singleton births (Schramm & Paprocki 2004).

The use of embryo splitting has also been proposed for human assisted reproductive procedures as a way to increase chances of implantation. Due to ethical implications,

this application is not allowed in all countries. However, a review of this topic falls beyond the scope of this introduction, and a more detailed discussion is presented elsewhere (ASRM 2004; Illmensee *et al.* 2010).

1.4.2.8 Embryo splitting limitations

Theoretically, it would be possible, via embryo splitting, to create as many embryos as there are blastomeres at the time of split. In practice, this is not possible due to some limitations of the technique. Splits seem to survive less well than intact controls (Willadsen & Polge 1981); additionally, apoptotic processes tend to be more prevalent in the splits, especially within the ICM (Chan *et al.* 2000).

In embryo bisectioning, it was observed that demi-embryos possess less than half the total cells of the original embryo due to a loss of cells in both the ICM and the TE (Skrzyszowska & Smorag 1989; Tao *et al.* 1995; Rho *et al.* 1998). The low cell count found in splits might explain a lower conception rates for embryo splits as compared to intact controls (Heyman 1985; McEvoy & Sreenan 1990).

Similarly, the decrease in survivability of cleavage stage splits might be caused by a reduction in the number of cells present in the embryo at time of compaction. Indeed, it is known that an insufficient number of cells at time of blastulation results in the formation of aberrant blastocysts deprived of an ICM, which cannot develop further (Gardner *et al.* 1973).

As a r

esult of these effects, the maximum number of viable embryos produced by splitting is restricted. Current reports suggest a limit of four twins might be difficult to overcome (Johnson *et al.* 1995; Chan *et al.* 2000). Interestingly, although embryo splits have a reduced cell count as compared to intact embryos, the offspring appear of normal size suggesting that embryos are capable of compensating for the initial cell number loss during development (Papaioannou *et al.* 1989).

1.4.2.9 Serial embryo splitting

The question arises as to whether it would be possible to perform successfully the splitting of an embryo derived from a previous split, the so-called “serial splitting”. A study that has investigated the effectiveness of serial splitting in mice found that the blastulation rate of first splits of 2-, 4- and 6-cell stage embryos was 74.3%, 75.0%, and 66.6%, respectively and was maintained to comparable levels for second splits (71.8%, 62.6%, 48.4%). However, blastulation rates fell sharply with the third split, which only yielded rates of 48.4%, 38.1%, and 10.6% resulting in a loss rather than an increase in the number of embryos produced (Illmensee *et al.* 2006).

While perpetual splitting seems definitively unachievable, serial splitting still holds promise to increase the number of viable blastocysts obtainable from each produced embryo. Nevertheless, a comprehensive comparison between single and serial splitting strategies has not been previously reported in the literature and the application of serial splitting to farm animal breeding is equally underreported.

1.4.3 Somatic cell nuclear transfer (SCNT)

Another method that is suitable for the artificial creation of clones is somatic cell nuclear transfer (SCNT). In brief, the technique requires the transfer of an intact nucleus derived from a somatic cell into an enucleated suitable cell that has the ability to reprogram the somatic nucleus. While it is possible to use zygotes and blastomeres as recipient cells, in most cases MII oocytes show the highest aptitude for reprogramming donor nuclei (Kato *et al.* 1998; Holker *et al.* 2005; Hall et al 2006). The nature of the somatic donor cell is also important, as some cell lines are more readily reprogrammed; for example, one study in cattle reported improved success with cumulus cells as compared to fibroblasts (Tian *et al.* 2003). It is perhaps not surprising that pluripotent cells like blastomeres and embryonic stem cells seem to support cloning efficiency to higher rates than somatic cells (Zhou *et al.* 2001; Eggan *et al.* 2002). The transfer of nuclei is normally operated by micromanipulation using highly specialised equipment (Li *et al.* 2004). However, protocols for the performing of the nuclear transfer by hand (the so-called “hand-made cloning”) have also been reported for cattle (Vajta *et al.* 2003) and pigs (Kragh *et al.*

2005). After the fusion of nucleus and cytoplasm donors, the construct must be activated either by an electrical pulse or chemically (reviewed in Campbel *et al.* 2007). The activated construct will then occasionally proceed to form a viable embryo with an identical genomic constitution as the somatic cell donor (Wilmut *et al.* 2002). The methodology of SCNT is further illustrated in figure 1.19.

Certainly, the most well known example of the application of SCNT to reproductive cloning belongs to Wilmut and colleagues: in 1997, the news of the birth of Dolly the sheep, the first animal born by SCNT, hit the major newspapers' headlines worldwide (Wilmut *et al.* 1997). However, nuclear transfer had previously been reported in the 1950s (Briggs & King 1952; Gurdon *et al.* 1958) and was perfected for use in mammals in 1984 when sheep embryos, resulting in live births, were constructed by fusing a sheep blastomere with a bisected oocyte (Willadsen 1986). To date, an ever-growing number of mammals have been cloned by means of nuclear transfer. Among them are the rhesus monkey (Meng *et al.* 1997), mouse (Wakayama *et al.* 1998), cattle (Kato *et al.* 1998), goat (Baguisi *et al.* 1999b), pig (Polejaeva *et al.* 2000), rabbit (Chesne *et al.* 2002), cat (Shin *et al.* 2002), mule (Woods *et al.* 2003), horse (Galli *et al.* 2003), dog (Lee *et al.* 2005), and more recently camel (Wani *et al.* 2010). However, the overall cloning efficiency has remained low with reports consistently indicating rates between 0 and 3% (Wilmut & Paterson 2015), even though several technical improvements have allowed for simplified protocols, reduced costs and better offspring survival (Campbel *et al.* 2007).

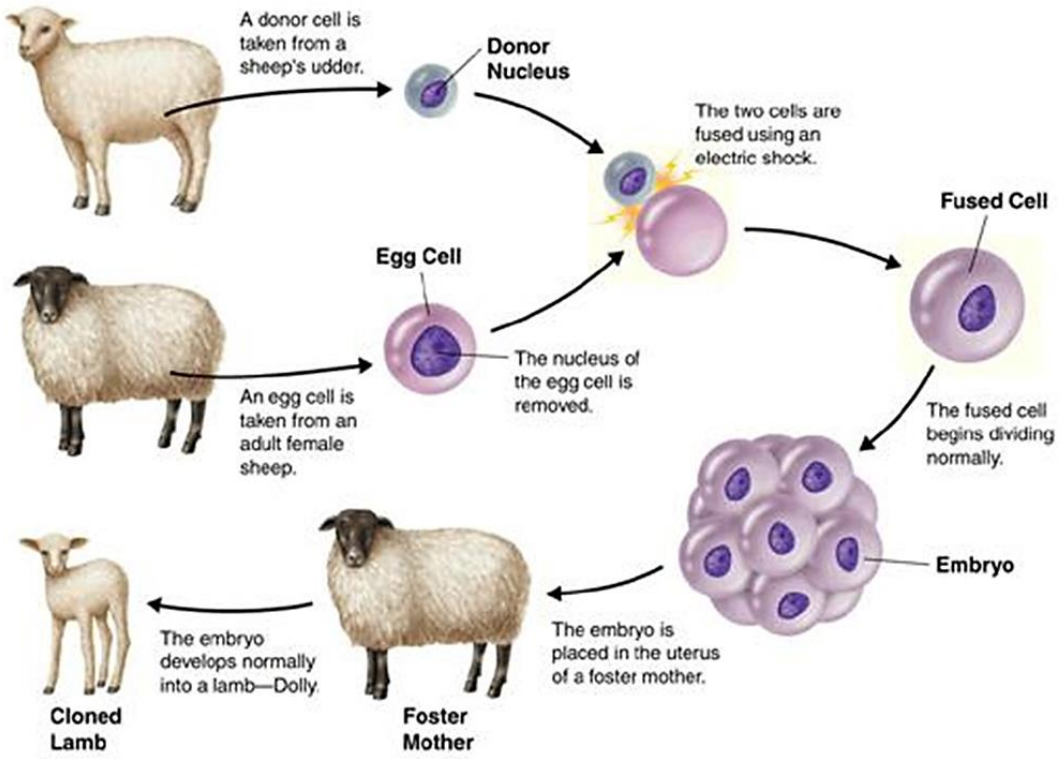


Fig. 1.19 – Somatic cell nuclear transfer (SCNT). A schematic representation of the SCNT method is presented. An enucleated oocyte is fused with the nucleus of an adult somatic cell. The activated embryo is transferred to a foster mother leading to the birth of an animal genetically identical to the somatic cell nuclear donor. (Image from Gifford & Gifford 2013).

1.4.3.1 Nuclear transfer in commercial breeding

SCNT might be used to multiply animals of proven genetic merit. One USA programme resulted in the transfer of 2.683 cloned cattle embryos (Faber *et al.* 2003) and recent reports suggest that meat and milk derived from cloned animals are safe for human consumption although public acceptance is not widespread (Yang *et al.* 2007).

Nonetheless, for SCNT to be applied for large-scale reproductive cloning some technical improvements appear mandatory (Smith *et al.* 2012). Disappointingly, a 2011 survey conducted in Japan that examined 3264 ET procedures with SCNT cattle embryos between the years 1998-2007 highlighted how the cloning efficiency had substantially remained stable over the decade with an average calving rate of 9.2% (Watanabe & Nagai 2011). Furthermore, for SCNT to be commercially viable, it is indispensable that clones can be produced at reasonable costs; however, current estimates suggest around

15,000-20,000 USD might be required per clone (Hasler 2014). For all these reasons, in the immediate future large-scale application of this practice seems very unlikely. However, SCNT could be useful to reproduce animals of exceptional value, such as top performing reproduction bulls (Bousquet & Blondin 2004) or elite horses (Lagutina *et al.* 2005).

1.4.3.2 SCNT in biomedical applications

Introducing modifications in the genome of a mammal has been possible since the 1980s by microinjection of DNA into a zygote; however, microinjection is subjected to several shortcomings yielding only a very low efficiency and imprecise control over the integration site of the transgene (Krisher *et al.* 1994). An alternative is applying SCNT using genetically modified cells as nucleus donors. Site-specific gene recombination in cultured cell lines coupled with SCNT has proven a powerful technique to produce transgenic animals with the exact desired genotype (Schnieke *et al.* 1997). Moreover, recent advancements following the development of the novel CRISPR/Cas9 system for gene editing have led to a much more precise control of the modifications induced (Whitworth *et al.* 2014; Zhou *et al.* 2015).

Transgenic animals can be exploited in a number of different ways. For example, it is possible to induce the production of desired proteins in the milk of a transgenic animal (Schnieke et al 1997). Reports suggest that good protein yields might be achieved by using this strategy, which could result in a commercial scale production of important molecules like insulin, anti-clotting proteins, growth factors, and cytokines (Baguisi *et al.* 1999b; Niemann *et al.* 2012). Similarly, it is possible to exploit transgenic animals' milk to supplement the human diet for example with important fatty acids not readily available otherwise (Wu *et al.* 2012). Moreover, transgenic animals might be uselful for xenotransplantation, the transfer of organs or tissues between two different species, thus alleviating the need for human organ donors. The main issue associated with xenotransplantation is the violent immunological response enacted by the transplant. However, the application of genetic modifications in the donor animal might provide for organs unlikely to be rejected, for example by removing epitopes from cell surfaces

(Sandrin *et al.* 1993). Due to the ethical concerns with the use of primates for xenotransplantation, and the higher risk to transmit infectious diseases, the use of different donor animals is desirable (Weiss 1998). Due to its physiology and size, the pig appears the most promising candidate (Ekser *et al.* 2012; Nagashima 2012). For example, the transplant of porcine islets cells might help diabetic patients to recover normal pancreatic functions (Groth *et al.* 2000; Nagashima 2012). Transgenic animals can also be designed to model certain human diseases in order to study their treatment, a well-known example being the creation of knockout mice (Capecchi 1989). However, larger animal models have the advantage of mimicking human physiology more precisely; for example, knockout mice are poor models of human cystic fibrosis while sheep provide a far better representation (Harris 1997). These models can be used for drug testing and assessment of novel therapies.

1.4.3.3 SCNT for wildlife conservation

SCNT might be used to recover wholly the nuclear genetic constitution of an animal of interest without the losses caused by hybridisation with related species/subspecies (Corley-Smith & Brandhorst 1999). Furthermore, SCNT could allow for the clonal propagation of animals that breed with difficulty in captivity, in view of their reintroduction in the wild (Tong *et al.* 2002). Some attempts, reaching various degree of success, have already been described in the literature. The first example was reported by Wells *et al.* who demonstrated the use of SCNT for the conservation of a nearly extinct subspecies: the Enderby Island cattle (Wells *et al.* 1998). A similar attempt was made in *Bos gaurus,* resulting in pregnancies but no live birth (Lanza *et al.* 2000).

1.4.3.4 SCNT limitations

Current technical limitations and lack of public acceptance have prevented SCNT from achieving all of its goals. Moreover, cloned offspring suffer from a number of abnormalities both during and after gestation, leading to increased mortality. It has been reported that only around 10% of cloned cattle embryos transferred to recipients develop to term owing to higher rates of pregnancy loss (Hill *et al.* 2000). The presence

of these abnormalities is well exemplified by the markedly higher prevalence of large offspring syndrome (LOS) in cloned animals as compared to other IVP controls (Liu *et al.* 2013). Other common abnormalities affect the placenta, the cardiovascular system, and the liver (Wilmut *et al.* 2002; Farin *et al.* 2006). These abnormalities are almost certainly caused, at least in part, by incomplete epigenetic reprogramming of the somatic nucleus after reconstruction (Rideout *et al.* 2001; Su *et al.* 2011); this claim is also supported by the observation that more readily reprogrammable cell lines result into a lessened prevalence of LOS (Liu *et al.* 2013). In addition, incompatibilities between the nuclear DNA and the mitochondrial DNA caused by the fusion might account for a proportion of the abnormalities (Hiendleder *et al.* 2004). Finally, certain SCNT techniques and certain media supplementations, like serum, are definitively associated with increased rates of abnormality providing room for further technical refinements (Campbel *et al.* 2007).

On the other hand, some encouraging reports have demonstrated that long-term surviving offspring have similar physiology to their original cell donors. In particular, one study found that the semen of a cloned bull has comparable fertilising potential to its original donor (Tecirlioglu *et al.* 2006). Furthermore, clones' offspring seems to be free from the typical issues faced by their parent suggesting a complete normal embryonic development (Abeni *et al.* 2012). Finally, it is a common conception that cloned animals face accelerated ageing due to telomere shortening (Blasco 2007) and Dolly the sheep was known to have shorter telomeres than its nuclear donor (Shiels *et al.* 1999). However, more recent reports suggest that complete genomic reprogramming leads to telomerase reactivation, producing clones with telomere lengths at least comparable to their donors (Alexander *et al.* 2007; Kishigami *et al.* 2008). In conclusion, SCNT still holds great promise for the future but further refinements seem crucial before its widespread application becomes possible.

1.5 Aneuploidy

Aneuploidy refers to the presence of an abnormal number of chromosomes within a cell, such as the lack of a homologue chromosome (monosomy) or the presence of an extra homologus chromosome (trisomy), representing a deviation from euploidy (Griffiths *et al.* 2000, Nagaoka *et al.* 2012). The clinical outcome of aneuploidy varies considerably based on the type of numerical aberration and on the specific chromosomes involved. For example, the vast majority of somatic chromosome trisomies are fatal during embryonic development. In humans, the only somatic trisomy leading to development past infancy is the trisomy of chromosome 21 which, however, is the cause of Down's syndrome, and is characterised by cognitive impairment, abnormal birth weight, and cardiomegaly (Hassold *et al.* 2007, Nagaoka *et al.* 2012). Aneuploidies of the sexual chromosomes, whilst better tolerated, are also associated with a variety of clinical outcomes ranging from only a modest cognitive impairment to skeletal problems and infertility, the most common examples being the Turner syndrome (45, X0) and the Klinefelter syndrome (47, XXY) (Gravholt *et al.* 1998; Lanfranco *et al.* 2004).

Importantly, aneuploidy is widely recognised as a leading cause of developmental arrest, miscarriage, IVF failure and infertility (Hassold & Hunt 2001; Hassold *et al.* 2007; Munné *et al.* 2007; King 2008; Hodes-Wertz *et al.* 2012; Mir *et al.* 2014). Consequently, the study of aneuploidy has found wide application in embryology. In humans, it has been shown that the majority of IVF embryos are affected by at least some degree of chromosomal abnormality (Lathi & Milki 2004; Baart *et al.* 2007; Franasiak *et al.* 2014), and several studies have attempted to characterise the frequency of aneuploidy on a chromosome basis across different developmental stages (Griffin 1996; Munné *et al.* 2004; Daphnis *et al.* 2008).

1.5.1 Origin of aneuploidy

The exact origin of aneuploidy is still an active research field (Handyside 2012) as several possible mechanisms have been described. Ultimately, aneuploidy is caused by the improper segregation of chromosomes (malsegregation) during either meiosis or

mitosis, which gives raises to a gain or a loss of a whole chromosome (Potter 1991; Cimini *et al.* 2005). Possible mechanisms of aneuploidy during mitosis include non-disjunction, anaphase lag and endoreplication. During non-disjunctions, sister chromatids remain attached during anaphase, leaving one daughter cell monosomic and the other trisomic (Fragouli *et al.* 2013; Baart *et al.* 2014). When anaphase lag occurs, sister chromatids segregate correctly at anaphase, but then a single chromatid fails to be incorporated in the reforming nuclear envelope, resulting in a monosomy in a daughter cell, but a normal chromosome number in the other daughter cell (Munné *et al.* 1994; Kalousek 2000; Coonen *et al.* 2004). A third mechanism is endoreplication, during which a cell cycle error or the initiation and abortion of mitosis might lead to the accidental replication of a chromosome without cytokinesis (Santos *et al.* 2010a; Mantikou *et al.* 2012; Taylor *et al.* 2014).

During meiosis, malsegregation can lead to different outcomes depending on the type of error and on whether it occurred at meiosis I or at meiosis II. In particular, a type of error that has been described in meiosis is premature predivision of sister chromatids. This is caused by sister chromatids becoming prematurely detached at meiosis I and then segregating independently, leading to a combination of possible outcomes, including balanced errors resulting in the production of a normal gamete, but also disomies and monosomies (Handyside *et al.* 2012; Ottolini *et al.* 2015), as illustrated in figure 1.20.

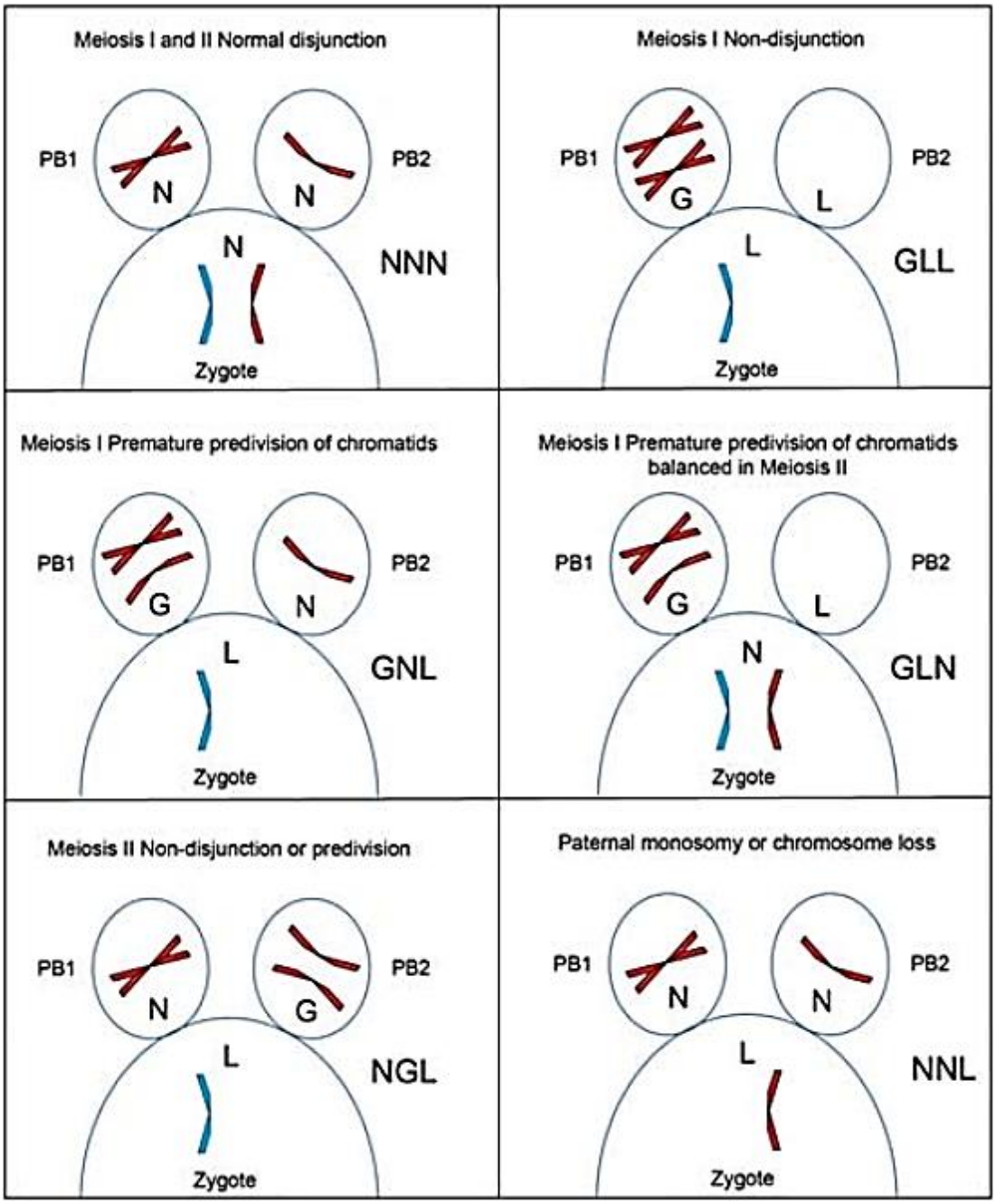


Fig. 1.20 – Examples of segregation patterns arising from normal disjunction, non-disjunction and premature predivision of chromatids. Maternal chromosomes in red, paternal chromosome in blue. PB: polar body. The net result of each segregation pattern is described on the right of each panel as gain (G), loss (L) or normal (N) copy number for PB1, PB2 and Zygote, respectively. Note that not all possible segregation patterns are represented and for premature predivision reciprocal patterns are possible leading to a balanced outcome. (Image from Handyside *et al.* 2012).

1.5.2 Aneuploidy in sperm cells

In humans, aneuploidy is thought to be a cause of infertility in males (Tempest 2011; Harton *et al.* 2012; Gibson *et al.* 2013). For example, high levels of disomy have been detected in infertile men (Tempest & Griffin 2004) and an association has been found between aberrant sperm parameters such as oligo-, astheno-, or terato-zoospermia and aneuploidy (Martin 2005; Mehdi *et al.* 2012). However, similar studies in farm animals have been much less comprehensive, partly because sub-fertile males tend to be simply excluded from breeding regimens. Only a few papers have been published detailing the aneuploidy levels in boar and bull spermatozoa, but used relatively small sample sizes and focused their screening on only a subset of chromosomes so their conclusions cannot be readily extended to the general population (Rubes *et al.* 1999; Rybar *et al.* 2010).

1.5.3 Aneuploidy in oocytes

The incidence of aneuploidy in human oocytes has been estimated to range between 2 to 14.5%, suggesting that aneuploidy is much more common in oocytes than in sperm cells (Hassold & Hunt 2001). A total aneuploidy rate of 30% has been described in cattle oocytes (Nicodemo *et al.* 2010) and a similar figure was discovered in pig oocytes, although these numbers are known to be affected by the age of the female (Lechniak *et al.* 2007).

Indeed, it is well accepted that a link exists between maternal age and aneuploidy incidence in oocytes, a phenomenon often described as maternal age effect (Hunt & Hassold 2008; Hassold *et al.* 2009; Chiang *et al.* 2012). Up to 33% of pregnancies in women over the age of 40 are affected by trisomies as compared to an incidence of just 2% in women under 25 years of age (Hunt 2006; Hassold *et al.* 2007; Loane *et al.* 2013). As described previously, the peculiar mechanics of oogenesis mean that oocytes are maintained in meiotic arrest at diplotene from early foetal life until recruited for ovulation, which might only occur after decades (see section 1.1.4). During all this time, chromosomes are bound together by kinetochores, but these structures are known to lose stability with age, which might account for an increased rate of segregation errors

(Chiang *et al.* 2010). Other possible mechanisms underlying the maternal age effect include an increase in meiotic non-disjunctions after meiosis resumption, and defective spindle assembly checkpoints (Chiang *et al.* 2012).

1.5.4 Mosaicism

In embryology, mosaicism is defined as the presence of at least two cells within the same embryo with a different chromosomal constitution. For example, the embryo could contain a mixture of euploid and aneuploid cells, or a mixture of different aneuploidies (Frumkin *et al.* 2008; Lebedev 2011). Mosaicism is caused by mitotic malsegregation errors that occur after the formation of the zygote and are therefore known as post-zygotic errors to distinguish them from meiotic errors, which do not give rise to a mosaic phenotype. All the mechanisms listed under section 1.5.1 for mitotic errors are considered as a possible cause of mosaicism; moreover, repair mechanisms attempting to restore euploidy following a meiotic chromosomal error during later mitotic divisions may also result in mosaicism (Taylor *et al.* 2014). Interestingly, mosaicism can also be caused by uniparental disomy (UPD), a mitotic event that creates no net loss or gain of chromosomes. In UPD, a cell inherits two homologous chromosomes from one parent and no chromosome from the other parent, often as the result of a trisomic rescue event (Robinson 2000; Conlin *et al.* 2010; Eggermann *et al.* 2015; Taylor *et al.* 2014).

Mosaicism is a common occurrence in human embryos, although the incidence of mosaicism reported in the literature varies considerably based on the investigation method. Harper *et al.* observed a mosaicism incidence of 15% in cleavage stage embryos (Harper *et al.* 1995), while Daphnis *et al.* reported a 90% incidence (Daphnis *et al.* 2005). Additionally, one particularly comprehensive study that analysed all the blastomeres derived from over 800 embryos reported an incidence of 73% (Van Echten-Arends *et al.* 2011). Similar high rates of mosaicism have also been detected in human blastocyst stage embryos (Liu *et al.* 2012). Interestingly, though, there has been some indication suggesting that mosaicism tends to be more prevalent in the TE rather than in the ICM (Taylor *et al.* 2014).

Since mosaicism is so common, it is likely that mechanisms are in place for embryos to tolerate it. Indeed, it has been suggested that a normal genomic constitution in at least 50% of the cells of an embryo would be sufficient to maintain developmental competence (Kuliev *et al.* 2004; Baart *et al.* 2005; Daphnis *et al.* 2005; Baart *et al.* 2014). Mosaic embryos have been known to exhibit normal morphology (Li *et al.* 2005; Frumkin *et al.* 2008), and some authors have suggested the existence of a self-correction process (Munné *et al.* 2005; Bazrgar *et al.* 2013; Scott *et al.* 2013).

Once again, while a wealth of information is available for the human model, only a few authors investigated the presence of mosaicism in cattle and pig embryos. For example, in cattle a study from Iwasaki & Nakahara (1989) found that 13.7% of the blastomeres examined carried aneuploidy, whilst a study published by Kawarsky *et al.* (1996) discovered abnormalities in over one thirds of the blastomeres examined. Two later studies detected chromosomal abnormalities in up to 80% of the observed cells (Yoshizawa *et al.* 1999) or in between 15 to 42% of cells depending on the exact developmental stage of the cleaving embryo (Viuff *et al.* 1999), and a figure of 38% was detected in bovine blastocysts (Iwasaki & Nakahara 1989). Whilst the results reviewed prove the existence of mosaicism in cattle embryos, there is no consensus on its incidence in the literature.

1.5.5 Aneuploidy and embryo morphology

A link between aneuploidy and embryo morphology has been described in the literature. For example, pronuclear morphology has been shown to be predictive of the ploidy status of the embryo (Gianaroli *et al.* 2003). Moreover, several studies have established a direct correlation between the presence of fragmentation and aneuploidy (Ziebe *et al.* 2003; Munné 2006; Chavez *et al.* 2012). Therefore, the availability of accurate methods for the description of embryo morphology might prove valuable for the selection of the best embryos for transfer in commercial IVPfuture test.

1.6 Methods for preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)

Preimplantation genetic diagnosis (PGD) indicates an array of testing methods for assessing the genetics of oocytes and embryos following their manipulation *in vitro* (Handyside 2011). PGD encompasses methods for the diagnosis of monogenic disorders, for human leukocyte antigen (HLA) typing, and for the detection of aneuploidy aimed at reducing the risk of congenital abnormalities or pregnancy loss (Braude *et al.* 2002; Frumkin *et al.* 2008; Harton *et al.* 2010b). The first reported PGD was performed in rabbit in 1968, where the aim was to select for embryos of a specific sex (Gardner & Edwards 1968). Conversely, the first human PGD cases were described in 1990 and used polymerase chain reaction (PCR) to select the sex of the offspring to be female in human couples carrying X-linked disorders thereby preventing the birth of children with the affected phenotype (Handyside *et al.* 1990). Shortly thereafter, in 1992, the first use of PGD for the detection of the recessive monogenic disorder cystic fibrosis was also published (Handyside *et al.* 1992).

In contrast, the term PGS is specifically restricted to the use of diagnostic procedures for the screening of aneuploidy in embryos. PGS is particularly used in cases of advanced maternal age or recurrent miscarriage when there is reason to suspect underlying aneuploidy issues (Delhanty *et al.* 1993; Geraedts & De Wert 2009; Geraedts 2010; Harper & SenGupta 2012). Importantly, in contrast with other screening methods like chorionic villus sampling and amniocentesis, PGS is performed before the establishment of a pregnancy (Stern 2014) and has, therefore, the unquestionable advantage of removing the need to terminate a pregnancy following an unfavourable diagnosis (Sermon *et al.* 2004). However, the use of PGS is only compatible with methodologies allowing the manipulation of embryos *in vitro*, such as IVF (Geraedts *et al.* 2002). Whilst the aims of PGD and PGS might be somewhat different, they largely share the same array of techniques, which are described hereafter.

1.6.1 Polymerase chain reaction (PCR)

The development of sensitive PCR methods allowed the application of PGD to single cell biopsies (Thornhill & Snow 2002; Handyside *et al.* 2004; Ben-Nagi *et al.* 2016). However, this procedure suffered from issues of sensitivity and contamination. In fact, the minute amount of DNA recovered from a single blastomere can be a cause of allele drop out (ADO), an error that occurs when one of the alleles of a heterozygous locus fails to amplify leading to a diagnosis of homozygosity instead. The introduction of nested PCR, which is based on two successive rounds of amplification using different, more internal primers for the second round, increased specificity and reduced ADO issues (Handyside *et al.* 1992). Following its development, several other improvements were published (Liu *et al.* 1995; Hussey *et al.* 1999; Ray *et al.* 2001; Dahdouh *et al.* 2015), the most notable of which was probably multiplexing, which allowed for the amplification of multiple loci in each reaction (Ao *et al.* 1998; Spits & Sermon 2009; De Rycke 2010).

Another important advancement was the development and application of quantitative real-time PCR (RT-qPCR) to PGS. In this approach, the samples undergo a preliminary amplification step followed by multiplex amplification of two regions on each chromosome arm for all chromosomes (Treff *et al.* 2012; Yang *et al.* 2015; Treff *et al.* 2016). By comparing the signal intensity from each probe to the intensity of a known control, it is possible to evaluate the chromosome copy number across the genome (Dahdouh *et al.* 2015). The fundamental advantage of this approach is that it can be directly applied to biopsy samples without need for preparatory steps. However, this also limits the application of RT-qPCR to TE biopsies only, due to the need to supply sufficient template DNA for reliable diagnosis (Treff *et al.* 2012; Dahdouh *et al.* 2015).

Later on, the availability of the genomic sequence of humans revealed the presence of many polymorphic markers that could be used for diagnostic procedures (Lander *et al.* 2001; Sachidanandam *et al.* 2001; Venter *et al.* 2001; International Human Genome Sequencing Consortium 2004). In particular, PCR reactions designed to amplify short tandem repeats (STRs) facilitated analysis and minimised the problems caused by ADO and contamination (Harton *et al.* 2010a; Thornhill *et al.* 2015).

1.6.2 Fluorescence *in situ* hybridisation (FISH)

Due to the limitations of PCR, many studies described the application of a different technique: fluorescence *in situ* hybridisation (FISH). In its beginning, FISH was used with sex chromosome specific probes (Griffin *et al.* 1991), which led to clinical application and live births (Griffin *et al.* 1992; Griffin *et al.* 1993; Delhanty *et al.* 1993; Griffin *et al.* 1994). Soon, though, the use of FISH was extended to the detection of aneuploidy and translocations becoming the most common form of PGS (Munné & Cohen 1993; Munné *et al.* 1994; Munné *et al.* 1996; Munné & Cohen 1998).

However, often only a limited panel of chromosomes could be screened through FISH (Munné *et al.* 1994; Munné *et al.* 1996) which led to some controversy in the field (Summers & Foland 2009). Moreover, the use of FISH was faced with several technical challenges, including accidental loss of nuclear material, overlapping or split signals, high levels of background, and hybridisation failure (Ruangvutilert *et al.* 2000; Fiorentino *et al.* 2011). In view of this, PGS by FISH with probe panels has been largely discontinued, and even though 24 chromosome FISH could be successfully applied in human embryos, technical burdens were considerable (Ioannou *et al.* 2011; Ioannou *et al.* 2012). Today the application of FISH is mostly limited to a research setting, and particularly to studies of cytogenetics (Yang & Graphodatsk 2017).

1.6.3 Comparative genomic hybridisation (CGH)

The successor of FISH was comparative genomic hybridisation (CGH). This method uses differentially labelled test and control DNA that are competitively hybridized to metaphase chromosomes (Theisen 2008), allowing for a more rapid assessment of chromosome copy number across the entire genome (Spelcher *et al.* 1993; Forozan *et al.* 1997) but with limited resolution (Kirchhoff *et al.* 1998; Lichter *et al.* 2000).

Later, CGH was adapted for use in a completely artificial environment, which did not rely on cells. In array CGH (aCGH), test and control DNA are hybridised to a panel of probes on a microarray chip. If test and sample DNA are present in equal measures, a 1:1 colour ratio will result at each locus. Deviation from parity might indicate losses or gains in

specific chromosomal regions arising from trisomy, monosomy, deletion, insertions or translocation (Le Caignec *et al.* 2006; Vanneste *et al.* 2009; Fishel *et al.* 2010; Traversa *et al.* 2011). Remarkably, though, this method is unable to detect UPD events due to the absence of change in copy number and is not well suited for the detection of duplications of the entire chromosome constitution such as triploidy.

1.6.4 Next generation sequencing (NGS)

Once again, though, technology has evolved and the use of aCGH has recently been superseded in clinical settings by the use of next generation sequencing (NGS) instead. In this approach, the DNA recovered from an embryo is cut into small 100-200 bp sections and analysed by massive parallel sequencing (Fiorentino *et al.* 2014). The data acquired can then be compared to a reference genome to detect mutations, and the inheritance of traits or genetic disorders can thus be investigated (Simpson *et al.* 2013). Moreover, the number of reads obtained for each section of DNA can be counted (binning) to obtain an indication of copy number as well (Fiorentino *et al.* 2014), leading to the detection of monosomy, trisomy or segmental alterations (Handyside 2013; Handyside & Wells 2013). NGS migh also be used to predict mosaicism in TE biopsies when intermediate copy numbers are detected (Vera-Rodríguez *et al.* 2016). However, in this context, the sensitivity of NGS has been shown to be highly dependent on the percentage of mosaicism present, and attempts to increase sensitivity have led to a parallel increase in false positives (diagnosig euploid embryos as mosaic) (Goodrich *et al.* 2016).

1.6.5 Single nucleotide polymorphism (SNP) analysis

A single nucleotide polymorphism (SNP) is a variation in a single nucleotide at a specific position in the genome. Interestingly, SNPs are bi-allelic, resulting from the inheritance of an ancestral point mutation (LaFramboise 2009; Habela *et al.* 2013). The two alleles for an SNP can be labelled with different fluorophores on array chips, and the detection of the resulting fluorescence ratio can indicate heterozygosity or homozygosity for either allele (Habela *et al.* 2013).

Specialised microarrays for the detection of thousands of SNPs are now commonly available commercially (Handyside 2015). With infinium technology (Illumina), a platform used for SNP typing, a SNP locus resulting in an uncertain genotype call is assigned a low "genecall" score, and readings with a genecall score beneath the no-call threshold are removed from analysis and result in a decrease in the sample's call rate (proportion of loci for which a genotype call above the threshold was generated) (Oliphant *et al.* 2002; Fan *et al.* 2003). Therefore, call rates represent an overall indicator of the performance of a DNA sample at SNP typing, a metric that is known to be sensitive to the quality of the starting sample (Kennedy *et al.* 2003).

Although the variability of SNPs is much smaller than that of STRs, they are remarkably widespread across the genome, with about 40 million SNPs identified in humans (Dahdouh *et al.* 2015). Thanks to their properties, the study of SNPs makes it possible to track the inheritance of specific chromosome sections when the parental genotypes are known, allowing PGD by way of linkage analysis (Rabinowitz *et al.* 2011; Treff *et al.* 2011).

1.6.6 Whole genome amplification (WGA)

An embryo biopsy sample will routinely require amplification in order to generate a sufficient DNA content for array-based analysis like SNP typing (Harper & Harton 2010). The most commonly used method for providing a sufficient DNA sample for analysis is whole gnome amplification (WGA) (Macaulay & Voet 2014). During WGA, the DNA is amplified by the use of specialised polymerase enzymes that can efficiently reproduce the DNA strands while operating at a constant temperature. Several commercial kits are on the market including GenomePlex (Treff *et al.* 2011), REPLI-g (Le Bourhis *et al.* 2011), or GenomiPhi V2 (Fisher *et al.* 2012). These kits have the ability to produce approximately 5 µg of DNA from a biopsied sample, representing at least a 40,000-fold increase in the initial DNA amount (Ponsart *et al.* 2014).

However, genotyping errors can arise when less than 30 cells are used for WGA for example due to some alleles escaping amplification (Fisher *et al.* 2012; Lauri *et al.* 2013).

Therefore, WGA can be a cause of misdiagnosis and strategies to reduce the impact of these ADO events should be emploied (Le Bourhis *et al.* 2011).

1.6.7 Karyomapping

Karyomapping is a recently established PGD methodology which can track the inheritance pattern of chromosomes through the recapitulation of haploblocks, where haploblocks are defined as sub-chromosome sections of DNA that are inherited together (Handyside *et al.* 2010). To achieve that, karyomapping takes the SNP fingerprint of a sample (most often an embryo) and compares it to the SNP fingerprint acquired from its parents and a sibling of known disease status identified as the reference. SNPs that appear homozygous in one parent and heterozygous in the other parent can positively identify the parental origin of a haploblock and are therefore called “informative” or “key” SNPs (Handyside *et al.* 2010). The genome of the sample and that of the reference are compared at each key SNP locus to establish whether the sample inherited the same or a different haploblock as compared to the reference as well as the parent of origin for that haploblock (Handyside *et al.* 2010). Because the reference is of known disease status, tracking haploblock inheritance will provide information on the disease status of the sample too, by exploiting the principle of genetic linkage (Handyside 2015).

Karyomapping can be applied for the detection of a vast number of abnormalities, for example single gene disorders, meiotic trisomy, monosomy, triploidy, parthenogenetic activation and UPD (Handyside *et al.* 2010; Natesan *et al.* 2014a; Natesan *et al.* 2014b). On analysis, monosomies and deletions appear as absence of haploblocks from one parent. Conversely, meiotic trisomies are visualised as a complex pattern involving frequent shifting between haploblocks. Furthermore, karyomapping can discriminate between error arisen during meiosis I or meiosis II depending on whether or not the centromere is involved in a double haplotype event (Handyside *et al.* 2010). An example of a clinical application of karyomapping is given in figure 1.21.

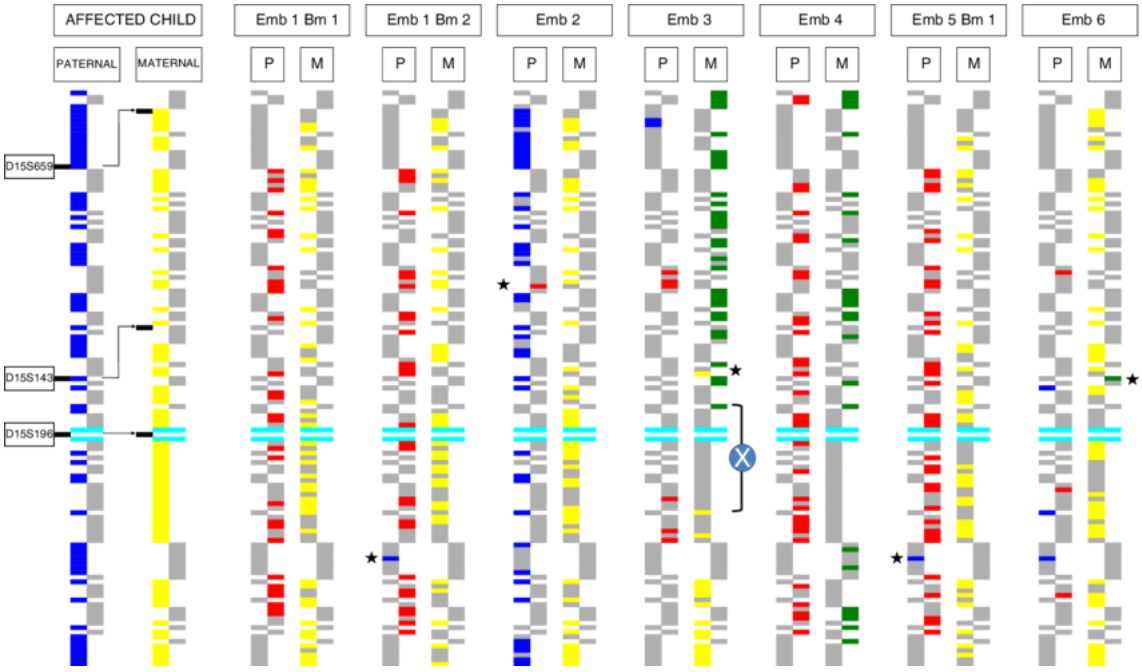


Fig 1.21 – Application of karyomapping in a case of preimplantation genetic diagnosis (PGD) in human embryos. The genotype of an affected sibling (reference) at specific single nucleotide polymorphism (SNP) loci was compared to 7 embryo biopsies. Two pairs of columns are given for each chromosome examined. P: paternal chromosome, M: maternal chromosome. Blue: paternal informative SNP concordant with reference; Red: paternal informative SNP different from reference; Yellow: maternal informative SNP shared with reference; Green: maternal informative SNP different from reference; Grey: absence of information. In embryos 3 and 6, the paternal chromosome is missing (paternal monosomy). (Image from Thornhill *et al.* 2015)

One of the key advantages of karyomapping is that it only relies on heterozygous genotype calls to establish haploblock inheritance, and this greatly reduces the chances of misdiagnosis associated with ADO (Griffin & Gould 2017). Nonetheless, in spite of the advantages of this method, karyomapping is also faced with several limitations. Firstly, the need to have a reference available, such as a sibling of known disease status, reduces the applicability of karyomapping to disorders that severely limit lifespan. Moreover, crossing over events that occur in the same or a similar region between the individual and the reference (juxtaposing crossovers) might lead to difficult data interpretation. Finally, karyomapping will not detect *de novo* mutations or traits that are not inherited by a Mendelian pattern (Rechitsky *et al.* 2015). Importantly, karyomapping is also unable to detect post-zygotic trisomies because these abnormalities do not cause any change in the haploblock inheritance pattern (Griffin & Gould 2017).

1.6.7.1 Future applications for karyomapping

In the future, karyomapping could be combined with methods for the measurement of allele intensity to allow for the detection of post-zygotic trisomies (Giménez *et al.* 2015), thereby overcoming one of its key limitations. Haplarithmisis is an extension of the karyomapping algorithm that takes into account allele frequency (Esteki *et al.* 2015). This method tracks the frequency of heterozygous calls across the genome to reduce the artefacts created by the process of WGA and detect the presence of numerical abnormalities as well as to distinguish between meiotic and post-zygotic errors (Zamani Esteki *et al.* 2015).

Whilst karyomapping has found use in clinical settings, its application to farm animal breeding is completely unreported in the literature. In the context of IVP, the availability of sibling embryos would simplify the application of karyomapping because a reference would often be readily available. Moreover, karyomapping could be used as a PGS method to select for the most chromosomally normal embryos for transfer.

1.7 Biopsy methods

Embryo biopsy is essential to produce a DNA sample for PGD and PGS. Biopsy can be operated at three levels: by recovering polar bodies (Verlinsky *et al.* 1990); by biopsying blastomeres from cleavage stage embryos (Handyside *et al.* 2004) or by removing a fraction of the TE from a blastocyst (Hahn *et al.* 2000; Bick *et al.* 2006). These approaches are demonstrated in figure 1.22.

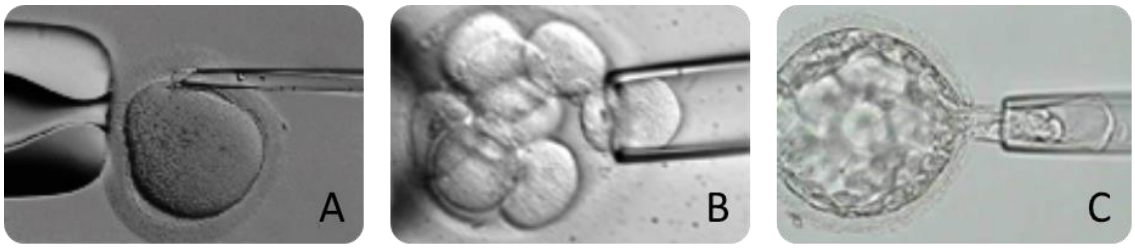


Fig 1.22 – Biopsy stages. A) Polar body biopsy. B) Cleavage stage biopsy, a single blastomere is being removed from an 8-cell stage embryo. C) Trophectoderm biopsy, a group of cells are removed from a blastocyst. (Image sources: A: www.cambridge.org; B: www.mitosis.gr; C: nordicalagos.org).

Polar bodies are extruded from the maturing oocyte as a byproduct of meiosis and contain genetic material that will not be required to form the female gamete. Because of this, polar body biopsy can be used to gather information on the genomic status of the oocyte without affecting embryo development (Verlinsky *et al.* 1990). Critically, however, this technique does not allow for the detection of aneuploidies of paternal origin nor of post-mitotic aneuploidies, and involves the manipulation of an early stage embryo which might not continue development causing a waste of resources. However, because a polar body biopsy can be obtained as soon as possible during development, this approach is well suited for the transfer of fresh embryos because it allows sufficient time to establish a diagnosis while the embryo develops (Geraedts *et al.* 2009).

Cells can be removed from cleavage stage embryos that have reached the 8-cell stage without significantly affecting its developmental competence (Hardy *et al.* 1989; Magli *et al.* 2004). With this method, a more comprehensive diagnosis on the genomic status of the embryo is achievable. However, because only one or two cells can be removed at this time, cleavage stage biopsy is thought to be more susceptible to ADO issues than biopsy at the blastocyst stage (Kokkali *et al.* 2007). Additionally, the sampling of such a limited subset of blastomeres carries the risk of misdiagnosis due to the presence of mosaicism, although the risk is mitigated when two blastomeres are removed instead of one (Kuo *et al.* 1998).

A final approach is to perform a biopsy at the blastocyst stage by removing a small portion of the TE. Because TE will not form part of the foetus, this approach is thought to be minimally invasive (McArthur 2014). Moreover, biopsy at the blastocyst stage allows for a greater number of cells to be sampled, reducing the issues associated with ADO and mosaicism and allowing better amplification by WGA (Kokkali *et al.* 2007; Harton *et al.* 2010b; Capalbo *et al.* 2013). Additionally, it has been found that the TE is a good indicator of the ploidy status of the ICM, with a concordance greater than 95% (Johnson *et al.* 2010; Cater *et al.* 2012; Capalbo *et al.* 2013). Interestingly, because of the existence of repair mechanisms, aneuploidy that affects cleavage stage embryos might not be present in blastocysts, suggesting that TE biopsy might provide for a better

indication of the future phenotype of the offspring (Capalbo *et al.* 2013). However, embryos biopsied at the blastocyst stage will often require to be cryopreserved while a diagnosis is established.

1.8 Genomic selection

Traditionally, breeding programmes aimed at improving the genetic merit of farm animals have been based on progeny testing (Henderson 1984), meaning that the desirability of an animal as a parent was judged not on its phenotype, but on the phenotype of its offspring, the assumption being that the performance of the offspring is a better representation of that specific animal's genotype (Robertson & Rendel 1950). Whilst progeny testing was effective and widely adopted, its key disadvantage was the long time required to judge the performance of the offspring; for example, a 5-year long observation period was common to fully evaluate a bull (Weller *et al.* 2017).

An alternative approach was described in the 1980s and postulated the selection of breeders based on DNA markers (Weller 2009). Many traits of commercial interest, such as milk yield or ease of parturition, are in fact polygenic, meaning that phenotypes reflect the joint action of several DNA segments, known as quantitative trait loci (QTL) (Georges *et al.* 1995). Early efforts in this field attempted to establish an association between QTLs and small subsets of known genetic markers (Geldermann *et al.* 1985; Cowan *et al.* 1990; Hoeschele & Meinert 1990; Bovenhuis 1992; Schutz *et al.* 1993), without necessarily needing to elucidate the nature of the genes underlying the variation in the traits of interest. In this context, the discovery of STRs was pivotal since it allowed to perform genotyping by PCR and expanded the availability of markers for association studies (Weber & May 1989).

More recently, though, the availability of a complete sequence for the genome of cattle (Elsik *et al.* 2009) and pigs (Groenen *et al.* 2012) has allowed the discovery of thousands of SNPs which can be used as evenly spaced markers across the genome for QTL association studies. The individuals used to discover associations are collectively known as the reference population. In order to obtain truly accurate predictions, association

studies must be completed on very large reference populations (Lund *et al.* 2011). However, after the association is established, SNP analysis can be used for the production of genomic estimated breeding values (GEBV) and for the selection of livestock based on genomic data alone, a process termed "genomic selection" (Meuwissen *et al.* 2001). The accuracy of predictions based on GEBV has been shown to be as high as 85%, (Meuwissen *et al.* 2001), comparing well with, or even exceeding the precision obtained with traditional methods (Hayes 2009).

Low-density chips for the simultaneous analysis of 5,000 SNPs in livestock were the first to become commercially available and are still in use today (Pryce *et al.* 2014a). However, the availability of high-density chips for the assessment of over 50,000 SNPs per sample is now widespread for both pigs and cattle (https://emea.illumina.com/products; accessed 04.09.2017).

In comparison to traditional breeding schemes, genomic selection allows an improved ability to select for poorly heritable traits, the evaluation of a higher number of candidate animals, and shorter generational intervals (Yudin *et al.* 2016). As a result, genomic selection is being adopted rapidly in the cattle breeding industry, with up to 50% of reproduction bulls sold across different countries now being evaluated solely on GEBVs (Pryce & Daetwyler 2012). Genomic selection has the potential to significantly increase rates of genetic gain (Schaeffer 2006), and a recent report confirmed that the adoption of genomic selection in Holstein cattle in the USA substantially decreased generational intervals, while rapidly increasing fertility, lifespan, and health (Garcia-Ruiz *et al.* 2016).

Remarkably, the methodologies of genomic selection can be applied to IVP embryos in order to establish which ones carry the most desirable traits and should be given preference for embryo transfer. The ability to combine IVP and genomic selection has the potential to magnify the advantages already identified for both techniques in terms of genetic gain, shortening of generational intervals and selection intensity (Humbolt *et al.* 2010; Ponsart *et al.* 2014).

1.9 Rationale for this thesis

IVP is currently a hot topic in reproductive medicine because the benefits it offers in terms of selection and distribution of superior genetics are thought to be a possible answer to the growing demand for highly efficient animal production systems. Therefore, the breeding industry would benefit from the availability of optimised methods for the production and transfer of large numbers of developmentally competent, high genetic merit embryos. At the same time, IVP embryos represent valuable models for the study of infertility and early embryonic development and the ready availability of twins would allow the design of specific biological studies. A successful IVP system is formed of several components: culture of developmentally competent oocytes, culture of embryos, and selection of the best possible embryos for transfer. The intent of the present work was to propose an optimised IVP protocol by pursuing improvements on each of these areas.

As described under section 1.2.2.2, cumulus cells play a pivotal role in the maturation of the oocyte and the morphology of the COC has long been used to select for the most competent oocytes for IVM. However, the current screening criteria might be wasteful, due to the general recommendation to discard oocytes with a reduced but not fully depleted cumulus in the interest of maximising developmental rates but at the cost of not achieving a maximal embryo yield. A comprehensive investigation of the actual developmental potentials of oocytes with a reduced cumulus investment could lead to an improved availability of IVP embryos derived from specific donors.

As mentioned under section 1.4.2.4, the ability to multiply the offspring of superior parents would increase selection intensity. This could be achieved by the application of reproductive cloning methods such as embryo splitting which would further increase such availability. Moreover, the ready availability of twins would allow for the design of stringent case-control studies for research purposes, and would be useful for the investigation of early mammalian development. However, a comprehensive comparison between different embryo splitting strategies has not been described in the literature

and indications on the suitability for splitting of embryos displaying asymmetry is largely underreported.

Morphological screening has proven to be an invaluable tool for embryologists to achieve high pregnancy rates after the transfer of the most promising embryos (refer to section 1.3.1). However, this assessment is often hampered by issues of subjectivity whilst the peculiar morphology of ungulate embryos makes the screening more challenging. Novel optical methods, like the combination of OCT and speckle variance analysis (see section 1.3.4.1), could be applied for the non-invasive investigation of the developmental competence of ungulate embryos for both research and commercial purposes. If successful, these methods would allow obtaining estimates of embryo viability in a rapid, non-invasive and objective way.

Finally, the manipulation of embryos *in vitro* paves the way to the application of PGS and genomic selection, which are expected to speed up dramatically the rate of genetic gain in livestock, as described under section 1.8. Novel tools for PGS, like karyomapping, have recently been developed for use in a clinical setting (refer to section 1.6.7). Their application to animal IVP could assist practitioners in selecting the most chromosomally normal embryos for transfer while SNP information can be used simultaneously for genomic selection. At the same time, the data generated from the application of these methodologies would provide insights on the process of meiosis in model organisms such as pigs and cattle.

1.10 Specific aims

With reference to what discussed above, the specific aims of this thesis were:

Specific Aim 1: To test the hypothesis that gilt oocytes with a depleted cumulus display similar developmental competence to oocytes clad by three or more layers of cumulus cells.

Specific Aim 2: To test a variety of embryo splitting strategies in the bovine model in order to identify the optimal methodology for the production of a high number of viable blastocysts.

Specific Aim 3: To apply optical coherence tomography (OCT) to investigate embryo structure and viability non-invasively.

Specific Aim 4: To demonstrate the application of karyomapping to blastocyst stage bovine embryos and characterise the type, level, and origin of chromosomal aberrations and rearrangements.

2. Materials and Methods

2.1 Materials

For the assessment of the developmental potential of porcine oocytes (specific aim 1), pig ovaries from unsynchronised gilts were sourced weekly from an abattoir (Dunbia, Mansfield, UK) and transported to the University of Kent in phosphate buffered saline (PBS) at 38 °C within 6 to 8 h from culling. Moreover, extended boar semen from sires of mixed breeds and proven fertility history was acquired from a commercial supplier (JSR Genetics, Southburn, UK) and shipped at RT in the post, then preserved at 17 °C for up to two days before use.

In order to produce the bovine embryos required for investigation (specific aims 2, 3 and 4), ovaries from cows of mixed breed and age were sourced weekly from a local abattoir (Charing Meats, Charing, UK). Additionally, frozen bull semen straws from several different Holstein sires were acquired from a commercial supplier (Semex, Monkton, UK) and stored under liquid nitrogen until use.

In order to investigate the viability of bovine embryos via OCT (specific aim 3), day 7 post-IVF bovine embryos were assessed through a SS-OCT experimental microscope assembled by Dr Ramona Cernat (School of Physical Sciences, University of Kent, Canterbury, UK). Furthermore, motion maps of the embryos were produced by applying a speckle variance algorithm developed by Dr Ramona Cernat and Miss Sophie Caujolle (School of Physical Sciences, University of Kent, Canterbury, UK).

For the production of haplotype maps from bovine embryos via karyomapping (specific aim 4), biopsies from bovine blastocysts 7 to 8 days post-IVF were either obtained from in-house produced embryos or supplied by Paragon Veterinary Group (Carlisle, UK). The in-house biopsies were performed by Dr Kara J. Turner (School of Human and Life Sciences, Canterbury Christ Church University, Canterbury, UK) using a standard laser-assisted method. The biopsies received from Paragon Veterinary Group were produced

by either a standard laser-assisted or a standard blade-assisted method by a single operator and were shipped on dry ice. In all cases, the biopsies contained an estimated 5 to 12 TE cells, were stored in 4 µl of PBS, and were preserved at -80 °C until required for WGA. Additionally, a DNA sample from each parent of the biopsied embryo was isolated from either blood, sperm or ovarian tissue as appropriate. For in house biopsied embryos, ovarian tissue and sperm samples were available from the regular sources detailed before (abattoir and commercial semen supplier). Blood samples and additional sperm samples were supplied by Paragon Veterinary Group (Carlisle, UK), were shipped to the University of Kent in the post on dry ice, and were preserved at -20 C°. Moreover, blood samples from calves born following the transfer of karyomapped embryos were supplied by Paragon Veterinary Group (Carlisle, UK) following the same way. The software used to create the karyomaps was BoVision (version 3), which was kindly provided by Prof Alan Handyside (Illumina Cambridge Ltd, Capital Park CPC4, Cambridge, UK).

Reagents and culture medium components were normally sourced from Sigma-Aldrich, (Gillingham, UK) unless stated otherwise. Additionally, FBS was only sourced from known Creutzfeldt-Jakob disease free territories and was supplied by Gibco (Grand Island, NY). A detailed summary of the culture media employed and of their specific methods of preparation is given under Appendix I.

2.2 Methods

2.2.1 Assessment of the developmental potentials of porcine oocytes

To investigate the developmental competence of porcine oocytes in relation to their cumulus investment, a number of functional tests were implemented which are described below.

2.2.1.1 Porcine COC retrieval, selection and IVM

Ovaries from unsynchronised gilts were sourced from an abattoir and transported to the University of Kent by a same day courier in PBS flasks at 38 °C within 6 to 8 h from culling. Follicles ranging between 3 to 8 mm were manually aspirated using a 5 ml syringe and a gauge 19 needle, which were primed with a small volume of Tyrode's Lactate (TL) – HEPES – poly vinyl alcohol (PVA) medium (Funahashi *et al.* 1997). Using a dissecting microscope, the COCs were collected, washed twice in TL-HEPES-PVA medium and divided in four morphological grades A - D according to the number and appearance of their cumulus cell layers: grade A had three or more cumulus layers, grade B had two intact cumulus layers, grade C had a single cumulus layer either complete or incomplete, and finally grade D were denuded oocyte (see fig. 2.1). The graded oocytes were *in vitro* matured in separate wells for 44 h in groups of 50 in 500 µl of NCSU-23 medium (Petters & Wells 1993) under 6% CO2 at 37 °C. During the first 22 h, the culture medium was supplemented with 10% porcine follicular fluid (pFF), 1:100 PG600, 0.8 mM L-cysteine, 10 ng/ml epithelial growth factor (EGF), 1 mM dibutyryl cyclic-adenosine monophosphate (db-cAMP) and 50 µM β-mercaptoethanol. The same supplements excluding PG600 and db-cAMP were used for the remaining 22 h.

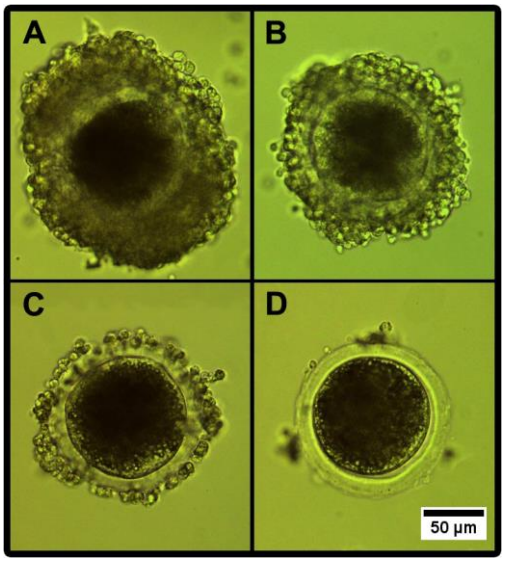


Fig. 2.1 – Grading of porcine cumulus oocyte complexes (COCs). Grades were assigned based on the extension of the cumulus complex. A) Three or more intact cumulus cell layers; B) two intact cumulus cell layers; C) one incomplete cumulus layer; D) denuded oocyte. Images captured with a Hoffman inverted microscope at x200 total magnification.

2.2.1.2 Porcine oocyte meiotic spindle immunostaining with anti α-tubulin

In order to visualize the meiotic spindle following IVM, oocytes were denuded by incubation for 10 min with 0.2% w/v hyaluronidase type VIII from bovine testis and passing them several times through a 125 µm wide tip (EZ-Tip, RI, Falmouth, UK), then fixed overnight at 4 °C in 4% paraformaldehyde (PFA) in PHEM buffer (60 mM 1,4-Piperazinediethanesulfonic acid (PIPES), 25 mM HEPES, 10 mM Ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 4 mM MgSO4, pH 7.0). The fixed oocytes were rinsed for 5 min in PBS three times and then permeabilized for 10 min in 1% Triton X-100 in PHEM buffer. After a further brief rinse in PBS, the oocytes were blocked in 20% FBS in PHEM buffer for 1 h at RT. The meiotic spindle was then stained with anti-α tubulin-Alexa 488 conjugated antibodies (ab195887, Abcam, Cambridge, UK) diluted 1:200 in 5% FBS in PHEM buffer. After a single rinse in PBS, the oocytes were counterstained in 0.05 mg/ml Hoechst 33342 in PBS and mounted on slides with the permanent anti-fade mounting medium Fluoroshield. The samples were then observed under epifluorescence microscopy using a BX60 Olympus microscope equipped with standard 4',6-diamidino-2-phenylindole (DAPI) and Fluorescein isothiocyanate (FITC) filters at a total magnification of x200, and their meiotic stage was classified as described by Ma *et al.* (2003).

2.2.1.3 CG staining with PNA-lectin in pig oocytes

After IVM, the oocytes were denuded as described in section 2.2.1.2, and then fixed in 4% PFA in PBS overnight at 4 °C. The CGs were then stained using peanut agglutinin (PNA). The staining was generally performed as previously described (Zhang *et al.* 2010a) with some modifications. Briefly, the fixed oocytes were rinsed three times in 0.3% BSA in PBS for 5 min and then permeabilised by immersion in 0.1% Triton X-100 in PBS for 5 min. After two washes in PBS, the oocytes were stained in 100 µg/mL PNA lectin-Alexa 488 (L21409, Life Technologies, Paisley, UK) in PBS. After three more 5 min washes in 0.3% BSA, 0.01% Triton X-100 in PBS, the oocytes were counterstained with 0.05 mg/ml Hoechst 33342, mounted, and observed as described in section 2.2.1.2. The oocytes were defined cytoplasmically mature if showing a clear, continuous ring of CGs close to their membrane instead of a dispersed pattern throughout the ooplasm.

2.2.1.4 Measurement of porcine oocyte GSH content

The oocytes were denuded as described in section 2.2.1.2, then washed 3 times in PBS to eliminate any possible thiol carryover from the culture media (mainly in the form of β-mercaptoethanol and L-cysteine). The GSH measurement was completed by measuring the speed of an Ellman's reaction (see figure 2.2) using a previously established methodology (Funahashi *et al.* 1994). Briefly, the oocytes were transferred to a microcentrifuge tube in groups of 30 in 5 µl of PBS, then mixed with 5 µl of 1.25 M orto-phosphoric acid, homogenised by vortexing for 30 sec and stored at -20°C until assessment. On thawing, each sample was supplemented with 700 µl of stock buffer (0.33 mg/ml NADPH, 10 mM EDTA, 0.2 M Na-phosphate, pH 7.2), 100 µl of 6 mM 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) in stock buffer, and 190 µl of water to give a final reaction volume of 1 ml. Finally, immediately before analysis, 0.25 U GSH reductase was mixed into the solution to initiate the reaction. A Biomate 3S spectrophotometer (ThermoScientific) was set for continuous reading at 412 nm and was used take Absorbance measurements every 20 sec for 2 min. Additionally, calibration curves were produced by using several GSH standards (1 nmol, 0.1 nmol, and 0.01 nmol GSH dissolved in 10 µl of PBS).

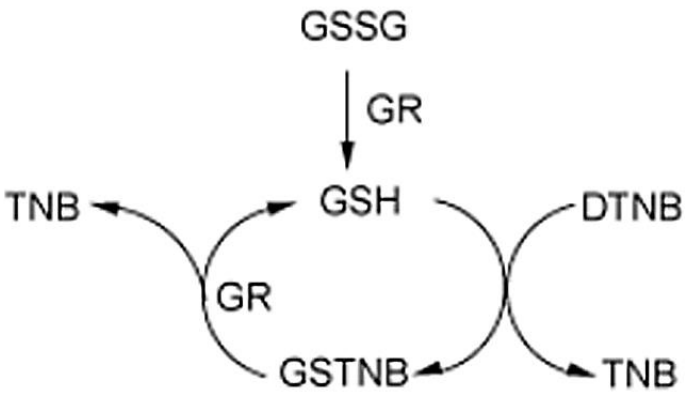


Fig. 2.2 – Principle of the Ellman's reaction. Glutathione disulphide (GSSG) is reduced by the enzyme glutathione reductase (GR) to glutathione (GSH). The Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reacts with GSH to form a new disulphide bond (GSTNB), releasing 2-nitro-5-thiobenzoate (TNB). Then, the GR reduces GSTNB releasing a second TNB molecule, thus allowing GSH to re-enter the cycle. The accumulation of TNB can be tracked by a spectrophotometer set at 412 nm. Assuming the reagents are provided in excess, the reaction rate is only limited by the starting concentration of GSH. (Image adapted from https://www.funakoshi.co.jp/data/datasheet/NWS/NWK-GSH01.pdf).

2.2.1.5 Boar sperm preparation by swim-up

In preparation for IVF, 1 ml of extended boar semen (JSR genetics, Southburn, UK) was diluted with 5 ml of sperm wash medium (154 Mm NaCl, 2 mg/ml BSA, antibiotics (Pen/Strep), pH 7.4) and spun at 475 g for 5 min. The supernatant was then discarded and the wash step was repeated a second time. The pellet was then resuspended in 5 ml of pre-equilibrated modified TRIS (mTRIS) buffered medium (Abeydeera & Day 1997) and spun as before. The supernatant was once again removed and the washed sperm pellet was resuspended in about 500 µl of remaining medium. At this point, sperm motility was confirmed by standard microscopy and only samples showing a total motile fraction of at least 50% were processed further. For the sperm selection by swim-up, 4 cell culture tubes were filled with 2.4 ml of mTRIS medium and allowed to equilibrate at 37 °C and under 6% CO_2 for at least 30 min. When ready, 100 µl of the sperm suspension was layered at the very bottom of each culture tube and incubated for 20 min at 37 °C and under 6% CO_2. After the required time, 2.1 ml of medium was removed from the top of each culture tube and pooled. The sperm suspension was then spun at 475 g for 7 min, the supernatant was removed and the pellet was resuspended in about 500 µl of the remaining medium and de-clumped by passing it several times through a gauge 21 needle attached to a standard 5 ml syringe. Finally, the concentration of the sperm

suspension was measured with a Makler chamber and the sperm was diluted to a final concentration of 10^4 motile cells/ml with mTRIS medium supplemented with 2 mM caffeine.

2.2.1.6 Porcine IVF and embryo culture

Shortly before the completion of the sperm selection by swim-up, the *in vitro* matured COCs (see section 2.2.1.1) were removed to 500 µl of mTRIS medium supplemented with 2 mM caffeine in groups of 50 and returned to an incubator set at 37 °C and 6% CO_2 pressure. When the sperm preparation was completed, the washed COCs were moved in groups of 50 to a well containing 500 µl of the sperm suspension to begin the IVF process. The gametes were then co-incubated at 37 °C and under 6% CO_2 for 4 h. After this time, the presumptive zygotes were removed to 500 µl of TL-HEPES-PVA medium at 37 °C and denuded by resolute pumping with a P200 Gilson pipette. The denuded presumptive zygotes were then washed once in pre-equilibrated NCSU-23 medium supplemented with 0.4% BSA and 0.8 mM L-cysteine, then cultured in 500 µl of this medium in groups of 50 at 37 °C and under 6% CO_2 until required. Cleavage rates were recorded by modulation contrast microscopy at x200 total magnification 72 h post-IVF.

2.2.1.7 Oocyte penetration rate and polyspermy assessment in the pig

The penetration rate and polyspermy assessments were carried out 16 h post-IVF by detecting the presence and number of pronuclei whithin zygotes. Since the presence of sperm cells still bound to the ZP could interfere with the pronuclei observation, the putative zygotes were washed for up to 30 sec in Acid Tyrode's Medium (8 g/L NaCl, 0.2 g/L KCl, 0.24 g/L CaCl dihydrate, 0.1 g/L MgCl hexahydrate, 1 g/L glucose, 0.4% BSA, pH 2.5) and immediately rinsed twice in PBS. The samples were then fixed in 4% PFA in PBS, stained with Hoechst 33342, mounted, and observed as described in section 2.2.1.2. The penetration rate was calculated as the fraction of oocytes showing at least 2 pronuclei, while the polyspermy rate was calculated as the fraction of penetrated oocytes showing more than 2 pronuclei.

2.2.2 *In vitro* production of bovine embryos

The same *in vitro* production protocol was used consistently to produce all the bovine embryos required for the present work, and is described hereafter.

2.2.2.1 Oocyte recovery from bovine ovaries

Abattoir sourced bovine ovaries were transported to the University of Kent's premises in warm (36-38°C) PBS in a thermos flask within 3 h from culling. The ovaries were rinsed several times in clean PBS to reduce their contamination load then maintained at 38 °C in PBS until ready for oocyte recovery. Follicles ranging between 3 to 8 mm were manually aspirated using a 5 ml syringe equipped with a gauge 19 needle and the aspirate was distributed between several 15 ml conical tubes taking care not to pool more than 4 ml of fluid in each container. The aspirate was then allowed to settle by gravity for about 20 min whilst being kept at 38 °C. After the required time, the supernatant was carefully discarded with the use of a Gilson pipette and replaced with 3 ml of 10% FBS in PBS. The pellet was then gently resuspended, removed to a 35 mm culture dish and kept on a heated stage. COCs showing homogenous ooplasm and at least 2 compact layers of cumulus cells, were selected under 20x observation and removed with a Gilson P10 to a second dish containing 3 ml of pre-warmed HEPES modified tissue culture medium 199 (TCM-199) supplemented with 10% FBS, 0.2 mM pyruvate and antibiotics (Pen/Strep, Gibco, Grand Island, NY).

2.2.2.2 *In vitro* maturation of bovine oocytes

An appropriate number of 35 mm culture dishes was set up in advance of oocyte retrieval (IVM dishes). Each dish contained seven 90 µl drops of TCM-199 which had been supplemented immediately before use with 10% FBS, 10 IU/ml pregnant mare serum gonadotrophin (PMSG) and 5 IU/ml human chorionic gonadotrophin (hCG) (PG600, Intervet, Milton Keys, UK), 0.2 mM pyruvate, and antibiotics (Pen/Strep, Gibco, Grand Island, NY). The drops were fully covered with embryo grade mineral oil and the dish was allowed to pre-equilibrate at 38.5 °C and under 6.5% CO_2 in air.

After retrieval and selection, COCs were washed through two drops of the IVM dish, then removed to a third drop and matured for 18 to 22 h in groups of 20 to 30 per drop at 38.5 °C and under 6.5% CO_2 in air. After the required time, COCs showing a homogenous ooplasm and an expanded cumulus were selected for fertilisation, while oocytes clad by a compact and/or dark cumulus and those that became strongly adherent to the bottom of the dish were discarded.

2.2.2.3 Bovine *in vitro* fertilisation setup

The IVF medium used was glucose-free Tyrode's Albumin Lactate Pyruvate (TALP), which was supplemented immediately prior to use with antibiotics (Pen/Strep, Gibco, Grand Island, NY), 10 µg/ml heparin and 1:25 penicillamine, hypotaurine and epinephrine (PHE) solution prepared as described by Miller *et al.* (1994). Appropriate numbers of 35 mm culture dishes were prepared each containing seven 90 µl drops of glucose-free TALP medium covered by embryo grade mineral oil, and were allowed to pre-equilibrate at 38.5 °C and under 6.5% CO_2 in air for at least 20 min. After the appropriate time, selected COCs were washed three times in this medium and kept in groups of 20 to 30 per drop at 38.5 °C under 6.5% CO_2 in air in preparation for their co-incubation with the sperm.

2.2.2.4 Bull sperm preparation and IVF

Frozen/thawed bull spermatozoa were selected using the commercial discontinuous density gradient system BoviPure™ (Nidacon, Mölndal, Sweden) according to manufacturer's instructions. Briefly, a discontinuous density gradient was created in a microcentrifuge tube by overlaying 590 µl of an 80% BoviPure™ solution with an equal volume of a 40% BoviPure™ solution. A frozen sperm straw was transported to the laboratory in liquid nitrogen and thawed by immersion for 30 to 60 sec in sterile water at 38 °C. After thawing, the contents of the straw were immediately layered on the top of the BoviPure™ density gradient and the tube was spun at 900 G for 6 min to favour the accumulation of normal sperm cells at the bottom of the tube. The supernatant was then discarded and the sperm rich pellet was removed to 1 ml of BoviWash™ (Nidacon, Mölndal, Sweden) and spun at 900 G for 2 min. The majority of the supernatant was

discarded and the pellet was gently re-suspended in the remaining 100 to 200 µl of BoviWash™. The motility of the sample was confirmed by x200 observation under modulation contrast microscopy and only samples with high (>70%) progressive motility were further employed. The sperm concentration was then estimated with the aid of an improved Neubauer chamber and an appropriate volume of the sperm preparation (usually between 2 to 4 µl) was added directly to the TALP drop containing the matured oocytes to give a final concentration of 10^5 motile sperm cells/ml. Finally, the gametes were co-cultured at 38.5 °C under 6.5% CO_2 in air for 18-22 h.

2.2.2.5 Bovine embryo culture

After IVF, the putative zygotes were removed to a 35 mm culture dish containing 3 ml of HEPES modified, glucose-free TALP medium at 38 °C and mechanically denuded with the assistance of a 125 µm wide tip (EZ-Tip, RI, Falmouth, UK). At this stage, the putative zygotes showing uneven cytoplasm or sign of apoptosis were discarded.

The medium used for embryo culture was synthetic ovarian fluid amino acids citrate inositol (SOFaaci) (Holm *et al.* 1999) supplemented immediately before use with 5% FBS, 5 mg/ml BSA and antibiotics (Pen/Strep, Gibco, Grand Island, NY). Seven 90 µl drops of SOFaaci were set up in each embryo culture dish, were covered in embryo grade mineral oil and were allowed to pre-equilibrate at 38.5 °C and under 6.5% CO_2 and 5% O_2 for at least 20 min.

The selected putative zygotes were washed three times in the SOFaaci drops, and then cultured in this medium in groups of 20 to 30 at 38.5 °C and under 6.5% CO_2 and 5% O_2 for as long as required. The culture medium was partially replaced after 48 h and again after 92 h of culture.

2.2.3 Embryo splitting

Depending on the stage of the embryo and the type of experiment performed (single or serial splitting) a variety of protocols were implemented. These are discussed below. Moreover, the strategies adopted are summarised under figure 2.3.

2.2.3.1 Pronase E stock preparation

Pronase E, also known as Protease from *Streptomyces griseus*, is a protease cocktail that has long been known to be able to digest the ZP without disrupting cell membranes (Mintz 1962). Lyophilised Pronase E was reconstituted in sterile PBS to a concentration of 2.5 mg/ml (equal to 0.25% w/v), then activated by heating at 56 °C for 15 min followed by cooling at 37 °C for 60 min. The cooled enzyme was filtered through a 0.45 µm membrane to remove any unwanted residues and tested on unfertilised eggs. If the enzyme preparation was able to thin visibly an oocyte's ZP under x20 observation within the first 5 min of exposure at 38 °C, the enzyme passed the quality control test and working aliquots of the 0.25% solution were stored at -20 °C until required.

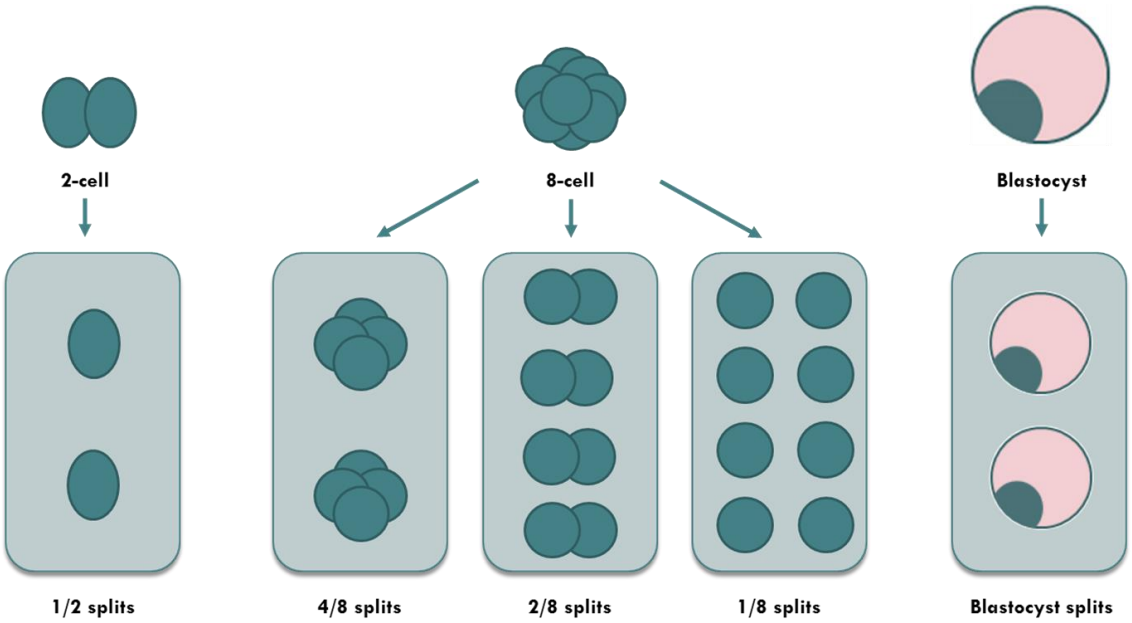


Fig. 2.3 – Embryo splitting strategies applied in this study. Several alternative protocols are shown. A 2-cell stage embryo is disaggregated into two 1/2 type splits. Alternatively, an 8-cell stage embryo can be split in several symmetric ways leading to the formation of either two 4/8 type splits, four 2/8 type splits or eight 1/8 type splits or even to a combination of these. Finally, blastocysts can be bisected leading to the formation of two blastocyst splits.

2.2.3.2 Blastomere separation

Cleavage stage embryos were disaggregated either 31 h or 72 h post IVF, when most embryos could be expected to be at the 2-cell stage or at the 8-cell stage, respectively. Depending on the experimental design, cleavage stage embryos found to possess asymmetrical cell numbers (between 7 to 14) at 70 h post-IVF, were processed following this same protocol and marked as "asymmetrical". All manipulations were carried out on a heated stage at 38 °C with the assistance of a 125 µm wide tip (EZ-Tip, RI, Falmouth, UK). The cleavage stage embryos were briefly rinsed in PBS to remove most Ca^{2+} and Mg^{2+} carryover from the culture medium. The embryos' ZP was digested by exposure for up to 7 min to 0.25% w/v pronase E in PBS. The denuded embryos were then immediately washed two more times in PBS to remove the excess enzyme and if necessary allowed to disaggregate fully by gentle agitation. Finally, the disaggregated blastomeres from each embryo were washed once in 10% FBS in PBS and once in pre-equilibrated SOFaaci. Then the blastomeres were cultured either alone or in groups as required by the experimental setup in commercial culture dishes designed to allow for up to 16 samples to be cultured under a WOW system (Primo Vision 16-well culture dish, Vitrolife, Göteborg, Sweden) in 90 µl drops of pre-equilibrated SOFaaci at 38.5 °C and under 6.5% CO_2 and 5% O_2. As illustrated in figure 2.3, the splitting of an 8-cell stage embryo allows for the pooling of separated blastomeres in groups of different sizes. The resulting embryos were named after the number of blastomeres received from the parent embryo and its original cell count. For example, the nomenclature 3/7 would indicate an embryo resulting from the grouping of 3 blastomeres harvested from an original embryo containing 7 cells (and therefore marked as asymmetrical).

2.2.3.3 Blastocyst bisectioning

Only grade I blastocysts (as defined by Nagashima *et al.* 1989) obtained 6 days post-IVF were used for this experiment. Firstly, embryos were rinsed once in PBS, and then positioned in a 50 µl drop of PBS under embryo grade mineral oil. A sterile, disposable P-730 ophthalmic scalpel with a 30° blade (Feather, Osaka, Japan) was directly assembled on an Integra TI micromanipulator platform (RI, Falmouth, UK). The P-730 blade was used to divide the embryo in two halves using a cutting technique previously

described (Rho *et al.* 1998). Briefly, the blastocysts were positioned so that the blade would divide their ICM in two even parts, then the embryos were bisected by gently lowering the surgical blade while slowly moving it sideways. An example of the cutting procedure is given in figure 2.4. After complete separation of the two halves, the PBS drop containing the splits was supplemented with 10% FBS to allow for the embryos to detach from the dish and improve their handling. The splits were then washed once in 10% FBS in PBS and once in pre-equilibrated SOFaaci. The splits were then cultured for a further 24 h in SOFaaci in single drops at 38.5 °C and under 6.5% CO_2 and 5% O_2

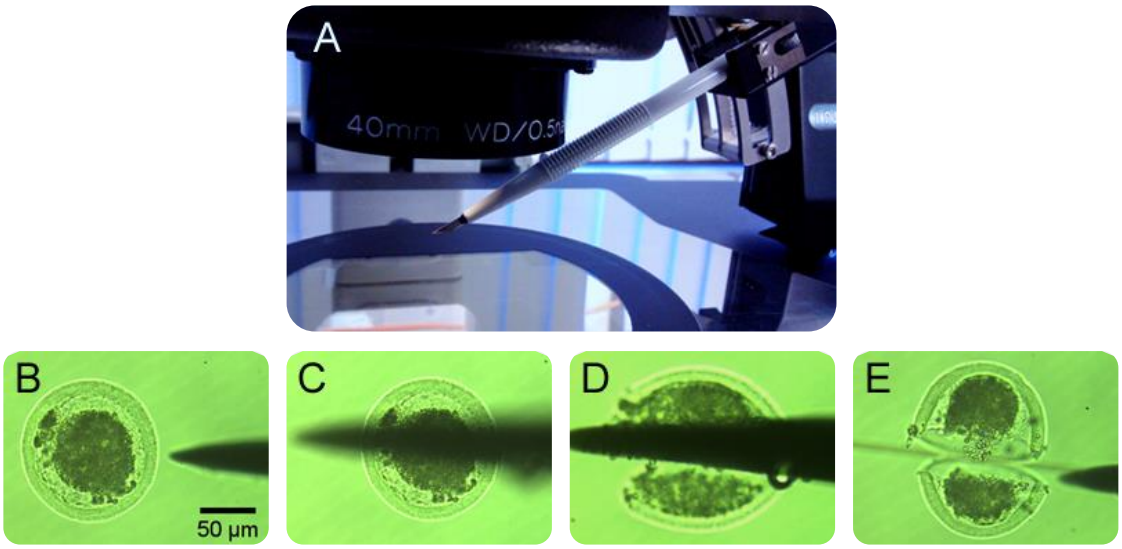


Fig. 2.4 – Bisectioning setup and demonstration. A) Assembly of a disposable P-730 microsurgical blade on an Integra TI micromanipulation rig. B-E) The image sequence (from left to right) demonstrates a blade bisectioning test performed on an arrested oocyte, which resulted in a neat cut. The microscopical images were captured with a Hoffman inverted microscope at x200 total magnification.

2.2.3.4 Serial splitting: blastomere separation and bisectioning (strategy A)

In this test, embryos were split twice in succession applying different methods as appropriate for their stage. Eight-cell stage embryos were disaggregated 70 h post IVF following the procedure described above. Blastomeres were then cultured in groups of two in 12.5 µl drops of pre-equilibrated SOFaaci at 38.5 °C and under 6.5% CO_2 and 5% O_2 for a further 70 h. After this time, the splits that had progressed to form a blastocyst were split a second time following the bisectioning protocol described above and were

then cultured for a further 24h in SOFaaci. A synopsis of this splitting approach is presented in figure 2.5.

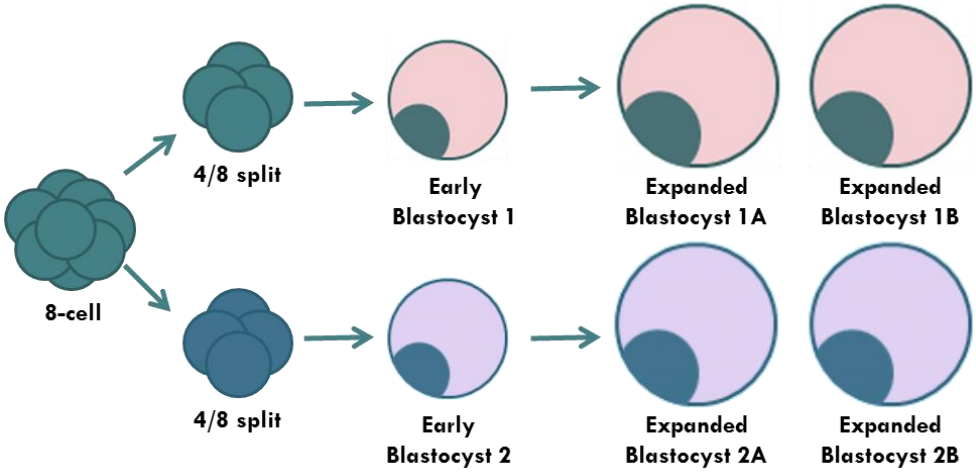


Fig. 2.5 – Schematic of serial embryo splitting by disaggregation and bisectioning. A potential way of performing serial splitting could be to bisect blastocysts derived from a previous blastomere separation experiment. In the diagram, an 8-cell stage embryo is divided in two 4/8 splits by blastomere separation. The resulting blastocysts are then bisected to produce up to four twins.

2.2.3.5 Serial splitting: serial disaggregation (Strategies B and C)

In this experiment, two-cell stage embryos were split for the first time 30 h post-fertilisation following the blastomere disaggregation protocol discussed above. These splits were cultured for 24 h in 12.5 µl drops of pre-equilibrated SOFaaci at 38.5 °C and under 6.5% CO_2 and 5% O_2. After this time, embryos showing cleavage were disaggregated again following the same protocol but omitting the pronase E step. In all cases, the blastomeres were segregated in two equal groups and cultured as before resulting in the production of second serial splits, which were then cultured in SOFaaci until day 7 post IVF (strategy B). In an alternative protocol (strategy C), second serial splits that showed cleavage after a further 24 h of culture were disaggregated a third time and the blastomeres from each embryo divided in two equal groups to form third serial splits which were cultured in SOFaaci until day 7 post IVF.

2.2.4 CDX-2 immunostaining

To obtain separate cell counts for the TE and ICM cell populations of some of the blastocysts in this study, a protocol for the immunostaining of the transcription factor CDX-2 was implemented.

2.2.4.1 Selection of suitable antibodies

A candidate anti-CDX-2 antibody for the immunostaining of bovine blastocysts was identified in the AB88129 (Abcam, Cambridge, UK) - a rabbit polyclonal antibody raised against a portion of the human CDX-2 protein. A preliminary confirmation of the suitability of this antibody was obtained by aligning the full amino acid sequence of the immunogen peptide used to raise this antibody against the known sequence of the bovine CDX-2 protein (Genbank accession number: NP_001193228) by using Clustal Omega (version 1.2.2, Sievers *et al.* 2011). The secondary antibody selected was AB6719 (Abcam, Cambridge, UK) - a standard polyclonal goat anti-rabbit immunoglobulin G conjugated to the fluorophore Texas Red (TR).

2.2.4.2 CDX-2 immunostaining protocol

Blastocysts from day 7 post-fertilisation were fixed in 4% PFA in PBS for 40 min at 4 °C under oil and were then washed three times for 5 min in 0.1% Triton X-100 in PBS. The permeabilization step was completed by immersion in a 1% solution of Triton X-100 in PBS for 10 min. To minimise aspecific anybody binding, the samples were blocked in 10% normal goat serum, 0.1% Tween 20 in PBS for 1 h at RT. After this step, the samples were incubated with a 1:400 dilution of the primary antibody AB88129 in blocking solution overnight at 4 °C. The following day, the embryos were washed in 0.1% Triton X-100 in PBS as before, then incubated in a 1:400 dilution of the secondary antibody AB6719 in 0.1% Tween 20 in PBS for 1 h at RT. Then, the embryos were washed a further two times in PBS for 5 min and a final time in a PBS solution containing 0.05 mg/ml Hoechst 33342 for counterstaining. Finally, the stained samples were mounted under a 10-mm round coverslip in 5 µl of an anti-bleaching mounting medium (Fluoroshield™). The samples

were observed under x200 magnification with an Olympus BX60 epifluorescence microscope equipped with standard 4',6-diamidino-2-phenylindole DAPI and TR filters.

2.2.5 Total cell number estimation in bovine blastocysts

Day 7 blastocysts (171 to 175 h post-IVF) were fixed overnight in drops of 4% PFA in PBS at 4 °C under oil. Nuclei were then stained with the fluorescent dye Hoechst 33342 by direct addition of Hoechst 33342 to the fixative to achieve a final concentration of 0.05 mg/ml. The embryos were then incubated in the dark for 5 min at RT, and mounted under a 10-mm round coverslip in a 5 µl droplet of an anti-bleaching mounting medium (Fluoroshield™). Cell counts were obtained under epifluorescence observation using an Olympus BX60 microscope equipped with a standard DAPI filter and a Hamamatsu ORCA03-G camera. The software used to collect and store the images was SmartCapture (version 3, Digital scientific, Cambridge, UK). To ensure that cells from the entirety of the embryo and from all focal planes were taken into account, each embryo was broken down into smaller sections using the tools provided with SmartCapture and cell counts from all planes were obtained for each section until the whole embryo was assessed. An example of this method is presented in figure 2.6.

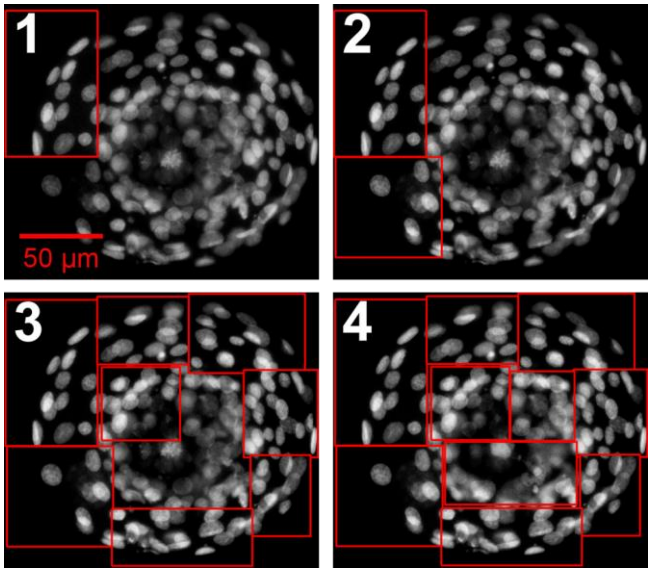


Fig. 2.6 – Total cell count estimation with SmartCapture 3 on Hoechst 33342 stained bovine blastocysts. To improve the accuracy of the cell count, the embryo was sequentially divided into smaller sections (red rectangles, panels 1 to 4) which could be individually brought into focus. Cell nuclei from the different focal planes were then counted in each section. The total magnification used was x200.

2.2.6 Time-lapse observation

Embryos cultured in the PrimoVision 16-well dishes were available for time-lapse observation. A PrimoVision EVO microscope was used together with the acquisition and analysis software provided by the supplier (Vitrolife, Goteborg, Sweden) to annotate the timing of cleavage, compaction and blastulation events. Observation began 22 h post-IVF or immediately after splitting for control and split embryos, respectively. The timing of these events was then compared between experimental groups and against known examples from literature (Holm *et al.* 1998).

2.2.7 Swept-source optical coherence tomography (SS-OCT)

SS-OCT is a microscopy imaging modality that can acquire both depth profile (B-scan) and cross-sectional (*en-face*) images of a sample. Coupled with image analysis algorithms like SV analysis, it is also capable of detecting fine differences between sequential images and can be used to track movement on a pixel level. The following sections describe the use of an experimental SS-OCT microscope to obtain static images of fixed bovine embryos and to quantify micron-scale movements in live bovine blastocysts.

2.2.7.1 SS-OCT system setup

The image acquisition was completed using an experimental SS-OCT microscope assembled by Dr Ramona Cernat (School of Physical Sciences, University of Kent, Canterbury, UK). A 1310 nm centre wavelength swept source (Axsun Technologies, Billerica, MA) was used to illuminate the sample with a bandwidth in the range 1256.6 to 1362.8 nm. The light from both sample and reference was collected by a photodetector (PDB460C, Thorlabs, Newton, NJ) and routed to a digitaliser (ATS9350, AlazarTech, Point-Claire, Canada). Axial and cross-sectional images were reconstructed by master/slave interpherometry following a procedure described before (Cernat *et al.* 2017). In air, the axial resolution of the microscope was approximatively 5 µm, while the transversal resolution was approximately 4.2 µm. A schematic of the system used is presented in figure 2.7.

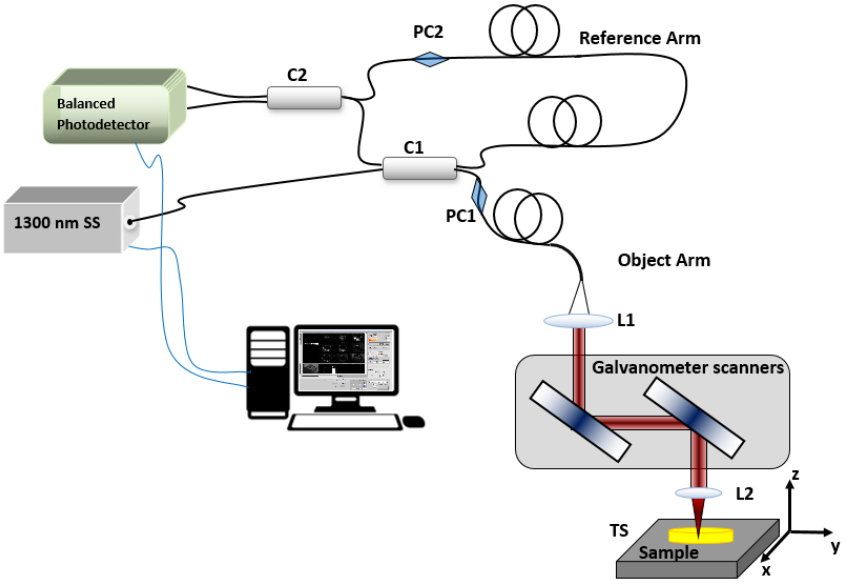


Fig. 2.7 - SS-OCT microscope experimental set-up. The dish containing the embryo was positioned on the microscope's translation stage for lateral scanning. C1-C2: optical couplers, PC: polarisation controllers, L1,2 – lenses, Is: isolator. This image was kindly provided by Miss Sophie Caujolle (School of Physical Sciences, University of Kent, Canterbury, UK).

2.2.7.2 Static SS-OCT imaging and 3D model reconstruction of fixed embryos

Bovine embryos of different stages were fixed in 4% PFA in PBS overnight, then washed in PBS and distributed, one per well, across a PrimoVision 9-well dish (Vitrolife, Goteborg, Sweden). The embryos were maintained in PBS under mineral oil and stored at -4 °C for up to a week until required for observation. For each embryo, approximately 300 cross-sectional images were acquired at different depths within its structure. The image stacks were then reconstructed into 3D models of the embryo by using the free software ImageJ (version 1.51n, Rasband W., National Institutes of Health, USA, https://imagej.nih.gov/ij).

2.2.7.3 Live embryo handling for SS-OCT microscopy

Bovine blastocysts from day 7 post-IVF were produces as described above. In preparation for observation, the embryos were distributed, one per well, across a PrimoVision 9-well dish (Vitrolife, Goteborg, Sweden) pre-warmed at 38 °C. To minimalize the effects of refraction, each well contained only a minimum volume of HEPES modified, glucose-free TALP medium which was overlaid with a thin mineral oil

layer to prevent rapid evaporation. After loading, the embryos were immediately transported to the nearby optics department in a portable hot box set at 38 °C. Because of the dish setup, the micro wells were not communicating stopping embryos from being displaced over transport. The image acquisition started within 30 min of live embryos being loaded into the dish.

2.2.7.4 Speckle variance analysis of embryo movements

For the detection and quantitation of micron-scale movements, embryos were observed for 10 min and a set of data was acquired every minute. Additionally, to establish a proof of principle, one embryo was continually monitored for 18 h acquiring a set of data every minute for 10 min followed by 20 min of rest. The SV analysis was performed on successive images at 1 min intervals, using an algorithm defined by Miss Sophie Caujolle (School of Physical Sciences, University of Kent, Canterbury, UK). To compensate the effects of Brownian motion, image sets from dead control embryos were also processed by SV analysis to establish a minimum variance threshold for a pixel to be considered in active motion. During the SV measurements on live embryos, all pixel values below this threshold were arbitrarily set to zero. The SV calculation from each optical plane considered was then used to highlight the portion of the embryo in movement during the observation period. For each optical plane, a quantitative assessment of motion was also obtained simply by summation all pixel values.

2.2.8 Karyomapping

In order to produce karyomaps for all the embryos and calves in this study, adequate DNA samples were derived from embryos, calves and their parents. For the embryos produced at the University of Kent, DNA samples were obtained from embryo biopsies, sperm and ovarian tissue as appropriate. While for the embryos produced at Paragon Veterinary Group, samples were obtained from embryo biopsies, sperm, and blood from either cow or calf as appropriate. The DNA obtained from embryo biopsies was incremented by application of a WGA step and in all cases the samples were genotyped

on an Illumina BovineSNP50 chip from which karyomaps could be produced. A detailed account of these protocols is presented hereafter.

2.2.8.1 Whole genome amplification of embryo biopsies

To produce a sufficient quantity of DNA for SNP typing, all embryo biopsies were processed by WGA using a REPLI-g Single Cell Kit (Qiagen, Manchester, UK), following manufacturer's instructions. In brief, a clean cabinet was sterilised with a 70% alcohol solution before starting the amplification protocol and used for all manipulations. The lyophilised buffer DLB was reconstituted by addition of 500 µl of water then used to prepare a sufficient volume of buffer D2 (0.25 µl dithiothreitol (DTT) 1 M, 2.75 µl reconstituted DLB per reaction) which was kept on ice. The embryo biopsies, which were contained in 4 µl of PBS in a sterile microcentrifuge tube and stored at -80 °C, were thawed on ice and received 3 µl of buffer D2 each. Then all samples were denatured for 10 min in a thermocycler (Mastercycler gradient S, Eppendorf, Stevenage, UK) set at 65 °C with a 105 °C heated lid. During the denaturation, a sufficient volume of the amplification master mix was prepared (9 µl water, 29 µl REPLI-g SC reaction buffer, 2 µl REPLI-g SC polymerase per reaction). Immediately after the denaturation step, the samples were put on ice to prevent DNA renaturation and each tube was supplemented with 3 µl of stop solution, which had the same function. Following this, 40 µl of the amplification master mix were mixed in each tube giving a final reaction volume of 50 µl. The amplification reaction was then carried out in a thermocycler (Mastercycler gradient S, Eppendorf, Stevenage, UK) set at 30 °C with a 70 °C heated lid for 4 h, which was followed by a single enzyme inactivation step carried out at 65 °C for 3 min. The concentration of the resulting amplified DNA samples was then tested by fluorometry using a Qubit 2.0 (Thermo Fisher Scientific, Loughborough, UK) and its dedicated dsDNA BR Assay Kit (Thermo Fisher Scientific, Loughborough, UK) following manufacturer's instructions. Briefly, a sufficient volume of Qubit working solution was prepared by a 1:200 dilution of Qubit dsDNA BR reagent in Qubit dsDNA BR buffer. Then, 1 µl from each amplified DNA sample was diluted in 199 µl of this working solution and briefly incubated at RT before taking a fluorometry reading. The sample DNA concentration was calculated by comparing the obtained reading against a 2-point calibration curve

obtained by using the standards provided with the kit and taking into account the appropriate dilution factor.

2.2.8.2 Blood DNA purification

A qualified veterinary practictioner (Paragon Veterinary Group, Carlisle, UK) drew a blood sample from the dams used by Paragon Veterinary Group a part of a routine procedure. Moreover, a blood sample from each calf born as a result of the transfer of one of the embryos biopsied during this study was also obtained in a similar manner. An aliquot from each of these blood samples was stored at -20 °C and then shipped to the University of Kent premises on dry ice in the post. At receipt, the blood was thawed at RT, and its DNA was isolated by using a QIAamp DNA Blood Mini Kit (Qiagen, Manchester, UK), following manufacturer's instructions. In brief, 200 µl of whole blood was dispensed in a microcentrifuge tube and was supplemented with 200 µl of buffer AL and 20 µl of proteinase K (Qiagen), then carefully mixed to initiate the lysis of the sample. The blood was then incubated at 56 °C in a water bath to complete the lysis step. After this time, 200 µl of ethanol was added to favour DNA precipitation and the mixture was loaded in a QIAamp Mini spin column and spun at 20,000 g for 1 min to allow the DNA to bind to the column. The ultrafiltrate was discarded and the column loaded with 500 µl of buffer AW1 and spun as before. The ultrafiltrate was again discarded and the column loaded with 500 µl of buffer AW2 and spun at 20,000 g for 3 min. The column was then removed to a clean microcentrifuge tube, received 50 µl of the elution buffer AE and was spun at 20,000 g for 1 min to release and recover the DNA. Finally, the eluted DNA sample was tested for concentration and purity by using a Nanodrop (ThermoScientific) and was judged suitable for karyomapping analysis if it had a minimum concentration of 20 ng/µl and an A260/280 between 1.60 – 1.90. All samples were stored at -20 °C until required.

2.2.8.3 Ovarian tissue DNA purification

All embryos produced at the University of Kent were derived from abattoir material. For each of the dams used at Kent, a DNA sample was extracted from ovarian tissue by using

a DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK), following manufacturer's instructions. In brief, a fragment (< 25 mg) of fibrous tissue from an ovary was cut using a sterile disposable scalpel and finely chopped, then placed in a clean microcentrifuge tube. The tube was then filled with 180 µl of buffer ATL and 20 µl of proteinase K (Qiagen), and the sample was mixed by vortexing. To complete the lysis of the tissue, the tube was incubated at 56 °C in a water bath and regularly vortexed until the fragments were completely dissolved. The lysed sample was then supplemented with 200 µl of buffer AL and 200 µl of ethanol to promote DNA precipitation and the mixture was loaded in a DNeasy Mini spin column and spun at 6,000 g for 1 min. The flow through was discarded, then the column received 500 µl of buffer AW1 and was spun as before. After discarding the flow-through, the column was loaded with 500 µl of buffer AW2 and spun at 20,000 g for 3 min. Finally, the DNA bound to the column was eluted by adding 200 of buffer AE and centrifugation at 20,000 g for 1 min. After elution, the ovarian tissue DNA was tested for concentration and purity by using a Nanodrop (ThermoScientific) and was judged suitable for karyomapping analysis if it had a minimum concentration of 20 ng/µl and an A260/280 between 1.60 – 2.00. All samples were stored at -20 °C until required.

2.2.8.4 Sperm DNA purification

For each sire in this study, surplus sperm aliquots left unused after sperm preparation for IVF (refer to section 2.2.2.4) were preserved at -20 °C in readiness for DNA extraction. Alternatively, similarly treated sperm aliquots were supplied by Paragon Veterinary Group (Carlisle, UK) and shipped in the post on dry ice. In all cases, the samples were thawed at RT, then 50 µl of the sperm suspension was aliquoted in a fresh microcentrifuge tube, supplemented with 1 ml of wash buffer (150 mM NaCl, 10 mM EDTA, pH 8.0) and carefully mixed. The sample was then spun at 6,000 g for 10 min to pellet the sperm cells while the supernatant was discarded. The pellet was resuspended in a further 500 µl of wash buffer and the sample was spun at 15,000 g for 2 min. The supernatant was again removed and the pellet was gently resuspended in 300 µl of lysis buffer (500 mM NaCl, 100 mM TRIS, 10 mM EDTA, 1% sodium dodecyl sulphate (SDS), 100 mM DTT, pH 8.0) and incubated at 65 °C for 90 min. After this time, 150 µl of

ammonium acetate 7.5 M was added to the mixture to precipitate dissolved proteins. The sample was spun at 15,000 g for 10 min, then the supernatant was recovered and transferred to a clean tube while the protein-rich pellet was discarded. To isolate the DNA in suspension, 900 µl of isopropanol was added and the sample was spun at 20,000 g for 10 min. The supernatant was then discarded and the DNA-rich pellet was washed in 500 µl ethanol then spun as before. Finally, all supernatant was discarded and the DNA pellet was briefly allowed to dry at RT. The sample was then re-hydrated overnight at 4 °C in 20 µl of TE buffer (1 mM EDTA, 10 mM TRIS, pH 8.0). Finally, the sperm DNA sample was tested for concentration and purity by using a Nanodrop (ThermoScientific) and was judged suitable for karyomapping analysis if it had a minimum concentration of 20 ng/µl and an A260/280 between 1.60 – 2.00. All samples were stored at -20 °C until required.

2.2.8.5 Illumina BovineSNP50 microarray analysis

The appropriate DNA samples were shipped by courier on dry ice to Neogen Europe (Auchincruive, UK) for SNP typing. The SNP typing was completed on a BovineSNP50 bead chip (Illumina, Cambridge, UK) following manufacturer's instructions. The microarray chip contained 47,843 polymorphic SNPs uniformly spaced across the target genome (median spacing approximatively 37,000 bp). As a quality metric, call rates were calculated for each DNA sample. Only samples showing a call rate of at least 80% were considered suitable for karyomapping and included in the final analysis. At each SNP locus tested, the genotype was reported as either AA, BB or AB according to the tested genome being homozygous for the reference nucleotide, homozygous for the alternative nucleotide or heterozygous, respectively.

2.2.8.6 Production and interpretation of the karyomaps

Data from the BovineSNP50 microarray analysis was exported on Excel (version 2016, Microsoft, Redmond, WA) then analysed by BoVision (version 3, Prof Alan Handyside, Illumina, Cambridge, UK). To complete the karyomap of each test subject, the genome of its parents together with that of a sibling were used to establish haplotype blocks.

The resulting karyomaps were interpreted following the instructions provided in "A Technical Guide to Karyomapping: Phasing Single Gene Defects" (revision C Jan 2015, www.support.Illumina.com). For chromosomal abnormalities, three independent operators reviewed each case. The number of crossover events and aneuploidy events were determined on a per chromosome basis for all the assessed samples. At least three consecutive informative SNPs had to be counted before a crossover event was recorded.

2.3 Statistical analysis

Wherever applicable, results were presented as means and error bars were shown as standard error of the mean (SEM), and confidence intervals (C.I.) were calculated at 95%. Additionally, when the result took the form of a proportion, confidence intervals were calculated as confidence intervals for proportions by applying the Wilson interval. For statistical analysis, appropriate tests were selected depending on the specific experiment following the guidelines presented in McDonald (2014), and the α value for statistical significance was set at 0.05.

T-student, chi-square and ANOVA test were carried out on Excell (version 2016, Microsoft, Redmond, WA). Kendal correlation tests were carried out on Kendall tau Rank Correlation (v1.0.13) in Free Statistics Software (v1.2.1) (Office for Research Development and Education, https://www.wessa.net). ANCOVA tests, linear regression tests, and logistic regression tests were carried out on VassarStats (Lowry R., version 2017, http://vassarstats.net).

2.4 Ethical approval

All the embryos produced at the University of Kent during the course of this project were derived from abattoir material and destroyed by day 8 post-IVF. Moreover, during 2016 and 2017, routine commercial IVP procedures were regularly undertaken by the Cumbria OPU team and the Penrith IVP lab (Paragon Veterinary Group, Carlisle, UK). A subset of embryos that were part of this commercial breeding programme were biopsied in view of karyomapping and then transferred on animals already committed to an IVP/ET programme by the breeder. Because the present work did not require a bespoke intervention on live animals, ethical approval was not deemed necessary.

3. Specific Aim 1: To test the hypothesis that gilt oocytes with a depleted cumulus display similar developmental competence to oocytes clad by three or more layers of cumulus cells.

3.1 Background

Substantial improvements of the protocols currently in place for the IVM of porcine oocytes are likely required to unlock the full potential benefits of IVP in pig breeding. One of the focal hypothesis in the current literature is that the poor developmental potentials exhibited by porcine embryos derived from IVM oocytes are a consequence of incomplete oocyte nuclear and cytoplasmic maturation; as a result, in recent years much effort has been dedicated to the optimization of IVM in the pig (Wu *et al.* 2011; Jeon *et al.* 2014; Appeltant *et al.* 2015; Lin *et al.* 2016; Yuan *et al.* 2017). However, this research has often been complicated by the fact that the vast majority of the abattoir population of pigs is constituted of peripubertal gilts, which in turn become the principal donors of oocytes for IVM. Indeed, it is well established that oocytes derived from gilts display reduced developmental potentials when compared to oocytes derived from sows (Marchal *et al.* 2001; Bagg *et al.* 2004; Bagg *et al.* 2006; Lechniak *et al.* 2007). Moreover, the average follicle size is smaller in gilts than in sows (Bagg *et al.* 2007) and oocyte *in vitro* developmental competence is known to increase linearly with follicle size (Marchal *et al.* 2002). In pigs, primary oocytes for IVP are normally retrieved from follicles ranging between 3 to 8 mm in size since full oocyte meiotic competence is only achieved in follicles with a diameter of at least 3 mm (Bagg *et al.* 2007). However, it is difficult for operators to judge follicle size accurately during collection (Lin *et al.* 2016) and the problem is made worse in gilt ovaries due to the prevalence of small follicles (Bagg *et al.* 2007). Therefore, efficient criteria for post-retrieval oocyte selection are essential.

Currently, the morphology of the COC is used as a key indicator to select suitable oocytes for IVM (Somfai *et al.* 2004; Alvarez *et al.* 2009). This practice is based on the observation that cumulus cells and oocytes share an intricate network of interactions (Gilchrist *et al.*

2004) and that the oocyte's ability to fully complete both nuclear and cytoplasmic maturation is strongly influenced by the number of cumulus cells present in culture (Nagai *et al.* 1993; Dang-Nguyen *et al.* 2011; Lin *et al.* 2016). The oocyte is maintained in meiotic arrest by a stable supply of the meiotic progress inhibitor cAMP supplied from the cumulus via gap junctions (Anderson & Albertini 1976; Racowsky 1985). The meiotic arrest is also reinforced by the supply from the cumulus of cyclic guanosine monophosphate (cGMP) which inhibits the PDE3A phosphodiesterase that would otherwise decrease cAMP levels by hydrolysis (Norris *et al.* 2009). Remarkably, cumulus cells also control meiosis resumption in the oocyte in response to the LH surge through a variety of mechanisms including Ca^{2+} dependent signalling, membrane potential depolarization, and gap junction closure forcing a reduction of cAMP levels in the oocyte (Mattioli & Barboni 2000; Norris *et al.* 2008).

Furthermore, the cumulus plays a key role in cytoplasmic maturation by promoting the migration of CGs to the periphery of the oocyte (Galeati *et al.* 1991). The exocytosis of the CGs in the perivitelline space immediately after fertilisation, a process known as the cortical reaction, causes the proteolytic removal of sperm specific binding sites from the ZP (Dandekar & Talbot 1992) and is one of the primary mechanisms employed by the oocyte to prevent polyspermy: the penetration of multiple spermatozoa in a single oocyte (Wang *et al.* 1997). Therefore, inadequate cytoplasmic maturation in IVM oocytes is regarded as a cause of the high levels of polyspermy typically found in porcine zygotes, which is still one of the greatest challenges limiting the application of IVP in this species (Han *et al.* 1999; Sun & Nagai, 2003; Grupen 2014). Cytoplasmic maturation can be improved by the presence of GSH, which is actively produced and supplied to the oocyte by cumulus cells (Maedomari *et al.* 2007; You *et al.*, 2010). In the oocyte, GSH acts as a ROS scavenger, (Tatemoto *et al.* 2000), and increases amino acid transport as well as protein synthesis (Lafleur *et al.* 1994). Additionally, the presence of GSH is important for correct fertilization as it plays a role in the correct male pronuclear formation (Niwa 1993) by assisting the decondensation of the sperm head through reducing the disulphide bonds between protamines (Yoshida *et al.* 1993).

From the literature, it appears that the current recommendation is to select for IVM only COCs formed of multiple (three or more) compact layers of cumulus cells (Rath *et al.* 1995; Long *et al.* 1999; Esaki *et al.* 2004; Sherrer *et al.* 2004; Bagg *et al.* 2007; Lee *et al.* 2012; Lin *et al.* 2015). However, this practice could result in wastage since it has been reported that COCs with of three or more layers of cells form only 39% of the total yield (Lin *et al.* 2016). In an effort to improve the utilization of the resources available for IVP in the pig, the aim of this chapter was to establish whether oocytes clad by fewer than three layers of cumulus cells display levels of maturational and developmental competence comparable to those of oocytes clad with three or more layers of cumulus cells.

3.2 Specific aims

With reference to the background above, the specific aims of this chapter were:

Specific aim 1a: To test the hypothesis that gilt oocytes clad by fewer than three layers of cumulus cells are able to achieve complete nuclear maturation to levels comparable to oocytes clad by three or more layers after IVM.

Specific aim 1b: To test the hypothesis that gilt oocytes clad by fewer than three layers of cumulus cells display levels of CG migration comparable to oocytes clad by three or more layers after IVM.

Specific aim 1c: To test the hypothesis that cumulus investment is directly correlated to the GSH content of gilt oocytes following IVM.

Specific aim 1d: To test the hypothesis that, in gilt oocytes, the sperm penetration and polyspermy rates following IVF are, respectively, directly and inversely correlated to cumulus investment.

Specific aim 1e: To test the hypothesis that gilt oocytes clad by fewer than three layers of cumulus cells have reduced embryo developmental potentials as compared to oocytes clad by three or more layers of cumulus cells.

3.3 Methods

Oocytes were separated into four experimental groups A-D representing oocytes clad by 3 or more, 2, 1 or 0 layers of cumulus cells, respectively (refer to section 2.2.1.1). A total of 3005 porcine oocytes were obtained by manual aspiration from 3365 follicles, indicating a retrieval rate of 89.3%. Following IVM, the nuclear and cytoplasmic maturation levels achieved by oocytes of different grades were established by α-tubulin immunostaining and PNA lectin staining (refer to sections 2.2.1.2 and 2.2.1.3). Additionally, post-IVM GSH levels per oocyte were estimated spectrophotometrically (refer to section 2.2.1.4). Following IVF, sperm penetration and polyspermy rates were measured on oocytes of different grades (refer to section 2.2.1.7), and finally, records of embryo development after 72 h of culture were obtained for each grade (refer to section 2.2.1.6).

3.4 Results

As a general observation, it was found that the oocyte yield was unequally distributed across the experimental grades (Grade A: 17.9%; Grade B: 26.3%; Grade C: 36.9%; Grade D: 18.9%; chi-square, χ^2_3=249.63, P=7.9x10^{-53}).

3.4.1 Specific aim 1a: To test the hypothesis that oocytes clad by fewer than three layers of cumulus cells achieve complete nuclear maturation to levels comparable to oocytes clad by three or more cumulus cell layers.

The nuclear maturation was evaluated on stripped oocytes by visualizing the meiotic stage of the cell via α-tubulin immunostaining. As depicted in figure 3.1, meiotic stages were classified as either Prophase I indicating an oocyte that made no progression on nuclear maturation, Metaphase I indicating an oocyte that initiated but did not complete nuclear maturation, Anaphase I indicating an oocyte very close to completing nuclear maturation, or Metaphase II indicating full nuclear maturation. Due to the transient

nature of the Anaphase I, oocytes in this stage were rarely detected (n= 9/289), and therefore, Anaphase I oocytes were pooled together with Metaphase II oocytes and considered fully mature for the purpose of statistical analysis.

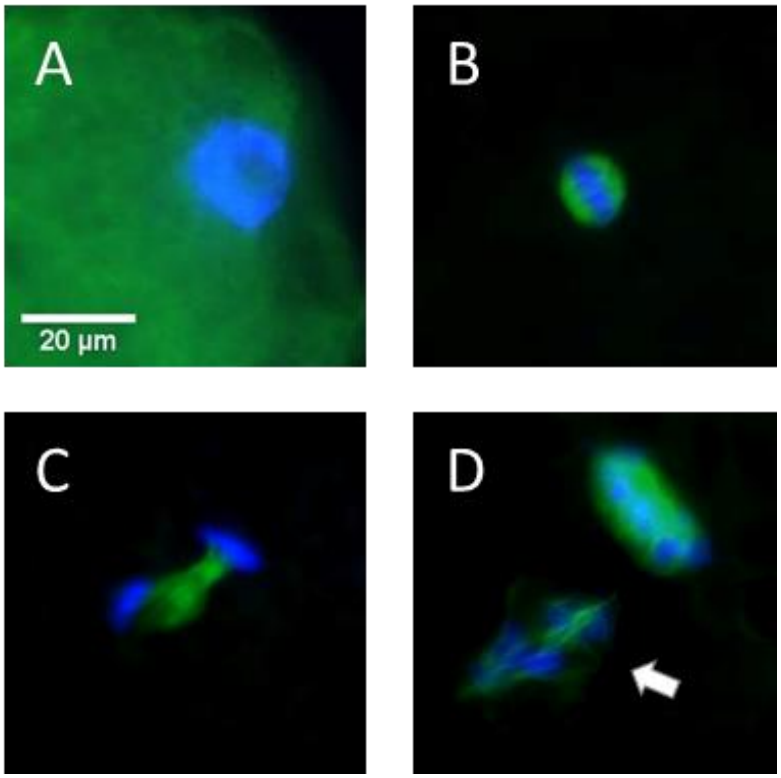


Fig. 3.1 – Meiotic stage of porcine oocytes visualized by α-tubulin immunostaining. Tubulin in green, DNA counterstained with Hoechst 33342 (blue). A) Prophase I, no spindle is detected. B) Metaphase I, a clear tubulin spindle has formed around the chromosomal compartment. C) Anaphase I, two sets of chromosomes are separated by a tubulin bridge. D) Metaphase II, two independent spindles can be detected belonging to either the oocyte or to polar body I (arrow). Images captured by fluorescence microscopy at x200 total magnification.

Generally, oocytes clad by fewer layers of cumulus cells were found to be arrested in Prophase I more frequently (Grade A: 48.0%; Grade B: 56.8%; Grade C: 69.8%; Grade D: 97.1%; chi-square, χ^2_3=45.15, P=8.6x10^{-10}). Nonetheless, the difference between grade A and grade B oocytes was not statistically significant (chi-square, χ^2_1=1.21, P=0.27), nor it was the difference between grade B and grade C oocytes (chi-square, χ^2_1=2.57, P=0.11). Interestingly, the proportion of oocytes in metaphase I was found to be consistent across grades A-C (Grade A: 16.0%; Grade B: 18.5%; Grade C: 19.1%; chi-square, χ^2_2=0.26, P=0.87). However, higher rates of MII oocytes were obtained from the COCs with the greatest investment (Grade A: 36.0%; Grade B: 24.7%; Grade C: 11.1%;

grade D: 0.0%; chi-square, χ^2_1=35.19, P=1.1x10^{-7}). This difference was statistically significant between grade A and grade C oocytes (chi-square, χ^2_1=11.42, P=7.25x10^{-4}), but it was not between grade A and grade B oocytes (chi-square, χ^2_1=2.36, P=0.12). Finally, denuded oocytes (grade D) were never able to achieve full nuclear maturation.

When a Kendall correlation test was performed to look more generally at the nuclear maturation pattern across grades, grade A and grade B oocytes appeared statistically indistinguishable (Kendall tau, τ=0.105, P=0.17), whilst a comparison between grade B and grade C oocytes resulted in τ=0.150 and P= 0.06. These findings are presented in figure 3.2.

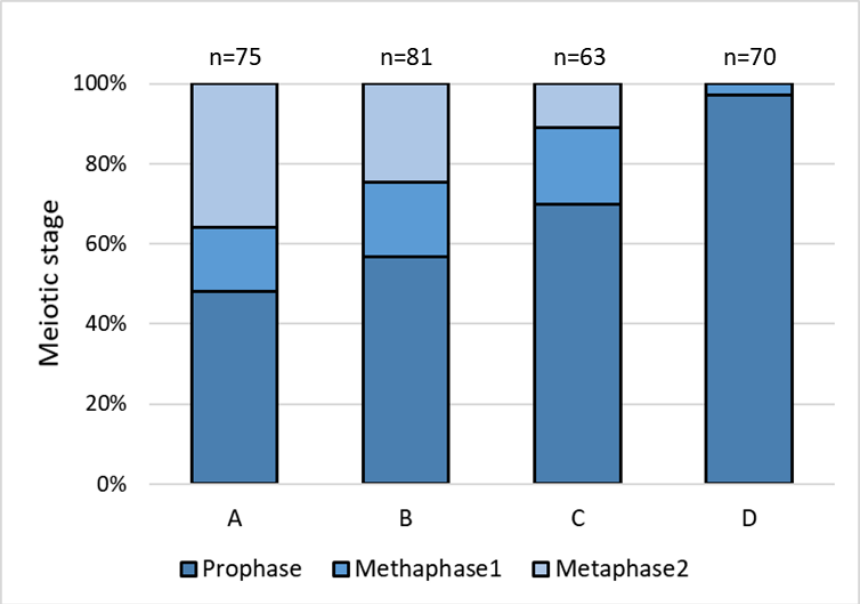


Fig. 3.2 – Meiotic stage of porcine oocytes of different grades after IVM. Oocytes clad by more cumulus layers were more likely to achieve full nuclear maturation (metaphase II). However, the number of oocytes in metaphase I appeared consistent across grades A-C. Grade D oocytes were found to be unable to complete nuclear maturation.

3.4.2 Specific aim 1b: To test the hypothesis that oocytes clad by fewer than three layers of cumulus cells display patterns of CG migration comparable to those of oocytes clad by three or more cumulus cell layers.

Cytoplasmic maturation post IVM was investigated on fixed oocytes by Alexa Fluor 488-PNA-lectin staining. Mature oocytes were expected to display a clear ring of CGs immediately beneath their ZP, as illustrated in figure 3.3. A total of 332 oocytes were used in this test.

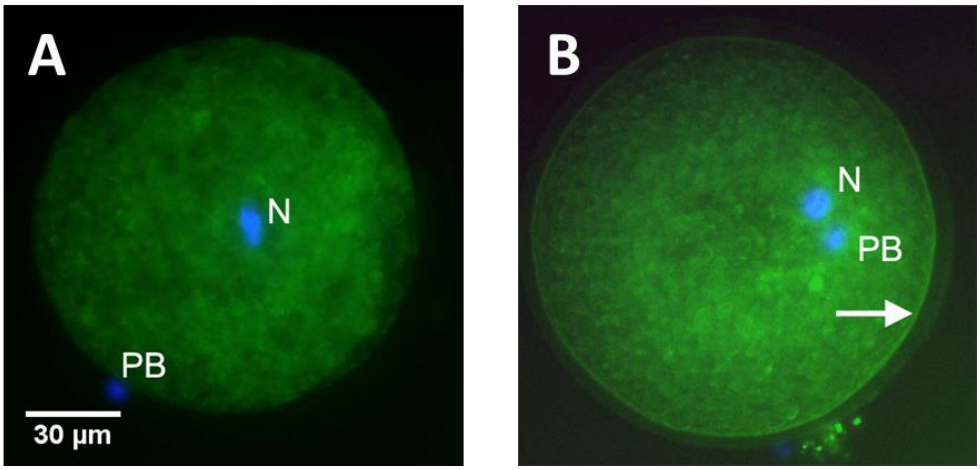


Fig. 3.3 – Cortical granules (CG) stain with PNA-lectin in porcine oocytes. CGs in green, DNA counterstained with Hoechst 33342 (blue). In the images, N indicates the oocyte's nucleus, PB indicates polar body I. A) A cytoplasmically immature oocyte, no clear CG distribution is present. The presence of a polar body indicates asynchrony between nuclear and cytoplasmic maturation in this cell. B) A cytoplasmically mature oocyte, a clear ring of CGs can be detected (arrow). Images captured by fluorescence microscopy at x200 total magnification.

Results seemed to support the hypothesis that COC investment linearly correlates with cytoplasmic maturation levels post IVM, with oocytes clad by more cumulus layers displaying higher rates of cytoplasmic maturation (Grade A: 58.9%; Grade B: 51.6%; Grade C: 29.0%; Grade D: 27.9%; chi-square, χ^2_3=26.64, P=7.0x10^{-6}). Specifically, oocytes in complex with two or more complete layers of cumulus cells (grades A and B) displayed a continuous peripheral ring of CGs more often than partially or fully denuded oocytes (grades C and D), whilst no significant difference was found between grade A and grade B oocytes (chi-square, χ^2_1= 0.87, P=0.35), or between grade C and grade D oocytes (chi-square, χ^2_1= 0.02, P=0.88). These results are summarised in figure 3.4.

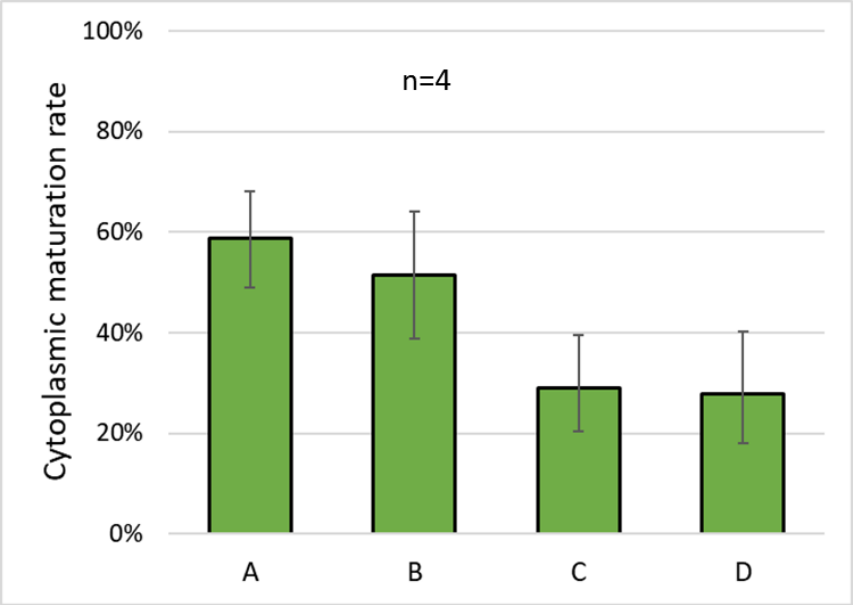


Fig. 3.4 – Rates of cytoplasmic maturation in gilt oocytes after IVM. Oocytes clad by either 2 or 3+ layers of cumulus cells (grades A and B) performed similarly in this test and completed cytoplasmic maturation more often than oocytes of other grades. Data given as mean ± S.E.M.

3.4.3 Specific aim 1c: To test the hypothesis that cumulus investment is directly correlated to the GSH content of gilt oocytes following IVM.

The intrinsic GSH content per oocyte was measured spectrophotometrically to investigate the oocyte's ability to cope with oxidative stress. Firstly, known amounts of GSH were used in triplicate experiments to establish a calibration curve by measuring the average increase in the A_{412} produced over the time interval considered (ΔA_{412}), as demonstrated in figure 3.5.

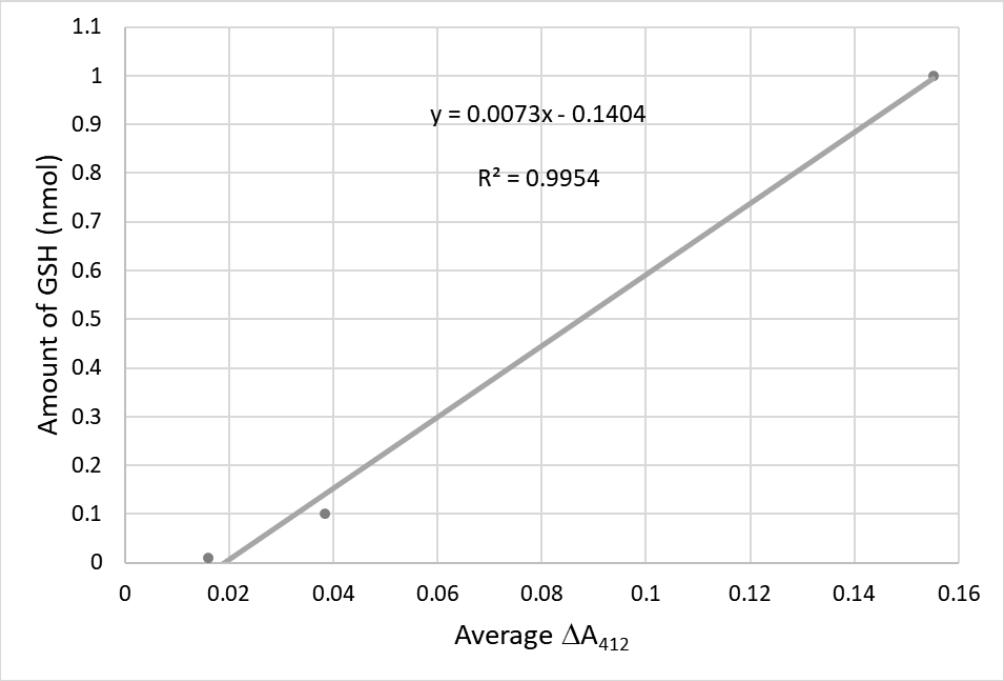


Fig. 3.5 - Calibration curve for the measurement of glutathione (GSH). Known amounts of GSH (0.01 nmol, 0.1 nmol and 1 nmol) were used to calculate the average increase in A_{412} for each 20 second interval. A calibration curve was then produced by linear regression.

Following calibration, a total of 120 oocytes for each grade were examined, 30 at a time, over four replicates. All the reaction rate curves produced showed high linearity (r^2 ≥0.95) and were therefore included in the analysis. A summary of the reaction curves recorded is given in figure 3.6, showing the average increase in the A_{412} of a sample as a function of time. The reaction rate across the different oocyte grades appeared to differ significantly (one-way ANCOVA, $F_{3,107}$=26.87, P<1.0x10^{-4}), suggesting that the COC investment has an effect on the GSH abundance in the oocyte following IVM. When a more detailed comparison was operated, it was found that grade A and grade B oocytes had statistically indistinguishable reaction rates (one-way ANCOVA, $F_{1,53}$=0.25, P=0.62), supporting the hypothesis that their GSH content is similar. Conversely, all the other individual comparisons between the reaction rates of the different test groups resulted in statistically significant differences (one-way ANCOVA, df=1,53, P<1.0x10^{-4}).

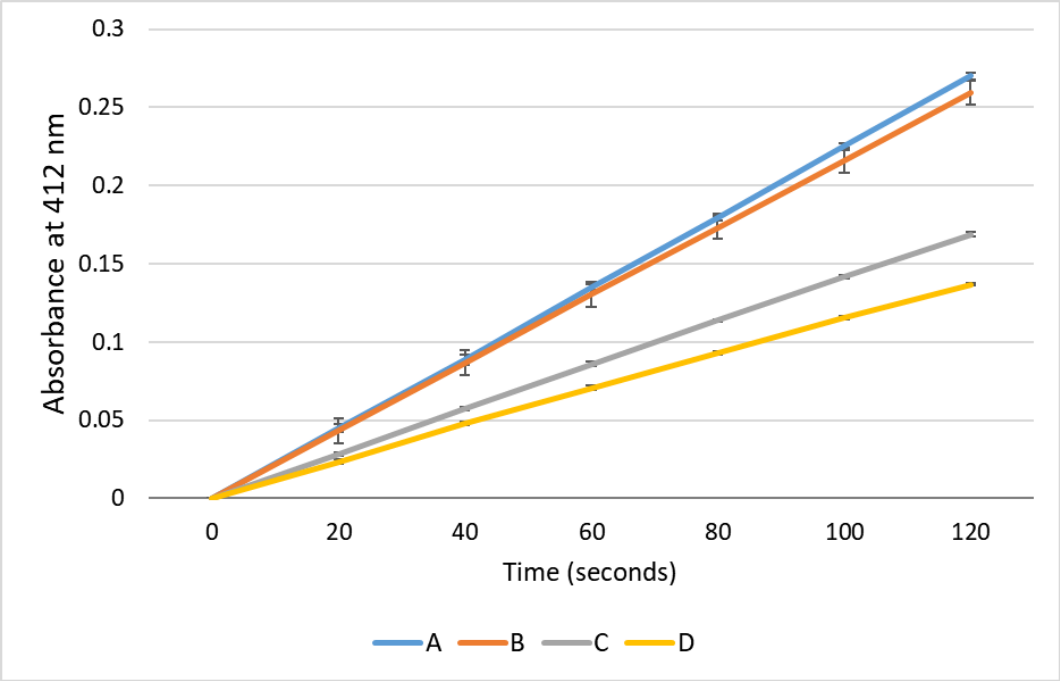


Fig. 3.6 - Reaction rate curves for the measurement of glutathione (GSH) levels in oocytes of different grades. The reaction rate was measured by tracking the increase in the A_{412} of each sample over time, a metric directly proportional to its intrinsic GSH content. Each reaction curve presented is the average obtained from triplicate experiments. Grade A and grade B oocytes showed very similar kinetics, whilst grade C and grade D oocytes displayed much slower reaction rates, indicating a reduced GSH content. Error bars given as S.E.M.

Finally, the total GSH amount per oocyte was calculated by comparing the slope of each reaction curve to the calibration curve. As shown in figure 3.7, grade A oocytes contained an average of 6.3 ± 0.5 pmol/oocyte of GSH which was similar to the amount calculated for grade B oocytes (5.8 ± 1.9 pmol/oocyte) but almost three times greater than the content measured for grade C oocytes (2.2 ± 0.3 pmol/oocyte). On the other hand, grade D oocytes appeared almost completely depleted of GSH with a content of just 0.9 ± 0.3 pmol/oocyte. Remarkably, grade B oocytes exhibited quite a high variability in terms of GSH concentration.

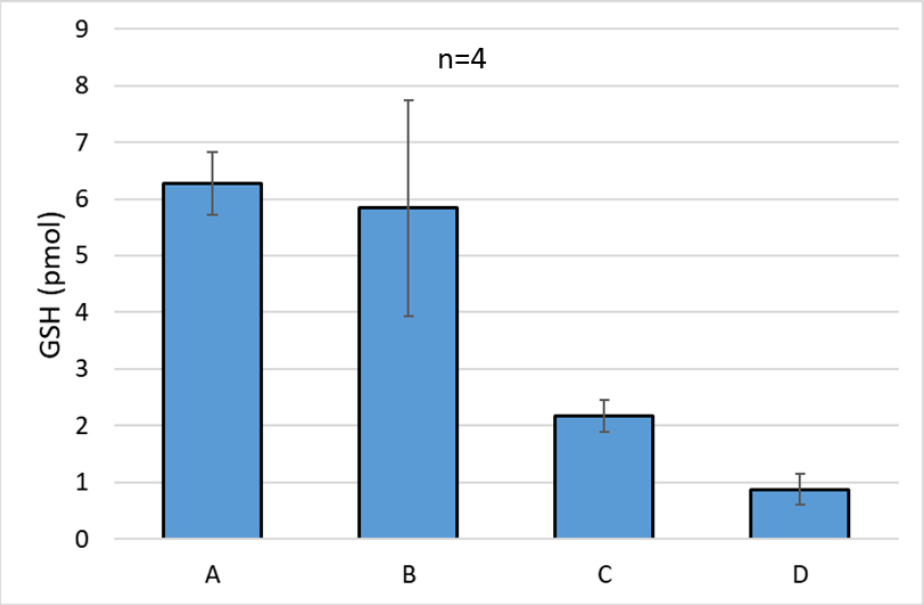


Fig. 3.7 - Total glutathione (GSH) content per oocyte according to grade. Oocytes clad by more layers of cumulus cells (grades A and B) were richer in GSH in absolute terms. However, the GSH content of grade B oocytes was found to be highly variable. Data given as mean ± S.E.M.

3.4.4 Specific aim 1d: To test the hypothesis that, in gilt oocytes, the sperm penetration and polyspermy rates following IVF are, respectively, directly and inversely correlated to cumulus investment.

A total of 297 presumptive zygotes derived from oocytes of different grades were examined by fluorescent microscopy to investigate sperm penetration and polyspermy rates. The oocytes were considered penetrated if showing at least 2 pronuclei (PNs); conversely, the polyspermy rate was calculated as the proportion of penetrated oocytes containing more than 2 PNs. An example of a polyspermic zygote is presented in figure 3.8.

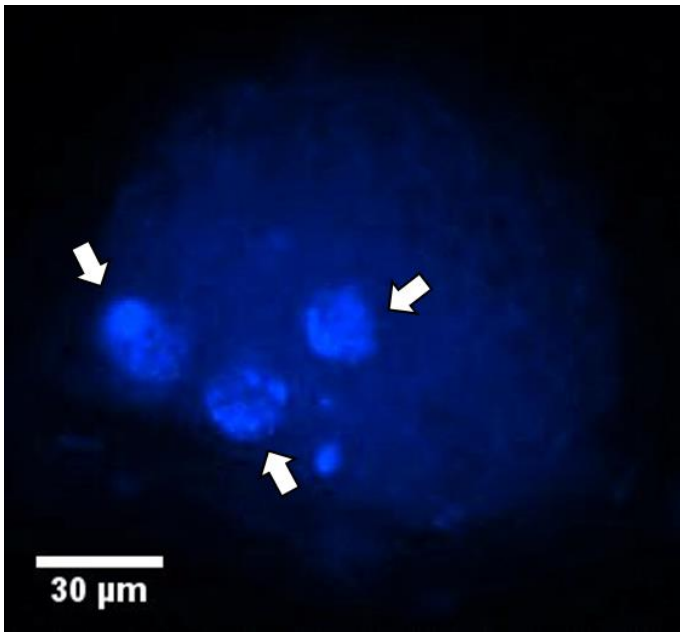


Fig. 3.8 - Polyspermic zygote. Three pronuclei can be distinguished (arrows), indicating this zygote has been penetrated by two separate sperm cells. DNA stained with Hoechst 33342, total magnification x200.

When assessed on the overall sample population, sperm penetrability appeared to be influenced by the oocyte's original cumulus investment (Grade A: 40.2%, Grade B: 38.7%, Grade C: 32.6%, Grade D: 16.7%, chi-square, χ^2_3=9.50, P=0.02). A closer analysis, however, showed that only grade D oocytes had a significant decrease in sperm penetrability (chi square, df=1, P<0.01), whilst oocytes from grades A-C showed statistically indistinguishable penetration rates. Moreover, all penetrated oocytes suffered from similar rates of polyspermy regardless of their original cumulus investment (Grade A: 60.6%, Grade B: 44.9%, Grade C: 71.4%, Grade D: 66.7%, Fisher's exact test, df=3, P=0.23). These findings are reported in figure 3.9.

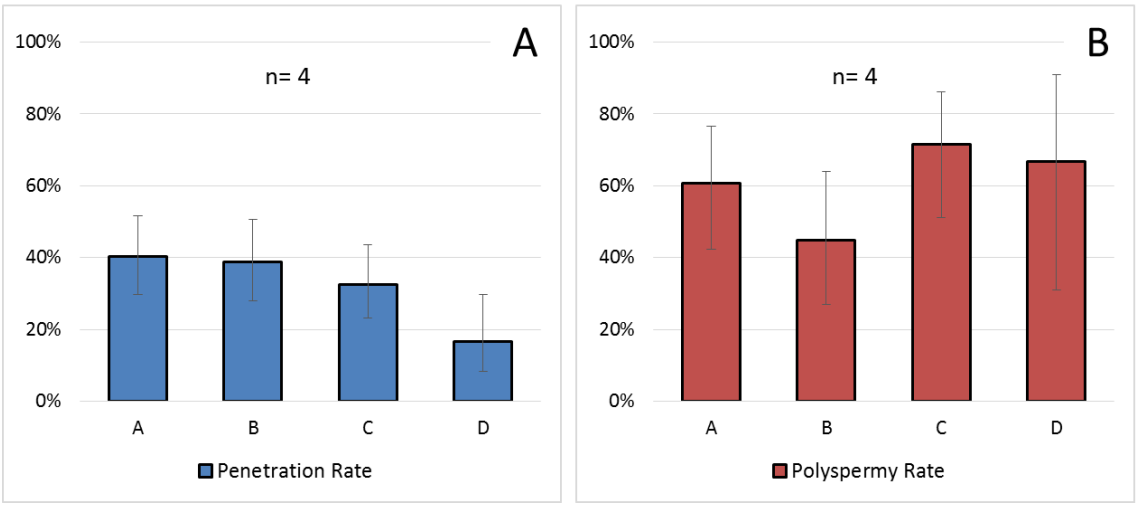


Fig. 3.9 - Penetration rate (A) and polyspermy rate (B) in zygotes derived from oocytes of different grades. A) The cumulus investment appeared to have only a moderate effect on sperm penetrability with only denuded oocytes (grade D) showing a reduction. B) Moreover, no difference was found in the polyspermy rates across the four experimental groups. Data given as mean ± S.E.M, statistical analysis performed with chi-square using α=0.05.

3.4.5 Specific aim 1e: To test the hypothesis that gilt oocytes clad by fewer than three layers of cumulus cells have reduced embryo developmental potentials as compared to oocytes clad by three or more layers of cumulus cells.

To conclude the evaluation, the developmental potentials of oocytes from different grades were evaluated by fertilising them through IVF and culturing the resulting embryos for three days. A total of 1609 oocytes across the different grades were used in this investigation. Examples of cleavage stage porcine embryos are given in figure 3.10.

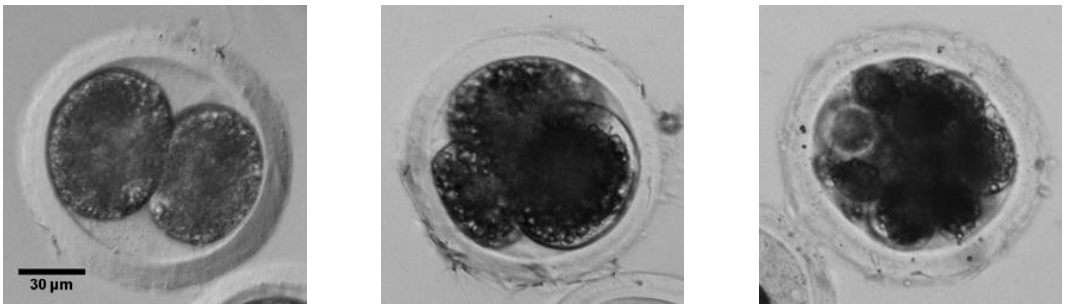


Fig 3.10 - Cleavage stage porcine embryos. Image captured with a Hoffman microscope at x200 total magnification

Unfortunately, the embryos rarely achieved the blastocyst stage regardless of the starting oocyte grade; therefore, the analysis of embryonic developmental competence had to be limited to the first three days of development.In accordance with the other findings presented in this chapter, the original COC investment appeared to be predictive of embryonic developmental rates, with oocytes clad by more layers of cumulus cells being able to produce cleavage stage embryos more often (Grade A: 46.3%, Grade B: 40.0%, Grade C: 22.9%, Grade D: 7.4%, chi square, χ^2_3= 122.6, P=2.1x10^{-26}), see also figure 3.11. Individual comparisons between the four experimental groups showed that grade A and grade B oocytes produced cleavage stage embryos with similar frequency (chi square, χ^2_1= 3.7, P=0.54), whilst all the other individual comparisons yielded statistically significant differences (chi square, df=1, P<1.0x10^{-6}).

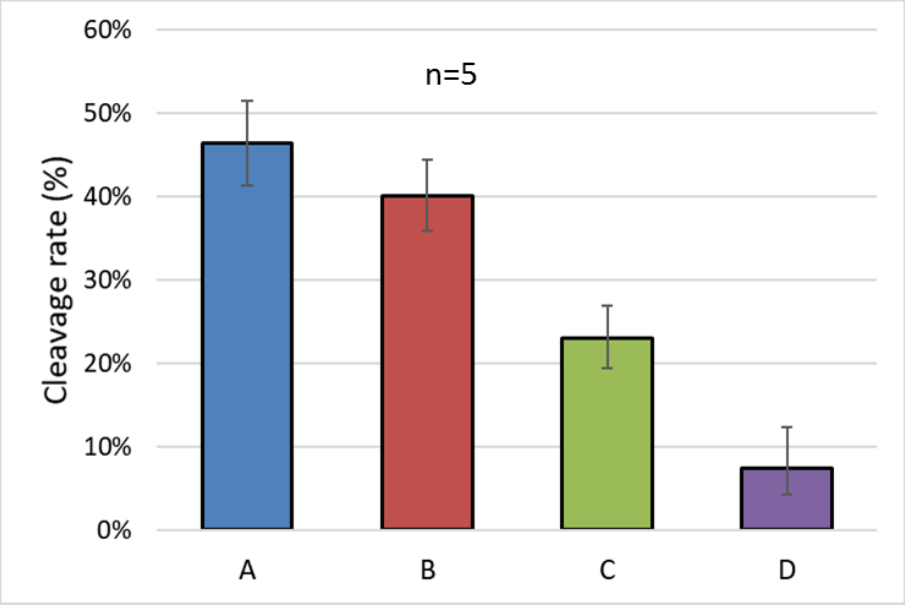


Fig. 3.11 - Cleavage rates achieved by fertilized oocytes of different grades. The original cumulus investment appeared to correlate with embryo developmental potentials. Oocytes clad by two or more layers of cumulus cells (grades A and B) produced cleavage stage embryos significantly more often, while denuded oocytes (grade D) only rarely produced embryos. Data given as mean ± S.E.M.

Following this initial analysis on overall cleavage rates alone, the final developmental stage achieved by each embryo was also taken into account, to investigate whether oocytes clad by more cumulus cells also tended to reach more advanced stages over the time frame considered. To this end, a rank correlation test was performed by assigning

higher ranks to embryos that reached later developmental stages. Again, no statistical difference was found between grade A and grade B oocytes (Kendall tau, τ=0.05, P=0.13) suggesting that not only did these two grades produce cleavage stage embryos with similar frequency, but also that the embryos produced will develop at comparable rates. Moreover, embryos derived from grade A and grade B oocytes tended to reach more advanced stages when compared with embryos derived from grade C or grade D oocytes (Kendall tau, $P<1.0\times10^{-6}$). Finally, the difference in the cleavage pattern of grade C and grade D oocytes was also significant (Kendall tau, τ=0.17, $P=1.8\times10^{-6}$). Figure 3.12 presents a visual summary of these findings by comparing the proportion of embryos that reached each successive cleavage stage for all the oocyte grades in this study.

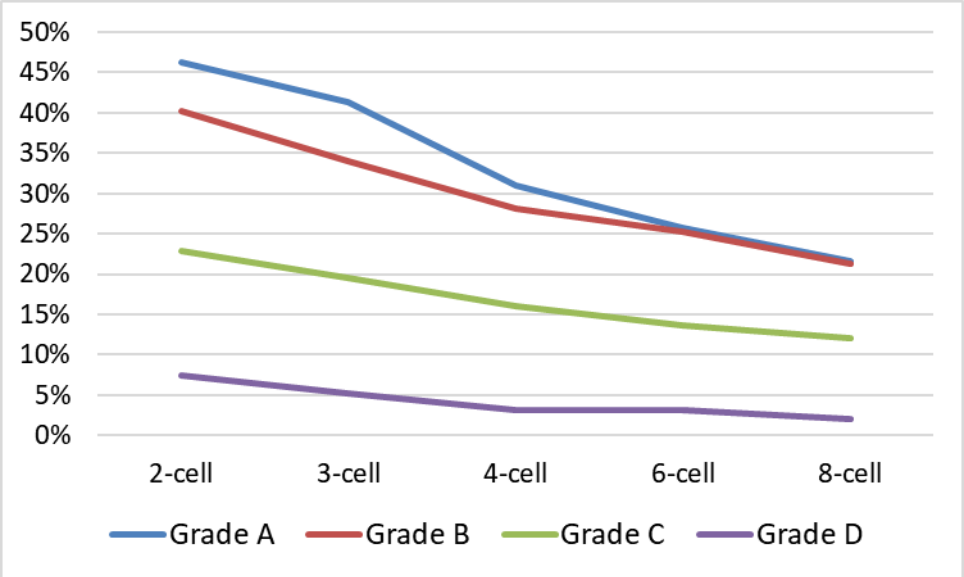


Fig. 3.12 - Cleavage pattern in embryos derived from oocytes of different grades. The lines portray the proportion of embryos reaching each successive cleavage stage. A pattern can be seen with embryos derived from grade A and B oocytes being both more abundant and tending to reach more advanced stages.

3.5 Discussion

The acquisition of adequate numbers of competent oocytes is, intuitively, a key element for successful embryo production. In line with another report (Lin *et al.* 2016), it was found that top grade COCs (intended as COCs with at least three compact layers of cumulus cells) form only a minority of the total yield from gilt ovaries, suggesting that the use of selection criteria that are too stringent risks further restriction of the number of IVP embryos available from the porcine model.

3.5.1 Nuclear maturation potentials

Overall, oocytes clad by more layers of cumulus cells were able to complete nuclear maturation more often. However, there was no difference in the nuclear maturation pattern of grade A and grade B oocytes. One interesting feature was that for grades A-C there was a noticeable subset of oocytes found in MI suggesting they were able to initiate nuclear maturation but did not complete maturation in the expected time. This finding could suggest that a proportion of gilt oocytes physiologically arrest during the maturation process.

Also, it is interesting to note that a Kendall correlation test was unable to discriminate between the nuclear maturation pattern of grade B and grade C oocytes resulting in a P=0.06. However, this finding should be interpreted with caution since the relatively small sample size and a result so close to the α value selected could have led to the retention of a false H_0 (a type II error). On the one hand, the comparison between the nuclear maturation pattern of grade B and grade C oocytes should be expanded to provide a more robust statistical answer. On the other hand, when the other evidence from this chapter is considered, grade C oocytes appear to be unlikely to possess high developmental potentials and therefore a more detailed investigation of their nuclear maturation behaviour would be unlikely to bring further benefits to pig IVP.

3.5.2 Cytoplasmic maturation potentials

The cytoplasmic maturation rate across the four oocyte grades could be divided in just two statistical populations: grade A and B oocytes which matured to higher levels and

grade C and D oocytes which matured to lower levels, supporting the hypothesis that COC investment influences cytoplasmic maturation post IVM. Of interest, was that fully denuded oocytes (grade D), which were never observed completing nuclear maturation, could occasionally progress toward full cytoplasmic maturation, providing further evidence that these two processes are not necessarily synchronous (Grupen *et al.* 1997). Moreover, previous studies have shown that denuded oocytes' developmental capabilities can be rescued by co-culturing with COCs (Luciano *et al.*, 2005). As some cumulus cells could have been present in grade D wells this may have been enough to induce some level of cytoplasmic maturation.

3.5.3 GSH content

To maintain a cellular environment free from oxidative stress and reduce ROS damage to the oocytes in culture, the IVM system used in this study included both cysteine and β-mercaptoethanol. Abeydeera *et al.* (1998) reported an average GSH content in pig oocytes of 7.9 ± 0.6 pmol/oocyte which increased to 10.4 ± 2.8 pmol/oocyte in the presence of 50 μM β-mercaptoethanol, while Yoshida *et al.* (1993) found that supplementing the maturation medium with 0.57 mM cysteine increased oocyte GSH from 4.0 ± 0.8 pmol/oocyte to 15 ± 0.3 pmol/oocyte.

In this work, it was found that grade A and grade B oocytes had levels of GSH compatible with previous reports (Yoshida *et al.* 1993; Abeydeera *et al.* 1998; Choe *et al.* 2010) while grade C and grade D oocytes appeared largely depleted of GSH. Therefore, it could be argued that the presence of cysteine and β-mercaptoethanol alone during IVM is not sufficient to increase intracellular GSH levels in pig oocytes as cumulus cells are likely required to complete the process. Moreover, the absence of sufficient quantities of GSH in grade C and grade D oocytes could well explain their reduced cytoplasmic and especially nuclear maturation potentials.

3.5.4 Sperm penetrability and polyspermy

A high polyspermy incidence has often characterized porcine IVP zygotes and can result in their reduced viability as compared to *in vivo* derived embryos (Funahashi *et al.* 2000).

Considering the role of CGs in the prevention of polyspermic events and considering that their distribution appeared more favourable in grade A and grade B oocyte as compared to oocytes of lesser grades, it was surprising to find no significant difference between the polyspermy rates of the four oocyte populations in study. However, polyspermy rates are known to vary on the basis of the individual boar used as a sperm donor (Sirard *et al.* 1993), and the use of caffeine as a capacitating agent in the IVF medium can lead to an increase in polyspermy rates due to the induction of a premature acrosome reaction (Funahashi & Nagai 2001). It is conceivable that these effects masked any difference between the populations in study. Additionally, grade D oocytes were rarely penetrated by sperm (n= 9/54); because of this, a reliable assessment of their polyspermy rate was difficult. To mitigate the issue, appropriate statistical methods for small samples (in this case, Fisher's exact test) were used to obtain a conservative comparison of their polyspermy rate against that of oocytes of other grades. However, a greater sample size and the use of the same frozen/thawed ejaculate for all replicates would probably be required to improve the ability of polyspermy rate assessment to describe an oocyte's maturational competence.

3.5.5 Embryo development

Taken together, the observations on the maturational competence of oocytes of different grades fit in line with the well-established link between COC investment and oocyte maturational competence (Marchal *et al.*, 2002; Nagano *et al.*, 2006; Bagg *et al.*, 2007; Alvarez *et al.*, 2009; Kim *et al.*, 2010; Lin *et al.*, 2016). Therefore, it is not surprising that the cleavage rates of the graded oocytes followed this trend. Interestingly, however, grade A and grade B oocytes showed statistically indistinguishable cleavage rates and embryonic developmental pattern, in contradiction with current literature which seems to favour the use of grade A-like oocytes over that of grade B-like (Esaki *et al.* 2004, Sherrer *et al.* 2004, Bagg *et al.* 2007, Lee *et al.* 2012, Lin *et al.* 2015), further supporting the hypothesis of a wasteful selection process currently in place. Interestingly, oocytes of lesser grades were still able to produce cleavage stage embryos, occasionally. However, both the quantity and the developmental pattern of cleavage stage embryos produced by grade C and grade D oocyte support the hypothesis that

reduced cumulus cell layers during IVM cause reduced embryo developmental competence.

3.5.6 Study limitations, reasons for caution

One of the greatest challenges in this work was establishing a convenient supply of gilt ovaries. Although a reliable supplier was found, the logistics of transportation were such that the abattoir material could not reach the laboratory before 6 hours from culling. It is difficult to find examples in the literature of porcine oocytes used for IVM after such a long interval, and information on the length of time considered acceptable after culling before starting IVM in the pig is scarce. Nevertheless, one study by Wongsrikeao *et al.* (2005) suggested that ovaries stored at 35°C for 6 hours are still likely to contain developmentally competent oocytes. However, the authors showed that a storage time of 3 hours resulted in a nuclear maturation rate of 72.4%, which fell to 44.8% after 6 hours and to 35.0% after 9 hours of storage. The latter two figures compare well with the results presented in this chapter for the nuclear maturation of grade A and B oocytes and suggest that, potentially, oocytes with higher developmental competence could be obtained by improvements in the logistics of ovary transportation.

Moreover, the difficulty in reliably producing blastocyst stage embryos detracts from a comprehensive evaluation of oocyte developmental potentials across the four groups in this study. While grade A and B oocytes outperformed grade C and D oocytes by day 3 of development in terms of cleavage stage embryos produced, it could be that a difference between grade A and grade B oocytes would only emerge past the 8-cell stage and was therefore missed in the current tests.

Furthermore, the IVM system in this study made use of pFF, which is known to enhance the maturation of porcine oocytes (Ocampo *et al.* 1993). However, being a physiological fluid extracted from abattoir material derived from animals of unknown health and fertility, pFF is an obvious source of experimental viability. Moreover, it has been shown that pFF extracted from follicles with a diameter of 3 mm or less contains maturation inhibiting factors such as hypoxanthine (Downs *et al.* 1985). Although the in-house

recovery of pFF was done with care, it was impossible for operators to ensure complete exclusion of follicles smaller than 3 mm, further adding to the variability of the pFF source. Recently, Yuan *et al.* (2017) have demonstrated that high levels of oocyte maturation in the pig can be achieved in a serum-free, fully defined IVM medium containing three key cytokines (FGF2, LIF and IGF1). Although the authors did not directly compare the performance of this new defined medium against a pFF supplemented variant, it is easy to imagine that the availability of a fully defined medium would be superior in providing at least a consistent testing environment. Therefore, its implementation would be an obvious future step to validate further the findings presented.

Finally, the grading system applied in this study was, by its own nature, subjective. Although two independent operators agreed on the final grading of the collected oocytes, a degree of interpretation could not be avoided. This could have caused a partial overlapping between the oocyte grades in this study, especially between oocytes belonging to the two central groups B and C. Whilst this could have contributed additional variability to the experimental setup, it did not seem to prevent a sound statistical evaluation of the four oocyte populations in this study.

3.6 Conclusions

As mentioned in the introduction, porcine IVM is still a very active field of research. Because the limitations in the embryonic development observed during this set of experiments made it impossible to test more complex IVP techniques, like embryo splitting, alternative methods to increase the yield of porcine IVP embryos were considered, such as the better utilization of the resources already in place.

It was found that oocytes clad by only two layers of cumulus cells (grade B) possess considerable developmental competence as they performed to levels statistically comparable to oocytes clad by 3+ layers of cells (grade A) in all investigations. The use of grade B oocytes in porcine IVP should therefore be encouraged, especially when embryo yield is a priority. Such practice would be expected to reduce wastage and

improve the number of embryos produced per donor. Exactly as it is the case for embryo splitting, this consideration becomes more relevant the higher the genetic merit of the donor. Additionally, the application of a single parameter oocyte screening method, such as testing on cumulus investment alone, would reduce inter-operator variability and simplify the collection process.

Based on the evidence presented in this chapter, then, pig oocyte screening for IVM should be simplified to a two-tier system with only oocytes clad by 2+ layers of cumulus cells being of interest for further manipulation. Taken together, the figures presented imply that, under the culture system here described, when only grade A oocytes are used for IVP, just about 8.3% of the starting oocyte population will result in a cleavage stage embryo. Nevertheless, this figure improves to 18.8% with the inclusion of grade B oocytes, a 230% yield increase. However, before accepting these results, a closer comparison between grade A and grade B oocytes should be performed after the establishment of a more reliable culture system, which would allow for a complete analysis of embryo developmental potentials.

4. Specific Aim 2: To test a variety of embryo splitting strategies in the bovine model in order to identify the optimal methodology for the production of a high number of viable blastocysts.

4.1 Background

Embryo splitting has the potential to offer significant benefits to cattle IVP by increasing the number of embryos available for transfer and therefore increasing embryo transfer success rates (Kippax *et al.* 1991). At the same time, it can multiply the offspring of the most valuable gamete donors, allowing for improved rates of genetic gain in the breeding population (Nicholas & Smith 1983). Additionally, the availability of IVP twin embryos could simplify PGS for sex selection or for estimating the breeding value of the embryo (Le Bourhis *et al.* 2010) since a single test would be simultaneously informative for each set of twins.

Bovine embryo splitting was firstly developed in the 1980s (Willadsen 1980; Willadsen & Polge 1981) and it has been successfully applied on both cleavage stage (Johnson *et al.* 1995) and blastocyst stage embryos (Lopes *et al.* 2001). Although, surprisingly, no previous study has compared the developmental potentials of cleavage stage and blastocyst stage embryos produced and split under the same IVP system, and such a comparison might be useful to identify the strategy most likely to produce the greatest yield of viable, transferable embryos. Moreover, a comprehensive evaluation of the developmental potentials of 8-cell stage bovine embryos split according to different ratios has not been reported in the literature, and a previous report failed to test 8-cell stage embryos split in more than two parts (Loskutoff *et al.* 1993).Furthermore, the suitability for embryo splitting of day 3 post-IVF embryos with asymmetrical cell numbers (7 to 14) is equally under-reported.

Additionally, a question arises as to whether it would be possible to perform successfully the splitting of an embryo derived from a previous split, so-called "serial splitting". The underlying hypothesis is that application of serial splitting could lead to the production of embryos with higher viability when compared to single splitting strategies due to the embryos having more time to recover after each reductional event. A study that has investigated the effectiveness of serial splitting in the murine model found that the blastulation rate of first splits derived from cleavage stage embryos was maintained to comparable levels for second splits, but fell sharply with the third split resulting in a loss in the number of embryos produced (Illmensee *et al.* 2006). However, a similar investigation has never been performed in cattle.

Moreover, as discussed in the introduction, in mice no difference has been found in the morphokinetic development of 2-cell split embryos as compared to control embryos, providing evidence for the existence of a "developmental clock" that synchronises early embryonic developmental events without necessarily accounting for cell numbers (Morris *et al.* 2012). A more recent study performed on human embryos found similar evidence (Noli *et al.* 2015). However, comparable reports for cattle appear absent in the literature and while the existence of this "clock" in mammalian embryos is acknowledged, its underlying mechanisms are not completely understood (Martinez Arias *et al.* 2013; Noli *et al.* 2015).

Intuitively, embryo transfers followed by live births are the only method to obtain a definitive evaluation of embryo viability (Van Soom *et al.* 1997). However, this level of evaluation requires extended periods of time and is burdened with considerable material and logistic costs (Amann 2005; Hansen 2006). As a result, researchers have employed a variety of both invasive and non-invasive methods to estimate the viability of IVP embryos. Among these, embryo morphology (Lindner & Wright 1983) and morphokinetics (Holm *et al.* 1998), blastulation rates (Chian *et al.* 2004), cell counts in the blastocyst (Loskutoff *et al.* 1993, Vajta *et al.* 2000) and different techniques for the assessment of TE/ICM cell count ratio (Handyside & Hunter 1984; Thouas *et al.* 2001). For the latter test in particular, methods for the detection of TE specific markers have

also been developed which offer the additional advantage of providing an assessment of the differentiation status of the tissues in the embryo. For example, CDX-2 is a nuclear transcription factor whose expression in the TE is essential for the proper differentiation of TE and ICM cells in the blastocyst (Niwa *et al.* 2005; Strumpf *et al.* 2005). Immunostaining for CDX-2 has been used to obtain separate TE and ICM cell counts in several mammals, including cattle (Ono *et al.* 2010, Su *et al.* 2012).

In an attempt to close some of the gaps identified in the literature review above, the aim of this chapter was to use blastulation rates and cell counts in blastocysts to identify the embryo splitting strategy likely to yield the highest number of viable embryos and to determine whether cleavage stage embryos with asymmetrical cell numbers are still suitable for splitting. Moreover, the developmental potentials of serially split embryos were also investigated in a similar way. Finally, to test the hypothesis that the developmental clock is affected by cell removal in bovine embryo splits, a time-lapse system (PrimoVision) was used to track the development of both control and split embryos.

4.2 Specific aims

With reference to the background above, the specific aims of this chapter were:

2a. To develop simple protocols for the splitting of bovine embryos at the 2-cell stage, 8-cell stage, and blastocyst stage.

2b: To test the hypothesis that embryo splits derived from symmetrical and asymmetrical cleavage stage embryos possess similar developmental potentials.

2c. To identify the single embryo splitting strategy likely to produce the greatest blastocyst yield under the same IVP system.

2d. To use cell counts to assess the viability of blastocysts produced according to different embryo splitting strategies.

2e. To test the hypothesis that embryo splits have reduced cell numbers when compared to intact embryos of the same age.

2f. To test the hypothesis that the serial splitting of bovine embryos would lead to the production of higher number of blastocyst stage embryos when compared to single splitting strategies.

2g. To test the hypothesis that embryo splits display an accelerated developmental rate as compared to unsplit control embryos.

4.3 Methods

In vitro produced bovine embryos (refer to section 2.2.2) were split at either the 2-cell stage, the 8-cell stage (refer to section 2.2.3.2), or the blastocyst stage (refer to section 2.2.3.3) in an attempt to determine the most effective splitting strategy. A grand total of 740 embryo splits were produced across different splitting methodologies and were derived from 239 intact embryos over n=21 IVP rounds, including at least 4 replicates for each test. To investigate the viability of blastocysts derived from embryo splits, cell counts were obtained by either CDX-2 immunostaining (refer to section 2.2.4) or by simple nuclear staining (refer to section 2.2.5) on both control and split embryos. In another set of tests, embryos derived from a previous split were split again (refer to sections 2.2.3.4 and 2.2.3.5) to explore the efficiency of serial embryo splitting. Moreover, split and control embryos were cultured in a PrimoVision system to annotate the timing of several developmental landmarks (refer to section 2.2.5).

4.4 Results

4.4.1 Specific aim 2a: to develop simple protocols for the splitting of bovine embryos at the 2-cell stage, 8-cell stage, and blastocyst stage.

Protocols for the splitting of embryos at both the cleavage and the blastocyst stage were drafted employing only a minimal range of components to allow for their potential application under field conditions. For both 2-cell and 8-cell stage embryo splitting, a relatively brief exposure to a Ca2+ and Mg2+ free medium, like PBS, was found to be sufficient to favour the complete disaggregation of the blastomeres after the zona digestion. This approach appeared to be reliable as 100% of the 2-cell stage embryos (n=44) and 97.5% (n=120) of the 8-cell stage embryos tested yielded at least one morphologically normal split.

Moreover, during the splitting of blastocyst stage embryos, PBS was found to favour cohesion between the blastocyst and the plastic dish, keeping the sample in place and eliminating the need for a holding pipette during the splitting procedure. Again, the splitting method appeared robust, with 95.7% of the bisections (n=46) appearing normal.

For serial splitting, 95.1% of the first split embryos (n=61 derived from n=29 intact embryos) survived to produce at least one serially split embryo, suggesting the protocols in place were indeed suitable. Examples of successfully split embryos are presented in figure 4.1.A breakdown of the methods employed has been presented under figure 2.3.

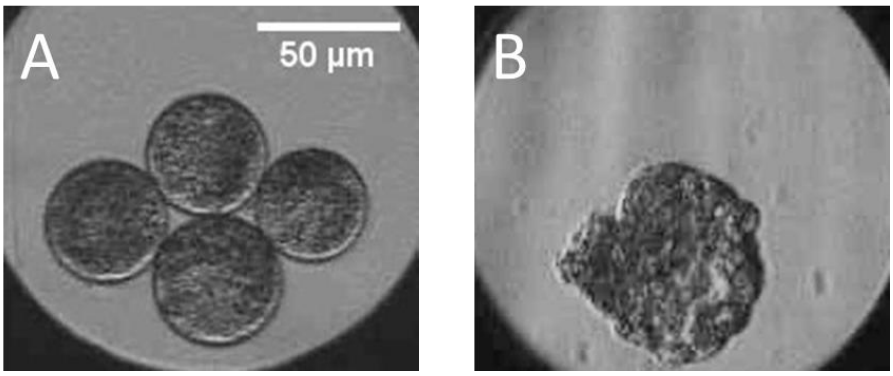


Fig. 4.1 – Embryo splits produced following different strategies. A) A 4/8 type split produced by blastomere separation from an 8-cell stage embryo. B) An embryo split derived from the bisectioning of a blastocyst, the embryo appears collapsed, a normal occurrence immediately after the cut. Images captured by phase contrast microscopy at x200 total magnification in a well-of-the-well culture system.

4.4.2 Specific aim 2b: to test the hypothesis that embryo splits from symmetrical and asymmetrical cleavage stage embryos possess similar developmental potentials.

A splitting ratio can be calculated by dividing the number of blastomeres pooled after disaggregation of cleavage stage embryos by the number of blastomeres originally present in the intact embryo. By this logic, all symmetrical 8-cell stage splits had a splitting ratio of either 0.125, 0.25 or 0.50 for the 1/8, 2/8 or 4/8 type splits, respectively. However, the actual splitting ratio range across all tests on 8-cell stage embryos fell between 0.08 and 0.57 when asymmetrical splits are taken into account. As presented in figures 4.2 and 4.3, preliminary regression tests were performed to investigate the effects of the splitting ratio on both blastulation rates and cell numbers in the resulting blastocysts. Because the formation of a blastocyst from an embryo split is a binary event, the effects of the splitting ratio on it were measured by logistic regression which showed a highly significant association (χ^2_1=29.6650, O.R.=59.9, $P<1x10^{-4}$). Conversely, the relationship between cell numbers present in a blastocyst and splitting ratio was investigated by linear regression, which showed a moderate but highly significant association (R^2=0.256, F=42.43, $P=1.7x10^{-9}$).

In view of this preliminary result, and to test the hypothesis that asymmetrical cleavage stage embryos had similar developmental competence as compared to symmetrical embryos, the asymmetrical embryos were divided in three groups based on which symmetrical splitting ratio they were closest to, then compared to their symmetrical counterparts. A summary of these test groups is presented in table 4.1.

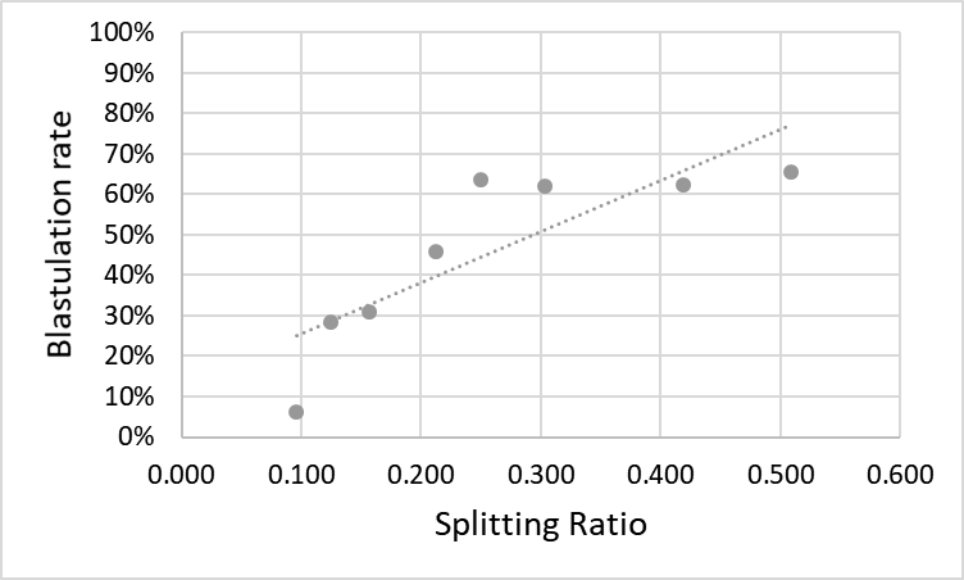


Fig. 4.2 – Variation in the blastulation rate of 8-cell stage splits in accordance with the splitting ratio. A significant correlation was detected by logistic regression between the two variables. For display purposes, in this graph grouping of data was operated and a trendline was given to guide the reader. However, logistic regression was performed on the raw, ungrouped data.

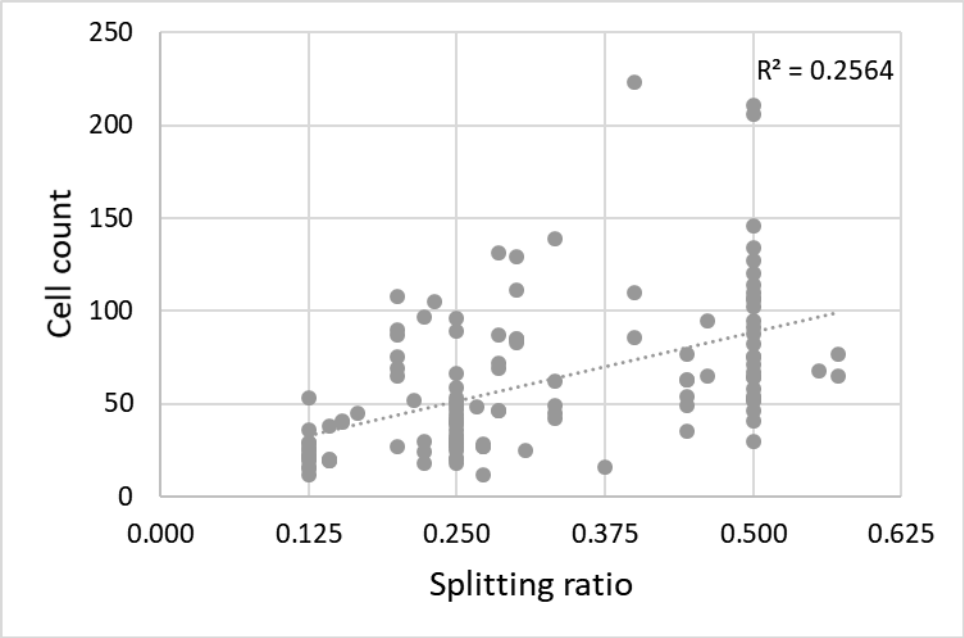


Fig. 4.3 – Variation in the cell count of blastocysts derived from 8-cell stage splits in accordance with splitting ratio. A direct correlation appears to be present between the two variables in study.

Group	Splitting ratio	Sample size	Group	Splitting ratio range	Sample size
4/8 symmetrical	0.50	45	4/8 asymmetrical	0.40 – 0.57	66
2/8 symmetrical	0.25	55	2/8 asymmetrical	0.20 – 0.36	95
1/8 symmetrical	0.125	53	1/8 asymmetrical	0.08 – 0.18	45

Table 4.1 – Symmetrical and Asymmetrical embryo split grouping. The different test groups are presented.

4.4.2.1 Comparison between symmetrical and asymmetrical 4/8

The embryos belonging to the 4/8 symmetrical group had an overall higher blastulation rate (75.5%) as compared to the 4/8 asymmetrical group (62.1%); however, this difference was not statistically significant (chi-square, χ^2_1=2.20, P=0.14). Furthermore, when cell counts were obtained for blastocysts belonging to the two groups, the 4/8 symmetrical and 4/8 asymmetrical embryos had an average count of 93.5 and 82.6 cells/embryo, respectively; but again, this difference was not significant (t-test, t_{41}=0.81, P=0.42).

4.4.2.2 Comparison between symmetrical and asymmetrical 2/8

In similarity to the previous test, embryos belonging to the 2/8 symmetrical group had an overall higher blastulation rate (65.4%) as compared to the 2/8 asymmetrical group (52.6%); but this difference was not statistically significant (chi-square, χ^2_1=2.34, P=0.13). However, when cell counts were obtained for blastocysts belonging to the two groups, the 2/8 symmetrical and 2/8 asymmetrical embryos had an average respective count of 40.7 and 64.0 cells/embryo, and this difference was found to be highly significant (t-test, t_{56}=3.12, P=0.003).

4.4.2.3 Comparison between symmetrical and asymmetrical 1/8

Furthermore, embryos belonging to the 1/8 symmetrical group had an overall higher blastulation rate (28.3%) as compared to the 1/8 asymmetrical group (22.2%); however, this difference was not statistically significant (chi-square, χ^2_1=0.47, P=0.49). Finally, the 1/8 symmetrical and 1/8 asymmetrical embryos had an average count of 25 and 30 cells/embryo, respectively; but again, this difference was not significant (t-test, t_{19}=1.06, P=0.30).

4.4.3 Specific aim 2c: to identify the embryo splitting strategy likely to produce the greatest blastocyst yield under the same IVP system.

Because the results presented in section 4.4.2 supported the hypothesis that asymmetrical embryo splits perform at least as well as symmetrical embryos in terms of blastulation rates and cell counts, symmetrical and asymmetrical embryos with similar splitting ratios were pooled together for the purpose of statistical analysis in this and the following sections.

On analysis, it was found that the embryos split at the 8-cell cleavage stage had a blastulation rate of 67.6%, 57.3%, and 25.5% for the 4/8, 2/8 and 1/8 type splits, respectively. Conversely, the blastulation rate of splits derived from 2-cell stage embryos was 61.9%. For embryos split at the blastocyst stage, recovery rates were recorded in place of blastulation rates and defined as the proportion of blastocyst bisections that

survived the cut and were able to re-form a blastocoel. In these tests, the recovery rate of the blastocyst bisections was 76.4%. Control experiments performed on 10 IVP rounds on n=520 zygotes, showed that the average cleavage rate achieved by intact control embryos was 81.0 ± 5.6% (C.I. 95%) and that 51.1 ± 6.7% (C.I. 95%) of those embryos that cleaved proceeded to form a blastocyst (n=208).

A chi-square test designed to investigate differences among the blastulation/recovery rates achieved by each test group as compared to standard IVP found a highly significant difference (χ^2_5=64.06, P=1.8x10^{-12}). To clarify which groups performed to statistically higher levels, further one-to-one comparisons were performed by chi-square analysis applying the Bonferroni correction for multiple tests. Embryos split at the 2-cell stage and blastocyst stage plus 4/8 type splits performed to statistically indistinguishable levels. Type 2/8 splits blastulated as often as type 4/8 and 2-cell stage splits but less often than blastocyst splits. Furthermore, 2/8 and 1/2 type splits blastulated as often as controls. Finally, 1/8 type splits were shown to consistently yield lower blastulation rates as compared to all other groups. These results are summarised in figure 4.4.

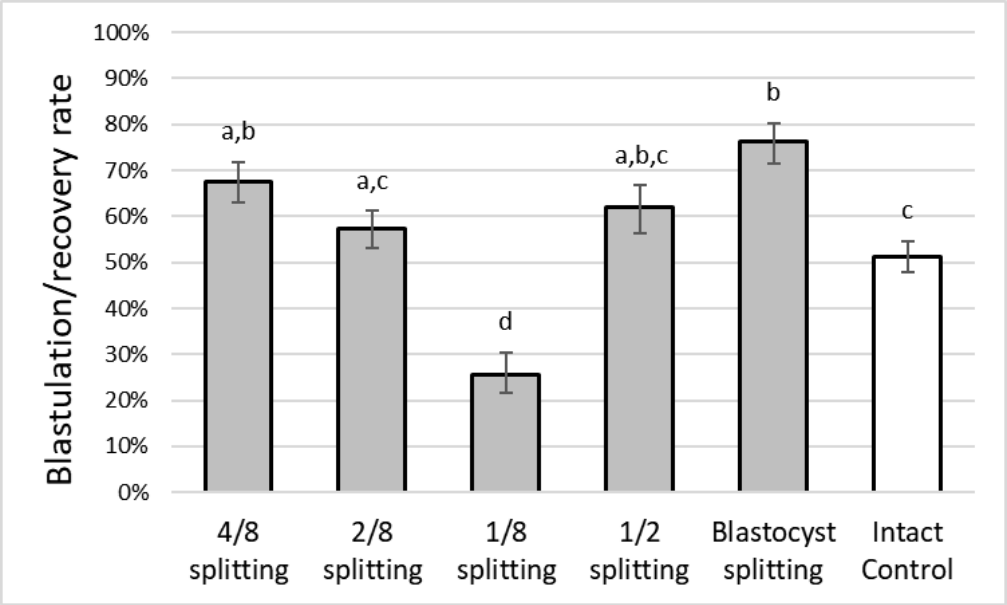


Fig. 4.4 – Blastulation and recovery rates achieved by embryos split following different strategies. While 1/8 type splits consistently underperformed, the other splitting strategies appeared to produce blastocyst stage embryos at similar rates. Averages with different superscripts differ significantly (Chi-square, P < 0.05). Data given as mean ± S.E.M.

However, it is important at this point to highlight that the potential blastocyst yield per original embryo differs according to the strategy used. While 1/2, 4/8 and blastocyst splits can form a maximum of only two blastocysts, type 2/8 splits can potentially result in the formation of up to four blastocysts and, similarly, 1/8 types splits can form up to eight. Therefore, a test group with a slightly decreased blastulation rate could still yield a greater net blastocyst output simply thanks to its greater splitting potentials. Indeed, after accounting for cleavage rates, splitting efficiency, and blastulation/recovery rates, the strategy with the greatest final blastocyst output per starting fertilised egg was the splitting of an 8-cell stage embryo in four parts (2/8 splits) which led to the production of 1.8 blastocysts per zygote, a 3.4-fold increase as compared to control IVP cycles which only produced 0.4 blastocysts/zygote. Remarkably, the 1/8 type splitting still produced 1.6 blastocysts/zygote, while 4/8 and 1/2 type splits showed similar rates of 1.1 and 1.0 blastocysts/zygote, respectively. Finally, blastocyst splitting performed only slightly better than unsplit controls, yielding 0.6 blastocysts/zygote. Theses finding are shown in figure 4.5.

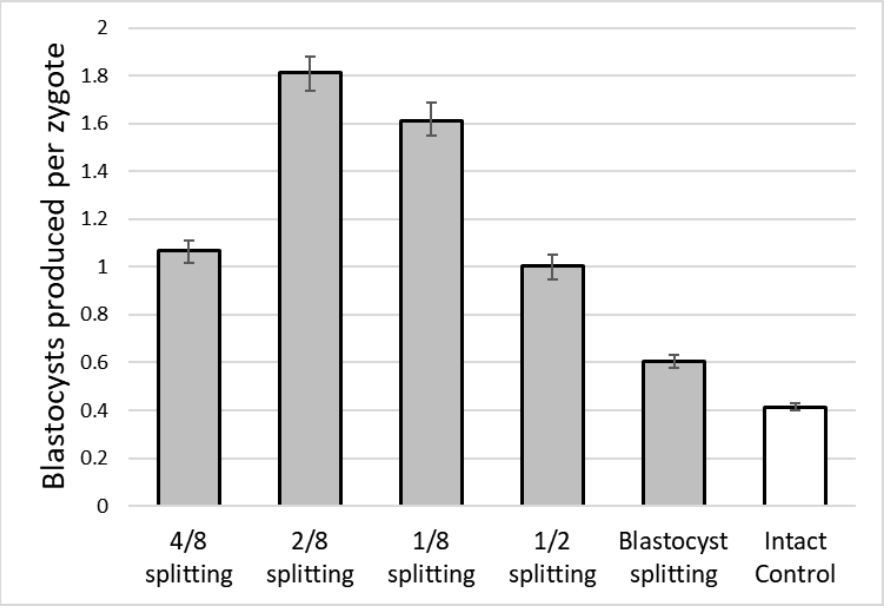


Fig. 4.5 – Projected blastocyst yield for different embryo splitting strategies. When the potentials of each different strategy and its efficiency were taken into account, a 2/8 type split was the strategy that yielded the greatest number of blastocysts per starting zygote. Data given as mean ± S.E.M.

4.4.4 Specific aim 2d: to use cell counts to assess the viability of blastocysts produced according to different embryo splitting strategies.

On a first approach, immunostaining for the nuclear transcription factor CDX-2 was used as a method to assess the number of cells present in a blastocyst and to attempt to obtain separate counts for TE and ICM cells. However, as shown in figure 4.6A, this method was not satisfactory since it failed to establish a clear differential stain between the two cell populations. Therefore, total cell counts were estimated instead by using a simple nuclear staining procedure (figure 4.6B).

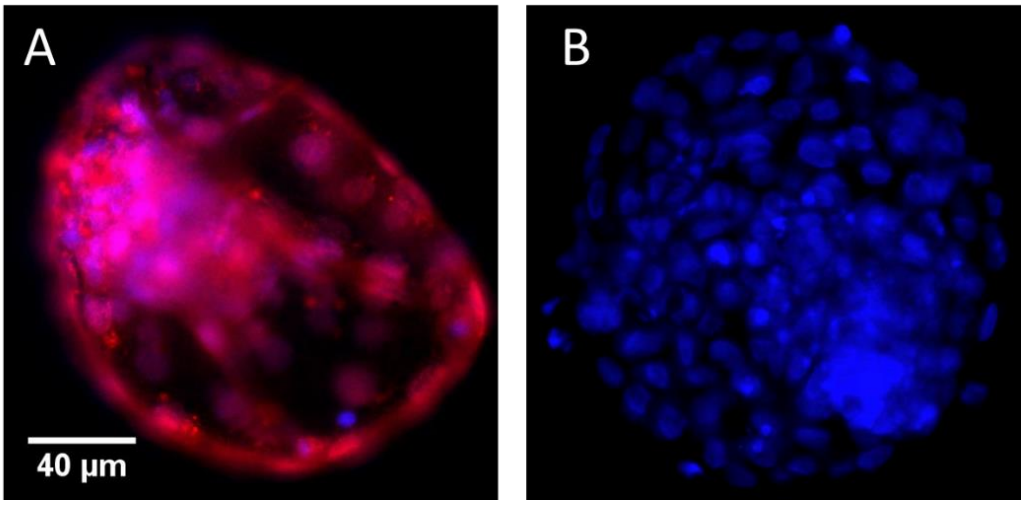


Fig. 4.6 – Blastocyst staining for cell count estimation. Blastocysts were stained using two alternative strategies. A) Immunostaining for CDX-2. Cells expressing CDX-2 were stained in red, and Hoechst 33342 (blue) was used a nuclear counterstain. CDX-2 appeared to co-localise with Hoechst 33342 in all cells indicating that the immunostaining had no ability to discriminate between trophectoderm and inner cell mass cells. B) Simple nuclear staining by Hoechst 33342, all nuclei stained in blue. This approach consistently allowed for total cell counts to be estimated. Images captured by fluorescence microscopy at x200 total magnification.

When total cell counts were obtained from n=82 unsplit control blastocysts 7 days post-IVF, it was found that, on average, they contained 122.4 ± 12.1 cells (C.I. 95%), compared to an average count of 87.7 ± 13.2 (C.I. 95%) for 4/8 splits, of 54.3 ± 7.7 (C.I. 95%) for 2/8 splits, of 27.0 ± 4.7 (C.I. 95%) for 1/8 splits, of 80.7 ± 8.6 (C.I. 95%) for 1/2 splits and finally of 70.7 ± 9.0 (C.I. 95%) for blastocyst stage splits as summarised in figure 4.7; moreover, examples of blastocysts derived from 4/8, 2/8 and 1/8 type splits are given under figure 4.8 These findings were compare statistically by using a one-way ANOVA test followed by a Tukey-Kramer *post-hoc* test. The ANOVA test showed a highly

significant difference between the six populations studied ($F_{5,308}$=30.1, P=6.4x10^{-25}) and the results of the *post-hoc* test are presented in figure 4.7. Briefly, no groups had as many cells as the intact control blastocysts, while 1/2, 4/8, 2/8 and blastocyst type splits performed to various degrees of similarity between them. Finally, 1/8 type splits had statistically fewer cells than any other group.

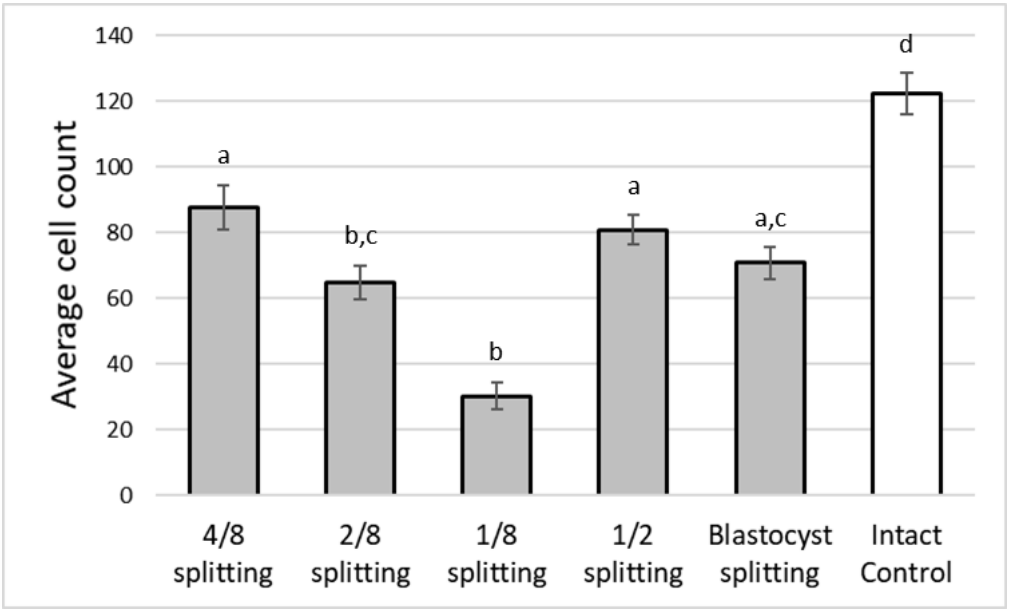


Fig. 4.7 – Average cell counts in day 7 post-IVF blastocysts produced according to different embryo splitting strategies as compared to unsplit controls. Averages with different superscripts differ significantly (Tukey-Kramer *post-hoc* test, P < 0.05). Data given as mean ± S.E.M.

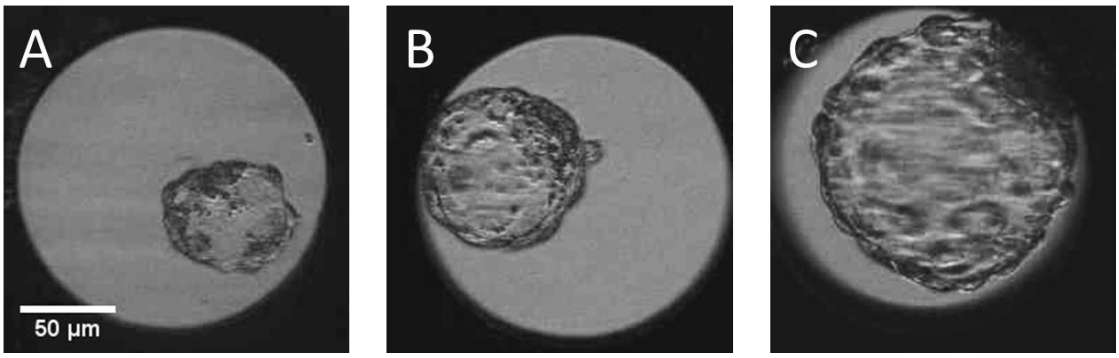


Fig. 4.8 – Example blastocysts derived from different splitting strategies. A) Blastocyst derived from a 1/8 type split. B) Blastocyst derived from a 2/8 type split. C) Blastocyst derived from a 4/8 type split. All the pictures were captured 168 h after IVF using a PrimoVision EVO microscope. The splitting ratio appears to have a clear effect on blastocyst size.

4.4.5 Specific aim 2e: to test the hypothesis that embryo splits have reduced cell numbers as compared to intact embryos of the same age.

To test the hypothesis that in blastocysts derived from embryo splits, cell counts are reduced as compared to unsplit controls by a factor identical to their splitting ratio (for example that a 4/8 type blastocyst with a splitting ratio of 0.5 possesses half as many cells as a blastocyst derived from an unsplit embryo), cell counts from split embryos were compared to cell counts from control blastocysts reduced by an appropriate factor (for example, by a factor of 4 when comparing against 2/8 type splits). When the groups were compared two by two by a classic student t-test, it was found that embryo splits had statistically greater cell counts than expected in all cases apart for blastocyst stage splits (T_{143}=1.78, P=0.08). The detailed results of this analysis are presented in figure 4.9.

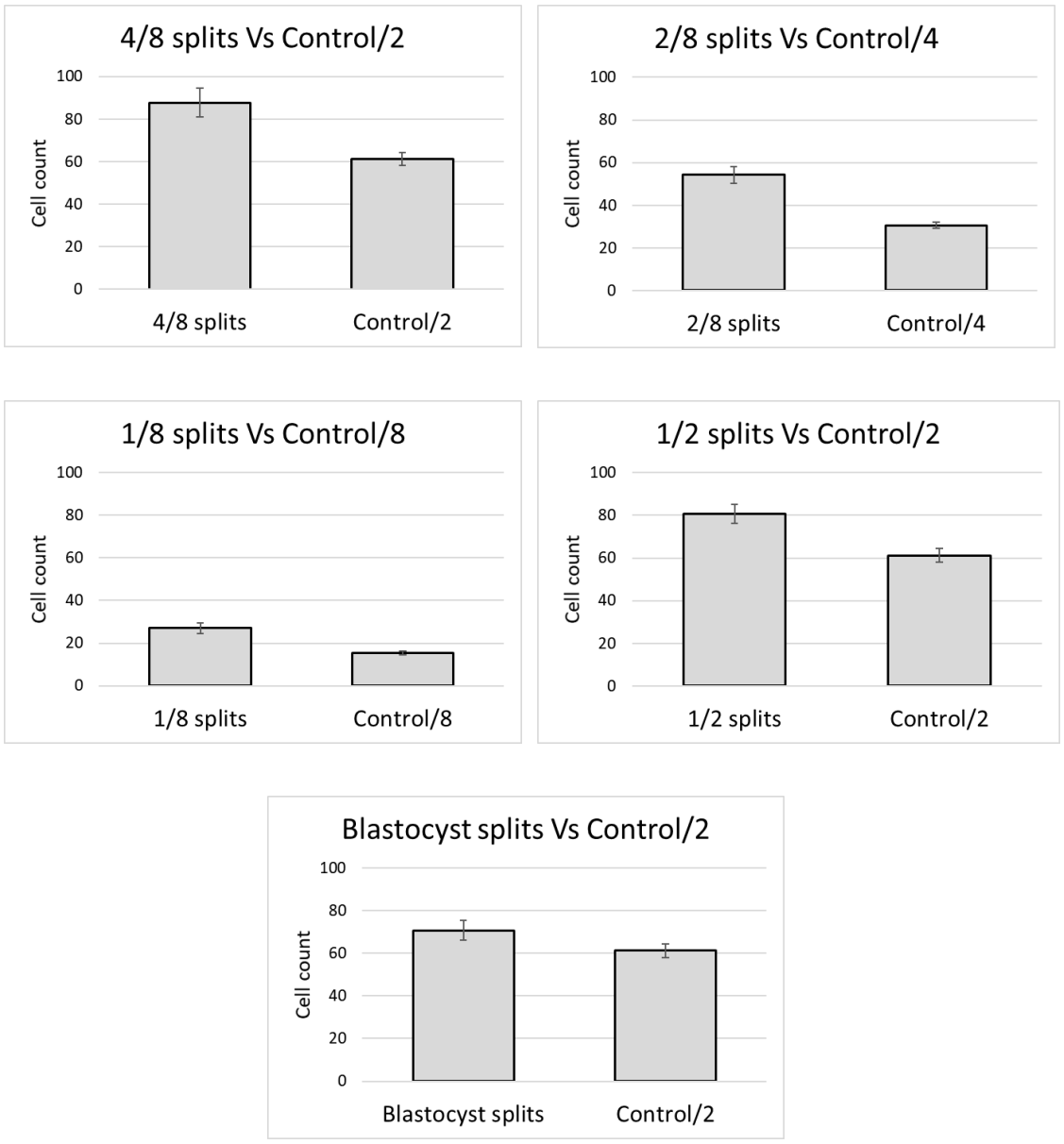


Fig. 4.9 – Series of ideal comparisons between cell counts in blastocysts derived from embryos split according to different strategies and control blastocysts the counts of which were reduced by an appropriate factor. Cell counts form intact control embryos were divided by 2 for comparison against 4/8, 1/2 and blastocyst type splits; by 4 for comparison against 2/8 type splits and by 8 for comparison against 1/8 type splits. Embryo splits had statistically more cells than these ideal controls in all cases but for blastocyst stage splits. Data given as mean ± S.E.M.

4.4.6 Specific aim 2f: to test the hypothesis that the serial splitting of bovine embryos would lead to the production of higher number of blastocysts stage embryos as compared to single splitting strategies.

Three different strategies for the serial splitting of bovine embryos were developed and tested. In strategy A, embryos derived from a 2/8 type split (1st serial split) would be split again at the blastocyst stage to produce 2nd serial splits. In strategy B, embryos were split at the 2-cell stage (1st serial split), then again after two cleavage events (8-cell sage equivalent) to produce 2nd serial splits then allowed to develop. In strategy C, embryos were split at the 2-cell stage (1st serial split), again after cleavage (4-cell sage equivalent), and again after the following cleavage (8-cell stage equivalent) to produce 3rd serial splits, then allowed to develop. For all strategies, the splits were performed symmetrically, and blastulation rates and cell counts were recorded.

4.4.6.1 Strategy A

Strategy A appeared largely unsuccessful. N=11 2/8 type splits that had reached the blastocyst stage were split again to produce n=23 serial splits. However, these serial splits showed a recovery rate of just 15% resulting in a loss, rather than a gain in the number of blastocysts produced. Because of this result, strategy A was abandoned in favour of testing strategies B and C instead.

4.4.6.2 Strategies B and C

The application of strategy B led to the production of n=67 2nd serial splits which blastulated with a frequency of 44.7%. The average cell count in the blastocysts produced was 37.9 ± 6.3 cells/embryo (C.I. 95%). Conversely, strategy C led to the production of n=116 3rd serial splits that developed to the blastocyst stage at a rate of 24.1%. On average, the blastocysts produced in this way only contained 15.0 ± 2.0 cells/embryo (C.I. 95%).

Because, potentially, strategy B could lead to the production of up to 4 blastocysts from a single embryo, and strategy C could lead to the production of 8; their performance was

compared to the 2/8 and 1/8 splitting strategies, respectively, which had identical potentials. When efficiency, cleavage and blastulation rates were accounted for, strategy B led to a production of 1.4 blastocysts per starting zygote, while strategy C produced 1.5 blastocysts/zygote. When appropriate statistical tests were run between single and serial split strategies, it was found that the blastulation rate in strategy B did not differ significantly from the blastulation rate achieved by 2/8 type splits (chi-square, χ^2_1=2.93, P=0.09); similarly, there was no difference between the blastulation rates of strategy C and 1/8 type splits (chi-square, χ^2_1=0.05, P=0.82). However, when cell counts were taken into account, it was found that both 2/8 type splits and 1/8 type splits had a greater number of cells at the blastocyst stage as compared to strategy B (t-student, T_{83}=2.64, P=9.7x10^{-3}) and strategy C (t-student, T_{43}=4.82, P=1.8x10^{-5}) produced embryos, respectively. These findings are summarised in figures 4.10 and 4.11.

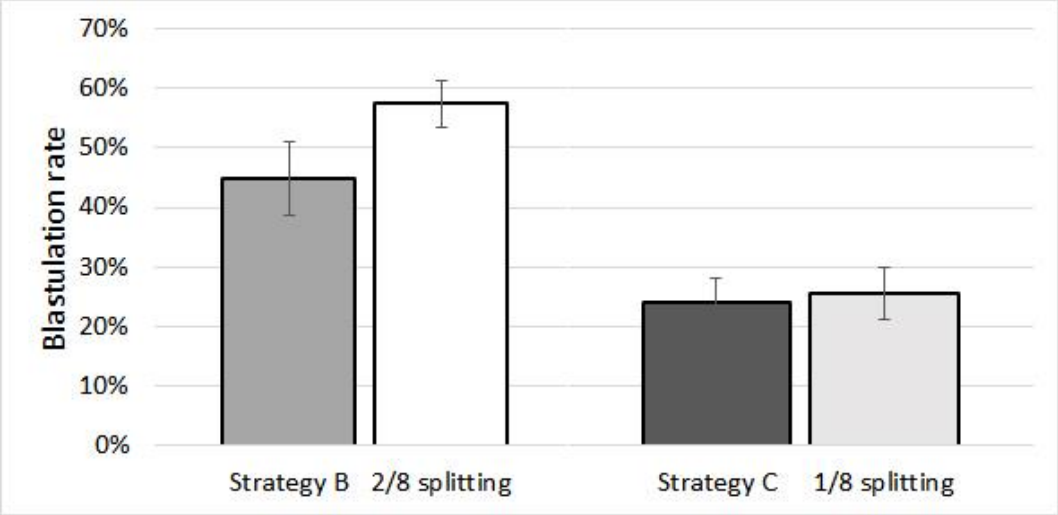


Fig. 4.10 – Blastulation rates for embryos produced by serial splitting as compared to embryos produced by single splitting strategies. Embryos split according to serial splitting strategy B and C blastulated to statistically similar rates to embryos produced by comparable single splitting strategies. Data given as mean ± S.E.M.

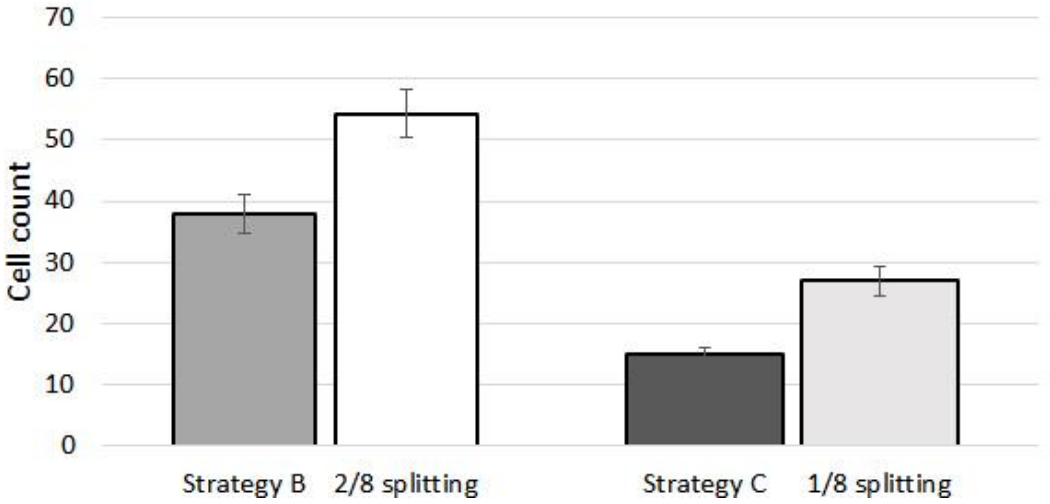


Fig. 4.11 – Average cell counts in blastocysts produced by serial splitting as compared to embryos produced by single splitting strategies. Blastocysts derived from single splitting strategies consistently showed higher cell counts when compared to embryos produced by serial splitting. Data given as mean ± S.E.M.

4.4.7 Specific aim 2e: to test the hypothesis that embryo splits display an accelerated developmental rate as compared to unsplit control embryos.

To test the hypothesis that cleavage stage embryo splits display a different development rate when compared to intact embryos, a morphokinetic analysis was completed on a number of developmental landmarks as appropriate for each group, which included timing of 2nd and 3rd cleavage, compaction, onset of cavitation, blastocyst expansion. It was found that the timing of 2-cell stage embryo splits compared well with intact control embryos up to the 3rd cleavage division (one-way ANOVA, df=1,57, P>0.05), however, 2-cell stage splits started the process of compaction earlier than both 8-cell stage splits and controls (one-way ANOVA, $F_{2,87}$=24.36, P=3.97x10^{-9} followed by Tukey-Kramer *post-hoc* analysis) and were similarly quicker than the other two groups in forming a blastocoel and then an expanded blastocyst (one-way ANOVA, df=1,57, P>0.05). While 8-cell stage splits compacted later as compared to controls, they formed a blastocoel and an expanded blastocyst with similar timing as controls (one-way ANOVA, df=2,87, P>0.05 followed by Tukey-Kramer *post-hoc* analysis). The results of this analysis are displayed in figure 4.12.

Additionally, the developmental timings of embryo splits derived from the same original embryo (twins) were compared to investigate whether twin embryos develop at the same rate. For each pair of twins (n=9 pairs for 2-cell stage splits, n=11 pairs for 8-cell stage splits) the difference in time required to achieve the same developmental stage was recorded. The results of this investigation are presented in table 4.2.

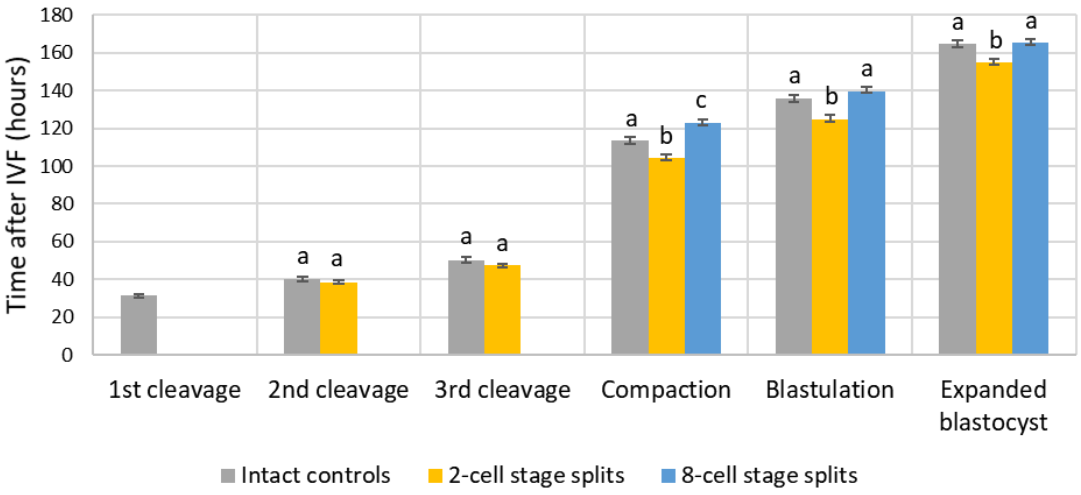


Fig. 4.12 – Timing of developmental landmarks in control and cleavage stage split embryos. For each event, columns with different superscripts differ significantly (Tukey-Kramer *post-hoc* test P<0.05). Data given as mean ± S.E.M.

	2-cell stage splits	8-cell stage splits
2nd cleavage	53.6 ± 48.2 min	n/a
3rd cleavage	70.0 ± 48.3 min	n/a
Compaction	495.8 ± 495.3 min	781.8 ± 299.8 min
Cavitation	469.3 ± 213.6 min	535.8 ± 273.8 min
Expansion	362.5 ± 138.5 min	595.8 ± 313.2 min

Table 4.2 – Difference in the time required by twin embryos to achieve the same developmental landmark. Results are given as average difference between two twins for each group and confidence intervals at 95% are shown.

4.5 Discussion

Only 2-cell stage and 8-cell stage embryos were employed for cleavage stage embryo splitting while 4-cell stage embryos were not. This decision was made based on the morphokinetics of bovine cleavage stage embryos. The first cleavage division is easy to identify and 2-cell stage embryos could be reliably found in culture about 30 h post-IVF. However, most 4-cell stage bovine embryos developed overnight, precluding a suitable window for testing. On the other hand, 8-cell stage embryos are known to become prevalent in culture about 50 h post-IVF and remain prevalent until about 100 h post-IVF (Holm *et al.* 1998), therefore this stage provided for a convenient, extended window over which to perform the splitting.

4.5.1 Protocol development for embryo splitting

The removal of the ZP is not known to affect the potential of the embryo to implant and develop (Seike *et al.* 1989) and has been shown to have no significant impact on the ability of bovine embryos to correctly develop when cultured under suitable conditions, for example by employing a WOW culture system (Vajta *et al.* 2000). The high technical efficiency demonstrated by the cleavage stage splitting reported here is in agreement with these previous studies and suggests that cleavage stage embryo splitting can be effectively performed without the need for fine micromanipulation of the blastomeres required to position them into surrogate zone pellucidae (Tagawa *et al.* 2008).

For blastocyst splitting, the same method described by Rho *et al.* (1998) was employed; for which the authors reported a 100% efficiency for their cutting strategy as compared to the 95.7% reported here. In both cases, a micromanipulation platform was used to deliver the cuts. While this approach has been successfully implemented under field conditions (Lopes *et al.* 2001), it restricts the use of this technique only to laboratories able to afford the expensive instrumentation required. However, it is possible to find examples of successful bisectioning protocols employing simple, custom-made splitting instruments (Müller & Cikryt 1989), while some authors hare reported hand-made

splitting techniques that did not require any micromanipulation tools (Wu *et al.* 1995, Velasquez *et al.* 2016) broadening the applicability of this method.

4.5.2 Developmental potentials of asymmetrical splits

The applicability of cleavage stage embryo splitting to embryos with atypical cell numbers has been largely underreported in literature. However, the results presented support the hypothesis that asymmetrical embryos are able to survive the splitting and develop to form blastocyst stage embryos as often as symmetrical embryos and that those blastocysts will be of similar or even better quality in the case of 2/8 type splits as judged by total cell counts.

On the other hand, the performance of embryos split at the 8-cell stage seems to be directly correlated to their splitting ratio, as shown in figures 4.2 and 4.3. Therefore, it is conceivable that asymmetrical embryos with a smaller splitting ratio would underperform as compared to symmetrical embryos and that asymmetrical embryos with a greater splitting ratio would outperform similar symmetrical splits, and that therefore the asymmetrical group would perform just as well as the symmetrical group, on balance. In summary, the results presented support the use of asymmetrical embryos for embryo splitting, but caution might be advisable when the resulting splitting ratio does not exceed that of the most similar possible symmetrical split.

4.5.3 Embryo splitting yield

While several studies have reported on the potential embryo production increase in cattle thanks to embryo splitting (Gary *et al.* 1991; Kippax *et al.* 1991; Lopes *et al.* 2001), no study so far has compared the potentials of a wide variety of strategies under the exact same IVP system.

In these experiments, the splitting of an 8-cell embryo in four parts was the strategy that produced the greatest blastocyst output. This result is in agreement with previous

findings suggesting that a limit of 4 identical twins from a single donor embryo would be difficult to overcome (Willadsen & Polge 1981; Johnson *et al.* 1995; Chan *et al.* 2000).

Interestingly, split embryos largely appeared to perform better than controls in terms of blastulation rates. This consideration could erroneously bring to the conclusion that embryo splitting is beneficial to embryo development. In reality, it is likely that embryo splitting amplifies the number of embryos derived from good quality embryos whilst having no effect on the development of poor quality embryos leading to an apparent increase in the blastulation rates of the splits as compared to the controls. However, in agreement with previous reports (Gray *et al.* 1991; Escriba *et al.* 2002; Tagawa *et al.* 2008), this finding suggests embryo splitting in its different forms is well tolerated by early stage embryos. Additionally, a recent study from Velasquez *et al.* (2016) demonstrated that embryo splits derived from blastocyst bisectioning show no difference when compared to control embryos in terms of developmental capability and gene expression; however, the authors discovered an alteration in the elongation of the split embryos after transfer. Nevertheless, a previous study found no difference in the pregnancy rates between split and intact blastocysts (Gray *et al.* 1991).

4.5.4 Cell counts in embryo splits and control embryos

The total cell count in a blastocyst is an indicator of embryo viability (Wurth *et al.* 1988) and is promptly reduced when embryos are cultured under stressful conditions (Hill & Gilbert 2008). Moreover, Loskutoffl *et al.* (1993) discussed how bovine blastocysts derived from quarter embryos containing about 40 cells could proceed to establish a pregnancy in 20% of cases after transfer, whilst blastocysts containing about 72 cells could establish a pregnancy in 35-40% of cases. Given the results presented in figure 4.7, it seems plausible that embryo splits derived from most of the strategies employed in the present work would preserve enough developmental potentials to yield acceptable pregnancy rates upon transfer. However, this might not be the case for 1/8 type splits which showed a total cell count of just 27 cells/embryo.

It is known that embryo splits possess, in absolute terms, fewer cells than intact control embryos (Heyman 1985; McEvoy & Sreenan 1990); however, the offspring derived from embryo splitting is of normal size (Papaioannou *et al.* 1989) suggesting that embryo splits will catch up on control embryos at some point during development. Interestingly, in these tests, embryos split at cleavage stage consistently demonstrated higher cell numbers than expected when an ideal experiment was performed and they were compared to artificially reduced cell counts from intact embryos. This finding seems to suggest that cleavage stage splits might have an increased mitotic index as compared to control embryos. An alternative explanation might be that the fractions of the embryo that survive are composed of cells of better quality perhaps due to mosaicism in the embryo (Iwasaki *et al.* 1992). However, this hypothesis does not explain well the high cell numbers observed in multiple twins derived from the same embryo. In order to clarify whether the morphokinetics of embryo splits is truly affected by the cut, time lapse-observations might be used. It is also interesting to note that blastocyst stage embryos had similar cell numbers to half of the intact controls. This finding is not necessarily surprising, since this type of splits were only cultured for a short amount of time before cell counts were obtained. However, the specific comparison between blastocyst stage splits and control embryos yielded a P value of 0.08, which is close to the significance threshold, perhaps providing for an indication that the embryo half that randomly received more cells after the initial cut survives more often to re-form a blastocoel.

4.5.5 Serial embryo splitting

Serial embryo splitting in cleavage stage bovine embryos has not been reported before in literature and only one study exists describing cleavage stage serial splitting in mice (Illmensee *et al.* 2006). However, a form of serial embryo splitting has been demonstrated before for bovine blastocysts which were bisectioned two times in short succession to produce quarter embryos (Rho *et al.* 1998). In the present work, it was found that triple serial splitting (strategy C) resulted in a marked decrease in the blastulation rate of the splits as compared with double serial splitting (strategy B), similar to what was described by Illmensee *et al.* (2006). However, in opposition to what

was described by that group, triple embryo splitting in bovine embryos produced an increase rather than a loss in the net output of blastocysts and both double and triple serial splitting performed to similar levels in this respect. Moreover, whilst Rho *et al.* (1998) demonstrated the feasibility of splitting a blastocyst derived from a previous cut, a similar application was unsuccessful in these tests (strategy A). A possible explanation could be that the embryos could not tolerate the cut as well after zona removal or this could have been caused by the increased difficulty in handling a zona-free blastocyst.

Interestingly, both double and triple serial embryo splitting appeared to have no negative effect on blastulation rates as both strategy B and C performed as well as, respectively, type 2/8 and 1/8 splits. However, cell counts were reduced in blastocysts derived from serially split embryos as compared to single splitting strategies. This finding supports the hypothesis that, while not lethal to embryos, serial splitting puts additional strain on them. This could be due to the successive manipulations operated on the same embryo causing it to spend more time outside of a stable incubator environment (Zhang *et al.* 2010b), or it could be due to the serial splits receiving fewer autocrine and paracrine factors which are known to enhance the growth of embryos (Gandolfi 1994) due to the repeated cell reduction and frequent forced media refresh after the splitting.

No information exists in the literature about the expected cell count in bovine blastocysts derived from serially split embryos. However, the impact of serial embryo splitting on the number of cells per blastocyst raises questions about the viability of the embryos produced following these strategies, especially in the case of triple serial splits. While the developmental potentials of blastocysts with much lower cell counts remains unexplored, with reference to the Loskutoffl *et al.* (1993) paper discussed above, it seems likely that the transfer of serially split embryos would establish pregnancies at unacceptably low rates.

As a result, the evidence presented suggests that serial embryo splitting performed in the way here described does not allow embryos enough time to recover from previous splitting events, in contrast with the original hypothesis. On the one hand, triple serial

splitting definitively appears to be a non-viable embryo multiplication strategy and therefore more ambitious serial splitting strategies are likely to fail as well. On the other hand, double serial splitting could be a useful IVP strategy; however, in these tests it still underperformed when compared to 2/8 splitting and had the disadvantage of requiring additional hands-on time.

4.5.6 Developmental clock in early bovine embryos

At time-lapse observation, the developmental timing of the intact control embryos closely matched that of a previously published study (Holm *et al.* 1998) for all the developmental landmarks reported (first three cleavage events, compaction, and blastulation) although the authors provided no information on the timing of blastocyst expansion. Interestingly, though, embryos split at the 2-cell stage displayed a significant increase in their developmental rate after compaction and, on average, produced blastocyst stage embryos 11 hours before the control embryos did.

It is also curious to notice that embryos split at the 8-cell stage compacted later than controls but blastulated with similar timing to them. From time-lapse observation, it appears that the blastomeres of 8-cell stage splits cultured in a WOW system required some extra time to come into contact with each other and to re-form the cell-to-cell junctions destroyed after disaggregation before they were able to proceed to compaction. Conversely, the blastomeres derived from a 2-cell split tended to stay always in contact with each other (they were all derived from cleavage of a single blastomere); so that compaction was only slowed down in 8-cell splits. This observation is in agreement with previous studies performed on mouse embryos, which showed that continuous cell-to-cell interactions are important for the fate specification of blastomeres, and that blastomeres likely require re-establishing these interactions after splitting (Johnson & Ziomek 1981; Lorthongpanich *et al.* 2012).

Moreover, 8-cell stage bovine embryo splits showed enough plasticity to be able to recover from the cell reductional event and blastulate with the same timing as intact control embryos. The finding that disaggregated blastomeres of 8-cell stage embryos

reconstructed in an embryo split in a random order were still capable to produce blastocysts with morphologically distinct cell populations supports both the inside-out (Tarkowski & Wroblewska 1967) and the cell polarity (Johnson & Ziomek 1981) embryo development models which postulate that the fate of blastomeres (ICM or TE) is determined by position-dependent mechanisms, while being in contrast with the pre-patterning model (Piotrowska *et al.* 2001; Piotrowska & Zernicka-Goetz 2001) which instead hypothesises that ICM and TE lineages are pre-determined due to the asymmetrical distribution of molecular determinants in the oocyte.

Finally, when the timing of developmental events between twin embryo splits was considered, the results suggested that the first three cleavage events are under a strict temporal control in bovine embryos, a finding that is in agreement with another study performed in human embryos (Noli *et al.* 2015). However, this stringent regulation seemed to relax after the third cleavage division, leading to twin embryos to behave in unconcordant ways from a temporal standpoint, in similarity to what has been previously reported for mouse embryos (Arav *et al.* 2008). The first blastomere fate decision is thought to become established only after the third cleavage event or possibly at compaction, when spatially oriented asymmetric cell divisions lead to one daughter cell being pushed peripherally (to become TE) and one internally (to become ICM) (Bruce & Zernicka-Goetz 2010; Lorthongpanich *et al.* 2012). The results here presented, then, might suggest that the developmental clock has a role in maintaining the whole embryo at the same pace until the moment of compaction, after which asymmetry becomes necessary to establish different fates in different cells. It might even be possible to imagine for early bovine embryos, a hybrid developmental model where the first few cleavage events are predetermined to happen at a certain pace (in similarity to the pre-patterning model) whilst later fate decision events are position-dependent (as per the inside-out and the cell polarity models). However, possible mechanisms for this interaction remain unknown.

4.5.7 Study limitations, reasons for caution

As discussed in the introduction, without the ability to perform embryo transfers, the results presented in this study must be considered preliminary only as embryo splitting has been only tested *in vitro*.

Moreover, it was impossible to obtain separate cell counts for the ICM and the TE of the produced blastocysts by CDX-2 immunostaining. While this could mean that, in the control embryos tested, CDX-2 was expressed at detectable levels in both TE and ICM nuclei; this would be in contradiction with current evidence suggesting that CDX-2 expression is restricted to the TE in bovine embryos (Ewart *et al.* 2008). A more plausible explanation would be a technical failure due to aspecific antibody binding to the intended target. While blastocyst viability could still be estimated by the use of total cell counts, it is known that embryo splits with very low cell numbers at compaction might develop into trophoblastic vesicles, non-viable structures that mimic the appearance of a blastocyst but lack an ICM (Gardner *et al.* 1973). Although it can be excluded that this phenomenon affected all cases in study, since a clear ICM could be often morphologically identified even with a simple nuclear stain, it is possible that some of these trophoblastic vesicles were erroneously considered blastocysts and included in the results presented, especially for embryos with lower splitting ratios.

4.6 Conclusions

In conclusion, embryo splitting holds promise to dramatically increase the availability of blastocysts for transfer, however the limited ability of the embryo to cope with cell reductional events makes perpetual splitting impossible and likely limits the application of embryo splitting to single splitting strategies and a maximum of four twins. However, the data presented strongly suggest that embryo splitting is a suitable technique to magnify the number of embryos available from a specific donor, which would be of interest to commercial breeders intending to disseminate some specific genetics more rapidly. Moreover, the availability of multiple identical twins, which tend to produce, overall, a greater number of cells at the blastocyst stage as compared to the intact embryo, would simplify PGS by providing multiple samples for genetic screening. Finally,

the availability of twins could benefit research by allowing for stringent case-control experimental designs.

Interestingly, the inability of embryo splitting to increase indefinitely the possible blastocyst yield suggests that this technique has no impact on the embryo's developmental clock.

5. Specific aim 3: To apply optical coherence tomography (OCT) to investigate embryo structure and viability non-invasively.

5.1 Background

To investigate the properties of early embryo development and improve selection of viable embryos for transfer, time-lapse systems have been introduced. Although widespread, the use of these systems has generated controversy in the literature with some studies reporting an increased ability to select for viable embryos (Mesequer *et al.* 2012; Campbell *et al.* 2013), and other studies reporting a limited or a lack of correlation between the morphokinetic parameters measured and clinical pregnancy outcomes (Racowski *et al.* 2015; Goodman *et al.* 2016). Time-lapse systems also require a long time to complete a morphokinetic assessment and suffer from a poor depth of view. The second problem is made worse in post-compaction embryos like blastocysts and in bovine embryos generally due to their unfavourable lipid distribution (Van Soom *et al.* 2003). Moreover, with conventional time-lapse, the embryo cannot easily be observed from different angles, the observation is difficult when the embryo presents overlapping blastomeres or is highly fragmented (Wong *et al.* 2010), and the operator cannot objectively quantify the percentage of fragmentation, which is known to affect embryo viability (Alikani *et al.* 1999).

Therefore, there is scope for the development of new imaging modalities, which can resolve the embryo structure in full depth, provide for a rapid and non-subjective assessment of embryo viability, and improve the understanding of embryonic development. OCT is a non-invasive optical method developed in the 1990s that has historically found application in ophthalmology (Hee *et al.* 1995). It can create static structural images of biological tissues by providing multiple cross-sectional images of a sample, enabling 3D modelling and potentially tracking the fate of specific cells in an embryo (Zheng *et al.* 2012). OCT shows a remarkable ability to penetrate biological tissues, which is unimpeded by the lipid content of some mammalian embryos (Zheng

et al. 2012). Moreover, recent advancements in the OCT field allowed for the display of *en-face* (cross-sectional) images in real time by using a swept source, simplifying sample positioning and image acquisition as compared to earlier OCT platforms and rendering the technique more similar to standard microscopy from an operator's point of view (Podoleanu & Bradu 2013; Cernat *et al.* 2017).

Interestingly, OCT can also be used to acquire functional images, one example being OCTA which is used to detect flowing blood cells thanks to algorithms like SV analysis which can detect and quantify the changes in the pattern of a sample associated with movement (Federici *et al.* 2015; Ruminski *et al.* 2015; Gorczynska *et al.* 2016).

OCT has already found some limited application in developmental biology in model organisms like *Xenopus laevis* (Drexler *et al.* 1999), and *Rattus norvegicus* (Larina *et al.* 2009a). Additionally, in a recent study, high-resolution intracellular imaging on live mouse and pig oocytes and embryos has been reported, representing the first application of OCT to time-lapse (Karnowski *et al.* 2017). In this context, the fundamental advantages of OCT are its ability to image embryos without fluorescent labels, and its use of low power light sources which reduces the chance of damaging the embryo during observation as compared to confocal systems (Zheng *et al.* 2012; Karnowski *et al.* 2017), or even to standard time-lapse systems (Ottosen *et al.* 2007). However, no study so far has applied the motion detection principles of OCTA to early stage embryos.

Here, an experimental SS-OCT microscope capable of real time *en-face* imaging display (Cernat *et al.* 2017) was used to produce a full depth structural characterization of bovine embryos and to measure micron-scale movements within live embryos to obtain a quantitative analysis of embryo viability over time and test the hypothesis that kinetic differences could be measured between live and dead embryos.

5.2 Specific aims

With reference to the background above, the specific aims of this chapter were:

3a. To test the hypothesis that SS-OCT can be used to obtain static images of bovine embryos and reconstruct 3D models.

3b. To test the hypothesis that speckle variance analysis can quantitate micron scale movements of blastocyst stage bovine embryos to infer their vital status.

3c. To test the hypothesis that SS-OCT can be used to obtain non-invasive cell counts from bovine blastocysts.

3d. To test the hypothesis that SS-OCT observation is not harmful to the embryo.

5.3 Methods

Bovine embryos were produced *in vitro* (refer to section 2.2.2). Fixed cleavage stage, and both fixed and living day 7 to 8 post-IVF bovine blastocysts were observed by SS-OCT and their structure and kinetic properties were evaluated (refer to section 2.2.7). Structural characterisation by SS-OCT was performed on n=5 cleavage stage embryos and n=15 blastocysts. Additionally, n=5 live bovine blastocysts were observed by SS-OCT and analysed by SV to obtain kinetic measurements (as per sections 2.2.7.3-4).

5.4 Results

5.4.1 Specific aim 3a: to test the hypothesis that SS-OCT can be used to obtain static images of bovine embryos and reconstruct their 3D models

Both cleavage stage and blastocyst stage embryos were imaged by an SS-OCT system with an approximate resolution of 5 µm axial x 4 µm transverse. From measurements on fixed embryos, the diameter of a cell in a blastocyst was estimated to range between 10 to 40 µm so the resolution available was estimated to be sufficient to resolve single

cells within any embryo. The system was able to produce multiple images from different transversal layers of the embryo simultaneously.

Figure 5.1 illustrates a single *en-face* image from the median layer of a 2-cell stage embryo. While not many features are evident, the image does display the presence of two distinct dark areas, which are indicative of the presence of nuclei. However, nuclei were evident in only two of the five cleavage stage embryos investigated in this way.

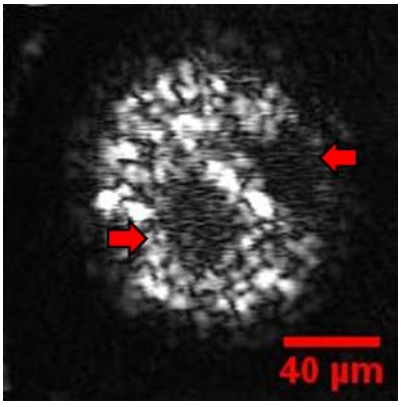


Fig. 5.1 – A 2-cell stage bovine embryo imaged by SS-OCT. While cell boundaries are not evident, the embryo clearly shows two dark areas (arrows) which are consistent with the presence of nuclei.

Conversely, figure 5.2 shows several cross-sections of a bovine blastocyst captured at different depths. In all the blastocysts analysed in this way (n=15) it was possible to identify the classic features of this type of embryo, namely an empty cavity, an inner cell mass and the trophectoderm ring. Additionally, the image stacks produced by OCT could be used to reconstruct 3D models of the imaged embryos. An example of this application is resented in figure 5.3.

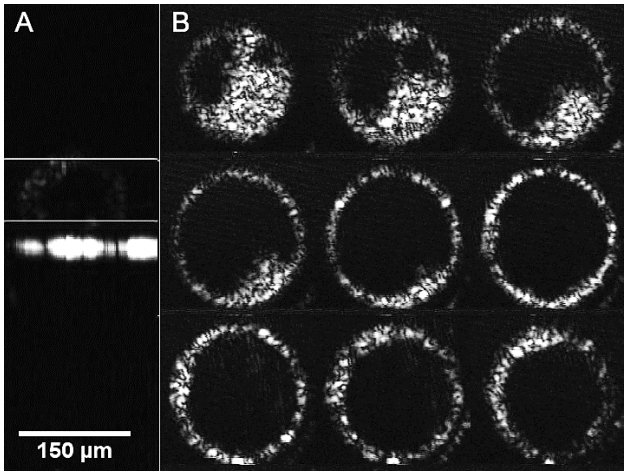


Fig. 5.2 – Bovine blastocysts imaged by SS-OCT. A) Axial view showing a section of the entire embryo. The white lines determine the boundaries used for the transversal analysis. B) Selection of transversal cross-sections at different depths, allowing distinguishing the size, shape, and distribution of the inner cell mass.

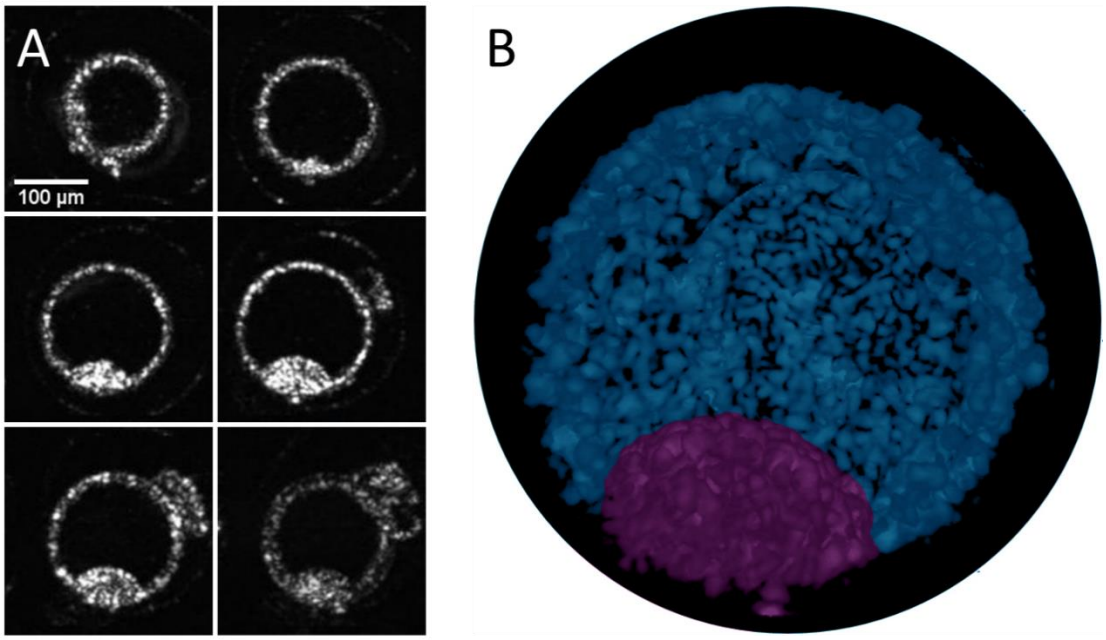


Fig. 5.3 – 3D reconstruction of a bovine blastocyst. A) A selection of *en-face* images captured at different depths in the embryo. B) 3D model of the embryo reconstructed on ImageJ from approximately 300 *en-face* images. For display purposes, images containing cumulus cells or reflections from the plastic dish were cropped, artificial colours were applied to the trophectoderm (blue) and the inner cell mass (purple), and a window was created to allow the visualisation of the inside of the embryo.

5.4.2 Specific aim 3b: to test the hypothesis that speckle variance analysis can quantitate micron scale movements of blastocyst stage bovine embryos to infer their vital status

Overall, n=5 live bovine embryos were observed short-term (10 minutes) and n=1 embryo was monitored long-term (18 hours, followed by an additional measurement at 26 hours). Motion maps were produced by speckle variance analysis on successive images at 1 min intervals showing which parts of the image were in motion over the interval and at the same time producing information on the intensity of the motion.

Figure 5.4 shows the speckle variance value for the median embryo layer in the five embryos monitored short-term (as a measurement of motion within that layer). An additional measurement obtained on a dead control embryo is also shown. Notably, the maximum value calculated for this control embryo was found to be at least 5 times smaller than the maximum value of any live embryo.

Conversely, figure 5.5 shows a more detailed examination of the motion pattern of a single embryo where quantity of movement was calculated at different depths simultaneously over long-term observation. Only movements exceeding the threshold set by the dead control embryo were included in the graph. An alternative representation of this information is given in figure 5.6, which shows the percentage of *en-face* images across the whole embryo in which movements could be detected at any given time over long-term observation.

A selection of images acquired over long-term observation is also given in figure 5.8 showing the appearance of the embryo at a fixed depth (fig. 5.7A) and the corresponding motion maps (fig. 5.7B). In this analysis, the blastocyst appeared to maintain its normal structure over the first 12 hours and started to collapse on itself by the 13^{th} hour. Interestingly, the complete collapse of the embryo's inner cavity was observed between 15 and 16 hours of culture, a detail that would have been impossible to notice with standard microscopy. After this point, however, the embryo failed to re-expand, an observation consistent with an embryo approaching the end of its life.

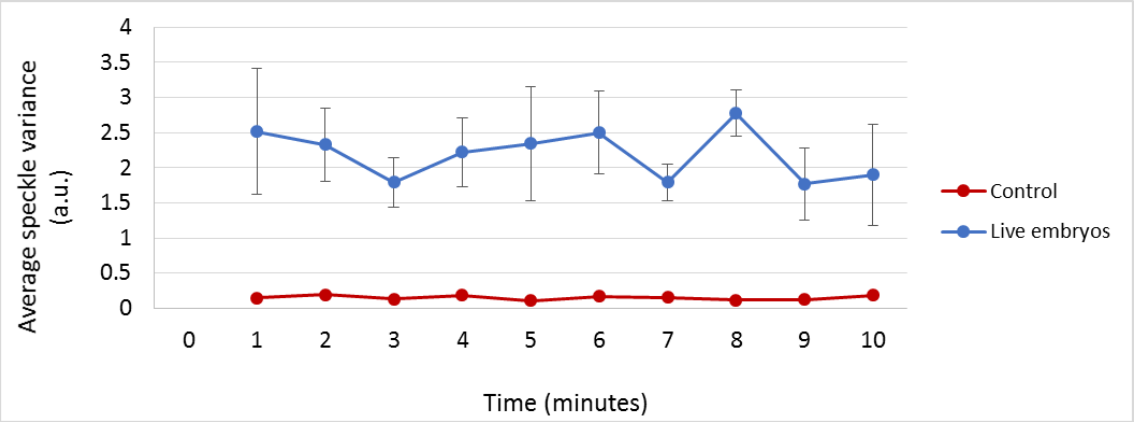


Fig. 5.4 – Average speckle variance measured on live bovine blastocysts at an arbitrary fixed depth over 10 minutes. Five live embryos were observed and one dead embryo was used as a control. Data given as mean ± S.E.M.

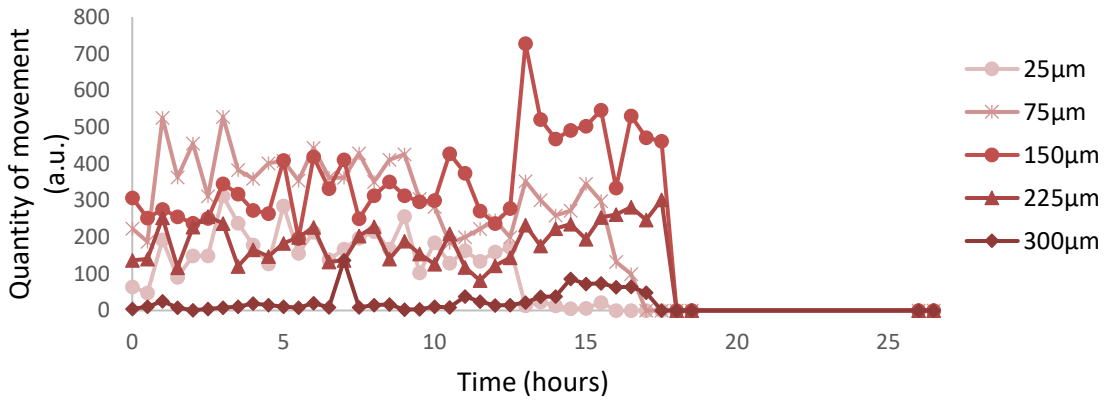


Fig. 5.5 – Total speckle variance (SV) for a single blastocyst at several depths over 26 hours. Movement was simultaneously detected by SV at multiple depths within the same embryo. In the graph, motion measurements at superficial (25 µm and 300 µm), intermediate (75 µm and 225 µm) and central layers (150 µm) are displayed.

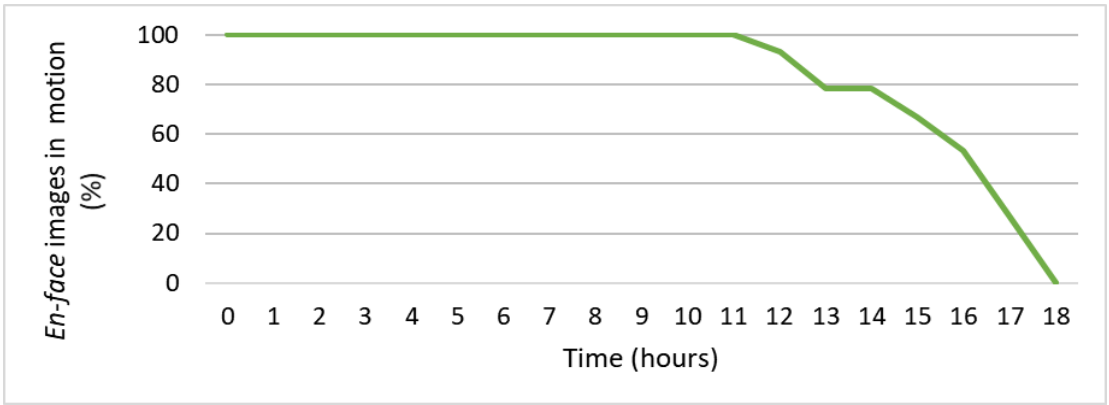


Fig. 5.6 – Percentage of cross-sectional (*en-face*) images displaying speckle variance values above threshold at any given time for a single blastocyst. Information from all depths within the same embryo was considered to track the decreasing motion of the embryo over time.

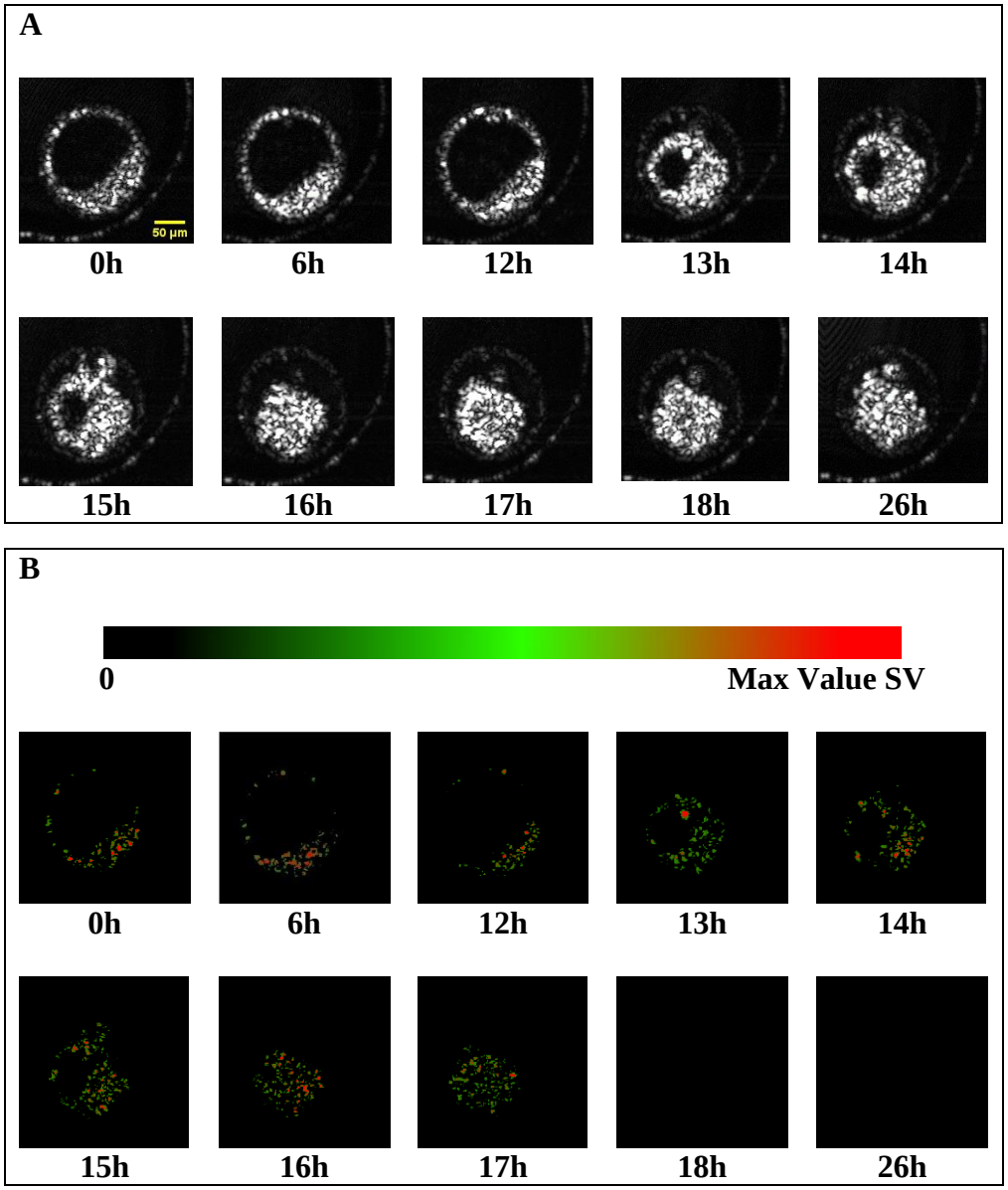


Fig. 5.7 – Cross-sectional SS-OCT images of a blastocyst over long term observation and corresponding motion maps. A) *En-face* SS-OCT images of the observed embryo over 26 h (actively monitored over the first 18 h) at a fixed depth (150 µm from the top embryo surface). B) Corresponding motion maps, black represents absence of movement while movement intensity is given on a Red/Green scale (red higher). Scale bar = 50 µm. The images were kindly provided by Sophie Cajoule (Applied Optics, University of Kent).

5.4.3 Specific aim 3c: to test the hypothesis that SS-OCT can be used to obtain non-invasive cell counts from bovine blastocysts

In this experiment, n=3 day 8 bovine blastocysts were imaged by SS-OCT and their cell count was non-invasively estimated by using the 3D objects counter feature of the software ImageJ (version 1.51n). After that, a control cell count was obtained by a standard fix and stain method (refer to section 2.2.4). When compared, the cell counts obtained with the two methods did not match. The results are shown in table 5.1, whilst figure 5.8 shows an example of this potential application.

	Standard method	3D objects counter	Error
Embryo1	79	87	+10.1 %
Embryo2	78	65	-16.7 %
Embryo3	46	92	+100.0 %

Table 5.1 – Cell counts in bovine blastocysts as estimated by standard fluorescence microscopy or by ImageJ 3D objects counter following SS-OCT.

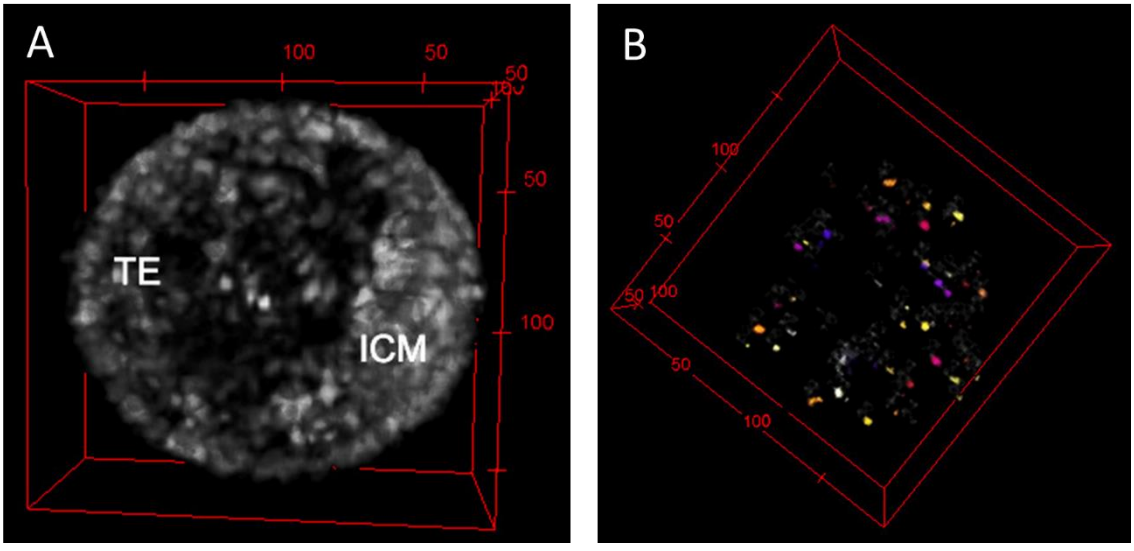


Fig. 5.8 - 3D reconstruction of a day 8 bovine blastocyst for automated cell count. A) 3D model of the embyo. TE: trophectoderm, IMC: inner cell mass. B) By using ImageJ, the 3D model was analysed and the position of each putative cell was highlighted with a dot of a different colour, then a count was produced.

5.4.4 Specific aim 3d: to test the hypothesis that SS-OCT observation is not harmful to the embryo

When n=6 live bovine blastocysts were observed by SS-OCT, all of them survived and could be cultured successfully for an additional 24 h post-observation. Moreover, n=3 bovine blastocysts of similar age and morphology were transported and handled in the same way as the SS-OCT samples but were never observed by OCT, these samples were labelled as “manipulation controls”. Two out of three manipulation controls survived and could be cultured successfully for an additional 24 h post-observation.

5.5 Discussion

Due to the experimental nature of the SS-OCT microscope used for this work, its limited availability, and the time required to adapt it for embryo imaging, it was impossible to use OCT to study the morphokinetic characteristics of embryo splits in order to study their developmental clock,. However, it was possible to explore the use of SS-OCT for the imaging of control embryos from both a static and a functional perspective. Some considerations on the potentials of OCT for non-invasive imaging are given below.

5.5.1 OCT for static embryo imaging

The results presented support the hypothesis that SS-OCT is a suitable technique to acquire structural images of bovine embryos, especially in the case of blastocysts. In cleavage stage embryos, the appearance of nuclei as darker structures is consistent with some previous reports (Zheng *et al.* 2012; Karnowski *et al.* 2017). However, the equipment setup employed did not have enough resolution to resolve the embryo in greater detail. On the other hand, as shown in section 5.4.2, SS-OCT could be successfully used to identify the gross morphologies within a bovine blastocyst on static observation and reconstruct its 3D model. Therefore, in future tests, this methodology could be used to obtain precise measurements of the shape and volume of a blastocyst’s ICM. These parameters have been shown to correlate well with implantation rates when measured by conventional microscopy (Richter *et al.* 2001). Because SS-OCT has far better depth resolution than standard microscopy, it could be used to measure

accurately the ICM structure to produce an accurate prediction of embryo implantation potentials. In a similar way, SS-OCT could be used to measure non-invasively the volume of a blastocyst's TE to obtain another metric of embryo quality: the TE/ICM ratio (Van Som *et al.* 1997) the assessment of which implies the destruction of the embryo with standard methods (Handyside & Hunter 1984; Thouas *et al.* 2001).

5.5.2 OCT for functional embryo imaging

To date, a limited number of reports have focused on applying functional OCT imaging to study early mammalian embryos (Larina *et al.* 2009b; Sudheendran *et al.* 2011; Karnowski *et al.* 2017). However, this is the first time SS-OCT and SV have been applied to a mammalian embryo to quantify micron-scale movement.

In agreement with the hypothesis that kinetic differences could be measured between live and dead embryos, over short-term observation, all live embryos consistently displayed higher SV values when compared with dead controls suggesting that a live embryo is likely to possess levels of intracellular activity detectable by OCT. However, the interpretation of the SV measurement became more complex over long-term observation. When the blastocyst collapsed (refer to figure 5.8A) after 12 hours of observation, a movement spike was detected at about 150 µm from the embryo's surface (close to the median point) as evident from figure 5.6. One possible explanation for this behavior is that the SV measurement could detect at the same time both genuine intracellular movement and the mechanical movement of the blastocyst's tissues caused by the deflation of the blastocoel, and that the summation of these two movements caused the SV surge displayed. Indeed, while the embryo could safely be declared dead after 18 hours of culture when completely blank motion maps were produced, the actual cessation of biochemical activity within the embryo could have occurred at an earlier time point between 15 and 18 hours. On the one hand, non-blank motion maps were obtained between 17 and 18 hours of culture after the complete disappearance of the blastocoel when mechanical motion seemingly ended, suggesting that the embryo more likely ceased all biochemical activity around the 18^{th} hour mark.

On the other hand, this might not be the only possible interpretation as spontaneous decaying processes, not necessarily linked with intra-cellular activity, could have been detected instead. Therefore, whilst the decreasing trend in figure 5.7 appears to be an effective indicator of embryo motion, it cannot not be used to guess the exact time of the embryo's death at the present state.

Moreover, as portrayed in figure 5.6, the embryo did not behave consistently across its depth from a kinetic point of view. This finding is not necessarily surprising since it is known that cells or cell groups in a blastocyst can become fragmented or even die (Hardy *et al.* 1989). This is also known to happen in embryos that are cryopreserved. Indeed, the cryopreservation of an embryo in liquid nitrogen is a standard procedure, however not all embryos survive the freeze thawing process and those that do are likely to suffer from various levels of cryodamage (Stinshoff *et al.* 2011). Cryodamage assessment on a per embryo basis is very challenging due to the invasive nature of the tests available. Very few papers have used OCT to investigate cryodamage in embryos (Zarnescu et al 2014; Zarnescu et al 2015) and none of them studied blastocyst stage embryos, which are commonly cryopreserved in bovine IVP schemes (Dobrinsky 2002). The SV analysis here described could become a useful tool to quantify the level of damage sustained by cryopreserved embryos within minutes of thawing and without need for extended culture. The results of this assessment would be easy to interpret since they would simply take the form of percentages of *en-face* images in motion. This assessment of the percentage of *en-face* images in active motion could also be expanded to take into account that, due to the spherical nature of the embryo, not all the *en-face* images contain the same number of cells so that a proportion of actively moving embryo volume could be calculated instead.

5.5.3 OCT for non-invasive cell counts

Potentially, the ability of SS-OCT to produce 3D models of embryos could be used to obtain cell counts, an approach that has been introduced by Zheng *et al.* (2012) who made use of a similar technique to SS-OCT, known as FF-OCT. In comparison to FF-OCT, the use of SS-OCT could permit real-time cell counts, thanks to the rapidity of its image

display process (Podoleanu & Bradu 2013). However, the limited number of embryos processed at present do not allow drawing any final conclusions on the current suitability of S-S-OCT to obtain cell counts.

From an anecdotal point of view, though, it is interesting to note that the embryo that produced a wildly different count by SS-OCT as compared to the standard method (embryo 3, +100.0%) was a collapsed blastocyst, which could suggest a limited ability of the method to segment amassed cells. Therefore, through implementing a SS-OCT system with a higher transversal resolution it could be possible to produce more refined 3D reconstructions that include robust non-invasive cell counts. If achieved, the ability to identify single cells in a blastocyst coupled with SV analysis would allow detection of dead cells instead of optical layers, stepping up the complexity of the analysis. This refinement could also lead to an accurate measure of the percentage of fragmentation (intended as cellular but immotile material) present in an embryo, a metric closely associated with viability, which is commonly only estimated subjectively (Alikani *et al.* 1999).

5.5.4 OCT safety

Although caution should be used because of the very limited sample size, current tests seem to suggest that OCT observation is not more damaging to embryos than mounting and transport. This finding, however, is not surprising since SS-OCT uses very low power light sources to illuminate samples (a 1300 nm infrared beam in this experimental setup) and is in agreement with previous similar reports (Zheng *et al.* 2012; Karnowski *et al.* 2017).

5.5.5 Study limitations, reasons for caution

The bovine embryos imaged by SS-OCT in this study were maintained at room temperature for the entire duration of the observation. Therefore, the results presented need to be interpreted conservatively from a biological standpoint. OCT systems have been adapted for use within incubators before (Larina *et al.* 2009a), and the SS-OCT

equipment reported here could be adapted for such use without major technical challenges for future projects (personal communication, Prof. Podoleanu A., School of Physical Sciences, University of Kent, Canterbury, UK, May 2017).

Additionally, the most obvious limitation for the results presented in this chapter for SS-OCT observation is the small sample size achieved. While this was largely due to the logistics of the experimental setup, it prevented a robust analysis of the properties of early bovine embryos to be delivered. Moreover, a final answer on the ability of SS-OCT to assess cell counts non-invasively or indeed on its safety cannot be provided in this work. However, the data presented does demonstrate several potential applications of SS-OCT to developmental biology and paves the way for exciting future interdisciplinary projects.

5.6 Conclusions

In conclusion, SS-OCT and SV analysis hold promise to become useful tools for the rapid identification of embryos with poor viability for example after freeze/thawing, and to obtain a number of viability metrics non-invasively. Moreover, the availability of a SS-OCT system built within an incubator would permit analysis of the developmental clock in bovine embryo splits in greater detail than what presented in Chapter 4 thanks to the ability to visualize the embryo in three dimensions, unimpeded by lipid content or overlapping structures. SS-OCT could become a valuable tool to confirm this finding whilst allowing for the viability of the embryo to be preserved so that embryo transfers could be performed as well.

6. Specific Aim 4: To demonstrate the application of karyomapping to blastocyst stage bovine embryos and characterise the type, level, and origin of chromosomal aberrations and rearrangements.

6.1 Background

Traditional breeding programmes aimed at improving the genetics of cattle focused around the selection of sires and dams based on phenotypic records of the individual and its relatives, and on progeny phenotyping (Henderson 1984). Later work, however, expanded on progeny testing and proposed a selection approach based on the assessment of QTLs, sections of DNA associated with variations in phenotypes of commercial interest such as milk production (Georges *et al.* 1995). More recently, the availability of a complete genome sequence for *Bos taurus* (refer to Elsik *et al.* 2009 for the most up to date draft), allowed the discovery of thousands of SNPs associated with QTLs which could be used as high-density markers for the production of GEBVs and for the selection of livestock based on genomic data, a process termed “genomic selection” (Meuwissen *et al.* 2001). The accuracy of GEBVs has been shown to be as high as 85% (Meuwissen *et al.* 2001), comparing well with, or even exceeding the precision obtained with traditional methods (Hayes 2009). It has been estimated therefore, that the application of genomic selection could double the rate of genetic gain for a given breed (Schaeffer 2006). Indeed, a recent report from Garcia-Ruiz *et al.* (2016) highlighted that genomic selection in Holstein cattle in the US over a time period of 8 years substantially decreased generational intervals, while rapidly increasing fertility, lifespan, and udder health.

As discussed in the introduction, IVP techniques hold promise to further reduce generational intervals and improve the dissemination of valuable genetics. Therefore, the ability to combine IVP and genomic selection could potentially lead to further

improved rates of genetic gain, through selective transfer of embryos that are proven carriers of desirable traits (Humbolt *et al.* 2010; Ponsart *et al.* 2014).

On the other hand, studies in several farm animals have suggested that the frequency of aneuploidy is increased in IVP embryos as compared to *in vivo* derived embryos (Viuff *et al.* 1999; Rambags *et al.* 2005; Coppola *et al.* 2007; Lechniak *et al.* 2007; Ullo *et al.* 2008; Hornak *et al.* 2015), and it is well established that aneuploidy is a leading cause of IVF failure (Hassold & Hunt 2001) and developmental arrest (Munné *et al.* 2007; King 2008). In humans, it has been shown that the majority of IVF embryos are affected by at least some degree of chromosomal abnormality (Lathi & Milki 2004; Baart *et al.* 2007; Franasiak *et al.* 2014). Moreover, in humans, aneuploidy is much more common in oocytes than sperm cells (Hassold & Hunt 2001) but similar information is lacking in cattle. However, levels of mixoploidy ranging between 25-90% have been discovered in bovine embryos (Viuff *et al.* 1999; Jakobsen *et al.* 2006; Garcia-Herreros *et al.* 2010; Destouni *et al.* 2016; Hornak *et al.* 2016), and an aneuploidy rate of 30% has been detected in bovine oocytes (Nicodemo *et al.* 2010).

A number of preimplantation genetic screening tools have been implemented in an attempt to improve embryo selection for patients with recurring IVF failure (Harper 2017). Among these tools is karyomapping, a screening method based on SNP typing in which the genotypes of an individual, its mother, father and a reference (often a sibling) are compared to describe the parental origin and haploblock inheritance of each chromosome (Handyside *et al.* 2010). Karyomapping can be used to detect simultaneously single gene disorders and chromosomal numerical aberrations, and to highlight regions of meiotic recombination in the parental karyotypes (Handyside *et al.* 2010; Thornill *et al.* 2015; Griffin & Gould 2017).

In cattle, the same SNP information used to construct GEBVs could also be used for chromosome copy number screening by employing the same karyomapping technique. In so doing, the combination of IVP, genomic selection and karyomapping in cattle embryos has the power to identify embryos with high expected breeding values, while

simultaneously selecting for chromosomally normal embryos in order to maximise pregnancy rates. This chapter describes this novel approach for optimising the delivery of superior genetics to the cattle breeding industry.

The first aim of this chapter, therefore, was to implement a successful karyomapping based PGS protocol for the screening of TE biopsies. The robustness of this analysis was investigated by testing the hypotheses that the performance of the biopsies at SNP typing as measured by call rates is not affected by the biopsy method, and that the SNP and karyomapping data obtained from embryo biopsies matches the information obtained from their respective calves borne after embryo transfer. Secondly, karyomapping was applied to investigate the type, level and parental origin of chromosome number aberrations in bovine IVP embryos and to estimate the recombination frequency in parental germlines.

6.2 Specific aims

With reference to the background above, the specific aims of this chapter were:

4a. To investigate whether SNP typing call rates are affected by the biopsy method or the biopsy operator

4b. To test the hypothesis that SNP genotypes and karyomaps obtained from embryo biopsy and the corresponding live born calf would be highly concordant

4c. To apply karyomapping to describe the frequency and type of chromosomal abnormalities in the trophectoderm of bovine blastocysts on a chromosome by chromosome basis

4d. To test the hypothesis that chromosomal aberrations in bovine embryos are more often of maternal rather than paternal origin

4e. To test the hypothesis that cross-over events happen with equal frequency in the maternal and paternal germline in cattle and estimate the average recombination distance

6.3 Methods

N=111 embryo biopsies were obtained either in-house or from Paragon Veterinary Group by either a blade assisted or a laser assisted method from n=15 IVF rounds and a total of 9 different sires and 11 different dams resulting in at least quadruplicate tests for each experiment (refer to section 2.1). The biopsies were processed by WGA (as per section 2.2.8.1) before being submitted for SNP typing (refer to section 2.2.8.5). DNA samples were also obtained from each of the embryo's parents (refer to sections 2.2.8.2-4) and submitted for SNP typing so that karyomaps could be produced and analysed (refer to section 2.2.8.6). Finally, DNA samples from calves born after transfer of karyotyped embryos were also recovered and used for SNP typing and karyomapping analysis.

6.4 Results

Overall, n=93 embryo biopsies were successfully amplified by WGA, n=89 were successfully SNP typed and n=61 fulfilled all the requirements for karyomapping (high enough call rate, availability of a sibling). Finally, n=5 calves were born following ET of karyotyped embryos.

6.4.1 Specific aim 4a: To investigate whether SNP typing call rates are affected by the biopsy method or the biopsy operator

In order to test whether the biopsy method (blade or laser assisted) or the biopsy operator (Kent operator or Paragon Veterinary Group operator) had an impact on the quality of the DNA presented for SNP typing, the call rates achieved by biopsies obtained with different methods were compared. Those biopsies that were successfully amplified

by WGA but failed to produce any result after SNP typing (n=4) were excluded from this analysis.

Results showed that the biopsy method had no significant effect on the performance of the DNA sample at SNP typing, with blade assisted biopsies scoring an average call rate of 88.4 ± 3.0% and laser assisted biopsies of 85.5 ± 4.0% (t-test, t_{87}=0.93, P=0.36). Additionally, because only samples producing a call rate ≥80% are considered of sufficient quality for karyomapping, the proportion of biopsies satisfying this requirement was also compared between the two biopsy methods. There was no significant impact on the proportion of samples with a call rate ≥80% between the two groups, with blade biopsies being found suitable in 87% of cases (n=26/30) and laser biopsies in 79% of cases (n=47/59) (chi-square, χ^2_1=0.66, P=0.41). These findings are displayed in figure 6.1.

Finally, when the call rates obtained from samples collected by two different operators were compared, it was found that samples collected by the Paragon Veterinary Group operator had an average call rate of 84.7 ± 1.7% (n=58), while samples collected by the Kent operator had a call rate of 89.8 ± 2.5% (n=31) which resulted in no statistical difference (t-test, t_{87}=1.72, P=0.09). However, it is worth noting that all the samples that completely failed to produce a result at SNP typing and were excluded from analysis were all derived from blade-assisted biopsies performed by the Paragon Veterinary Group operator.

Therefore, the results presented seem to suggest that TE biopsies will perform similarly in terms of suitability for karyomapping analysis, regardless of the method chosen to obtain them. Moreover, inter-operator variability appeared limited.

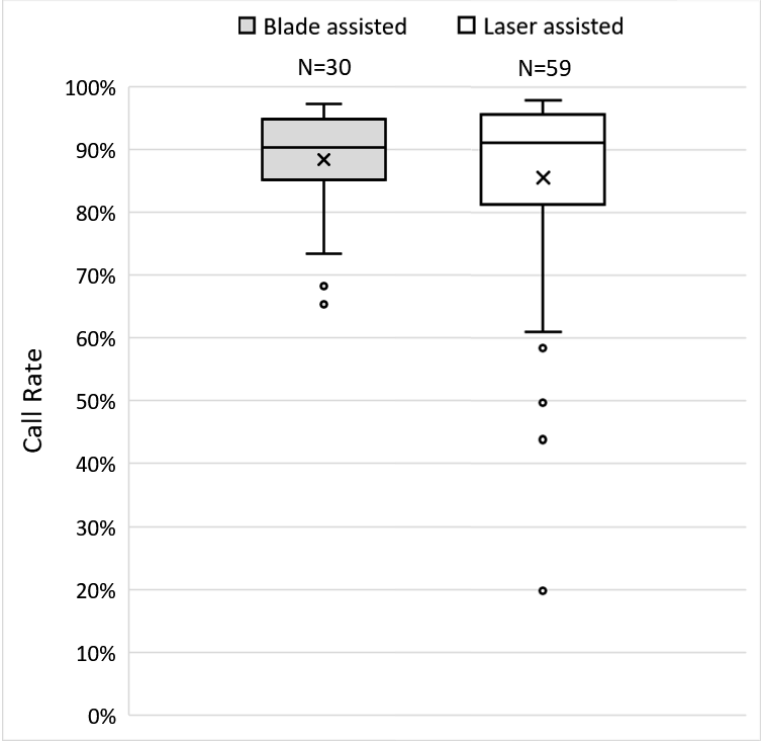


Fig. 6.1 – Call rates obtained from blade assisted or laser assisted biopsies. After whole genome amplification, the embryo biopsy samples obtained with either method were submitted for Single Nucleotide Polymorphism (SNP) typing and the call rates achieved by each group were recorded. No statistical differences were evident between the two methods (P>0.05).

6.4.2 Specific aim 4b: to test the hypothesis that SNP genotypes and karyomaps obtained from embryo biopsy and the corresponding live born calf would be highly concordant

To date, the birth of five calves has been recorded following 61 embryo transfers; however, some pregnancies were still ongoing at the time of writing. A portrait of Cookie Four, the first calf born from a karyomapped embryo is shown in figure 6.2, and the complete karyomap produced from its embryo biopsy is shown under Appendix II.

To verify whether the SNP typing and karyomaps produced from embryo biopsies were a true representation of the embryo's genotype, the results obtained from the biopsied embryos that later resulted in live births were compared against the SNP typing and karyomaps obtained from the respective live born calves.

As expected, all five embryo biopsies appeared euploid at karyomapping, a result that matched the live born calves. Moreover, when the SNP results were compared, it was found that the biopsy and the live born were concordant for 98.2 ± 1.4% of SNP loci. ADO events (where one sample was homozygous and the other heterozygous) represented 1.8 ± 1.4% of loci and complete mismatches (one sample homozygous for allele A, the other sample homozygous for allele B) affected between 0% and 0.02% of loci. However, it is important to note that for 10.8 ± 4.5% of the SNP loci assessed, no call could be generated in either the biopsy, the live born or both and these loci were excluded from the analysis.

Fig. 6.2 – Birth of the first karyomapped calf in the world. Cookie Four is the daughter of the sire Cinderdoor (Semex) and the dam Crossfell Uno Cookie (Paragon Veterinary Group) and was born in 2017 in Cumbria following the transfer of a karyomapped embryo.

6.4.3 Specific aim 4c: to apply karyomapping to characterize the frequency and type of chromosomal abnormalities in the trophectoderm of bovine blastocysts on a chromosome by chromosome basis

Following karyomapping analysis of trophectoderm biopsies, 68.8% of the embryos (n=42/61) appeared euploid while the rest displayed one or more abnormalities, which included monosomy, trisomy, triploidy, uniparental disomy and parthenogenetic activation. Table 6.1 reports the number of embryos displaying such abnormalities by type and, when appropriate, parent of origin, whilst figure 6.3 summarises the number of abnormalities discovered per embryo. A trend appeared to be present with embryos

displaying either up to three abnormal chromosomes or a large set of abnormalities with no in-between clusters. The appearance of these abnormalities on karyomapping analysis is also shown in figure 6.4.

	Paternal	Maternal
Euploid	49	46
Monosomy	3	6
Trisomy	4	6
Triploidy	-	1
Parthenogenetic	-	4
Uniparental disomy	1	

Table 6.1 – Types and number of chromosomal aberrations in embryo biopsies (n=61) by parent of origin. Numbers are given per parental haplotype. Haplotypes carrying multiple abnormalities are considered separately in each row.

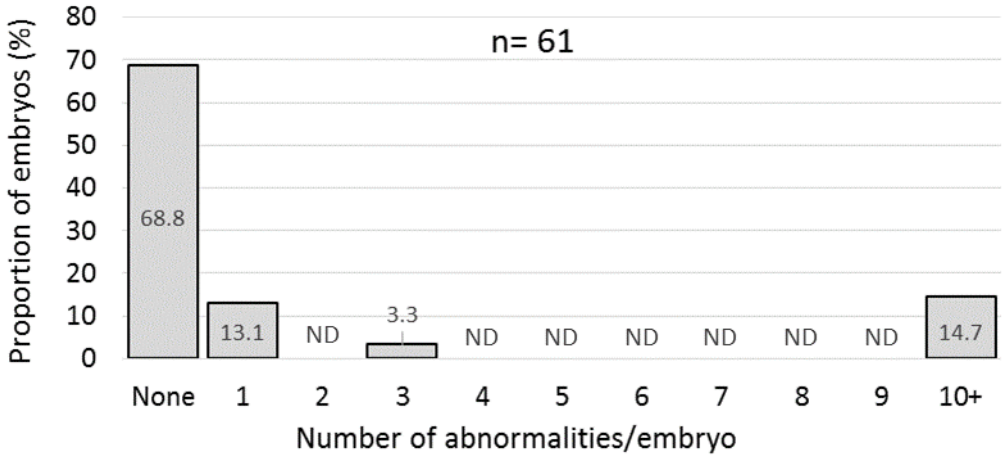
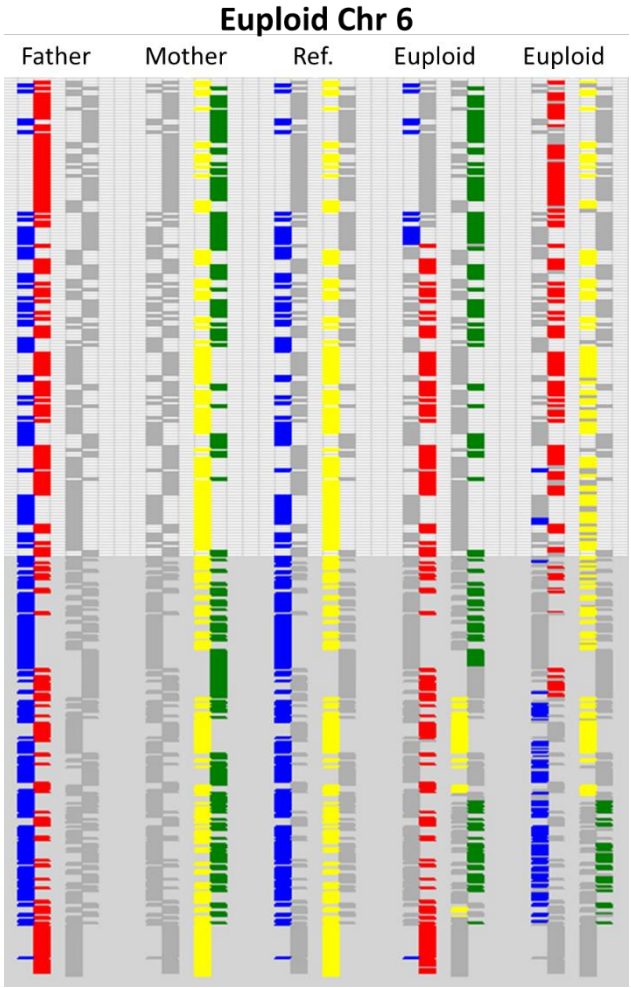
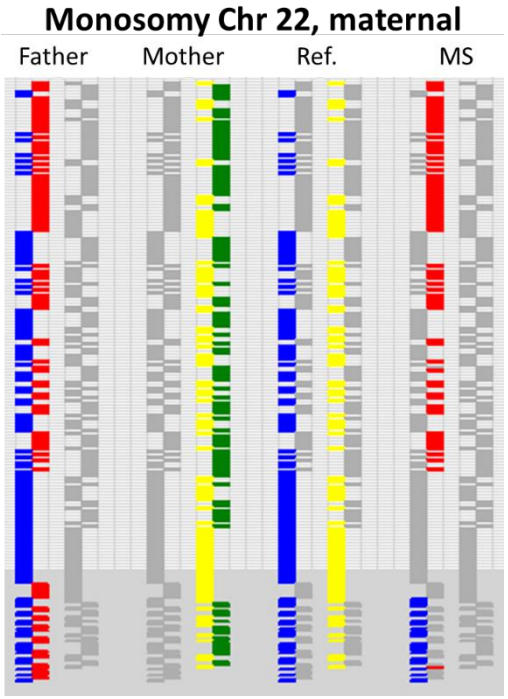
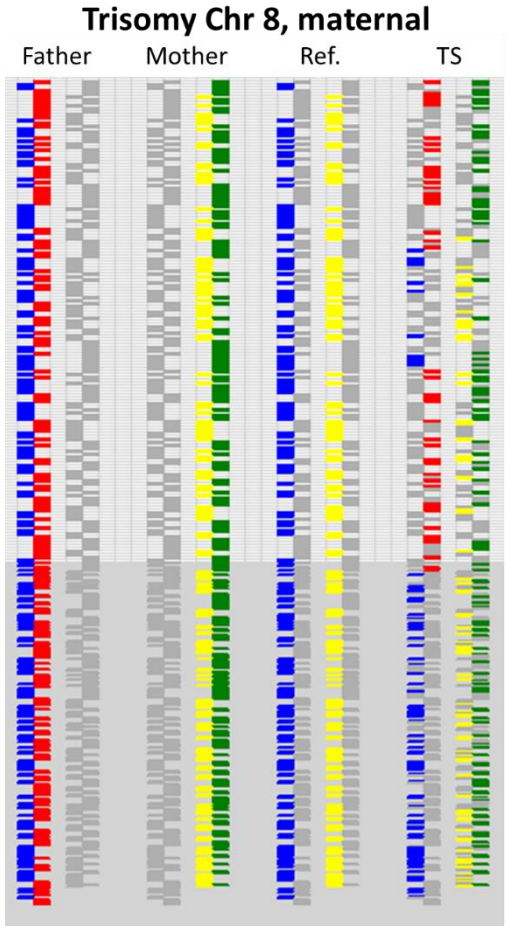


Fig. 6.3 – Number of embryos displaying zero, one or multiple chromosomal abnormalities. Interestingly, the affected embryos had either few or many chromosomal errors with no in-between clusters.


Euploid Chr 6
Father
Mother
Ref.
Euploid
Euploid


Monosomy Chr 22, maternal
Father
Mother
Ref.
MS


Trisomy Chr 8, maternal
Father
Mother
Ref.
TS

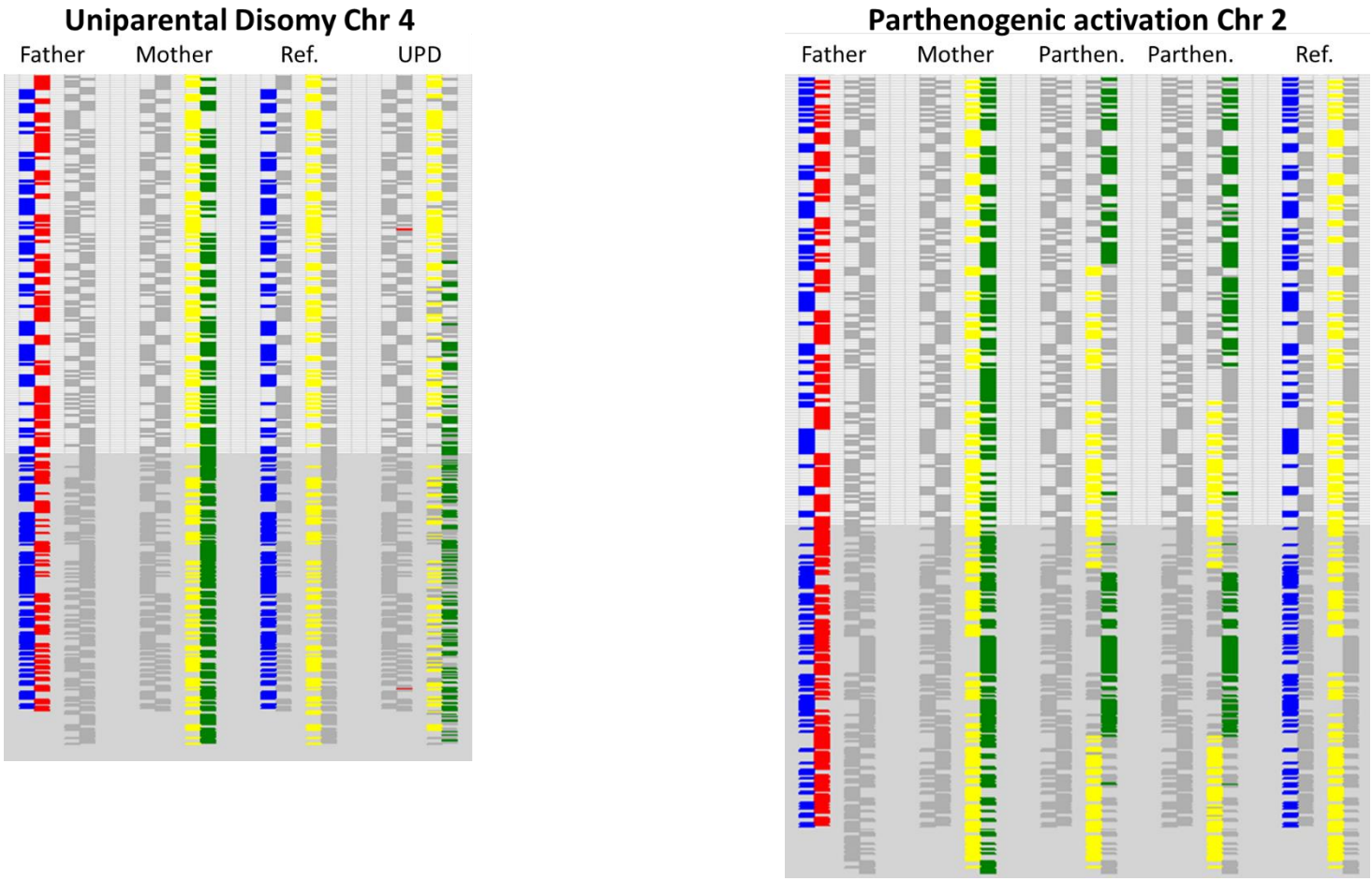


Fig. 6.4 – Example karyomaps. A series of karyomaps are presented from both normal and abnormal cases. Gray bands imply absence of information. Euploid chromosomes are characterised by few, large alternating blocks of blue/red (for paternal) or green/yellow (for maternal) bands, representing alternating haplotypes. Monosomies appear as complete or almost complete lack of information for a full chromosome, while trisomies appear as frequent and short blocks of alternating haplotypes. Uniparental disomies appear as a monosomy for one chromosome and a trisomy for its homologue, and, finally, parthenogenetic activation events appear similar to a monosomy of paternal origin at all loci. Chr: Chromosome; Ref.: Reference sibling; MS: Monosomy; TS: Trisomy; UPD: Uniparental Disomy. These karyomaps were produced by BoVision (version 3).

Additionally, karyomapping was employed in order to characterise the frequency and type of numerical abnormalities on a chromosome by chromosome basis. Parthenogenetically activated embryos were excluded from this analysis to avoid overrepresenting paternal monosomy events. Moreover, triploidy and uniparental disomy were only detected once and therefore could not be investigated further from a statistical point of view.

Overall, trisomies were detected with a frequency of 2.10% (n=68/3248 chromosomes) while monosomies with a frequency of just 0.31% (n=10/3248 chromosomes) resulting in a statistically significant difference between these two groups (chi-square, χ^2_1=43.6, P=4.0x10^{-11}). Figure 6.5 illustrates the number of monosomies and trisomies detected for each autosome and for the maternal X chromosome. While in these tests chromosomes 13 and 29 appeared to be affected by numerical abnormalities more often than other chromosomes, and chromosomes 20 and 21 were never affected, the rarity of these errors resulted in overall non-significant differences among all chromosomes (Fisher's exact test, df=58, P>0.05).

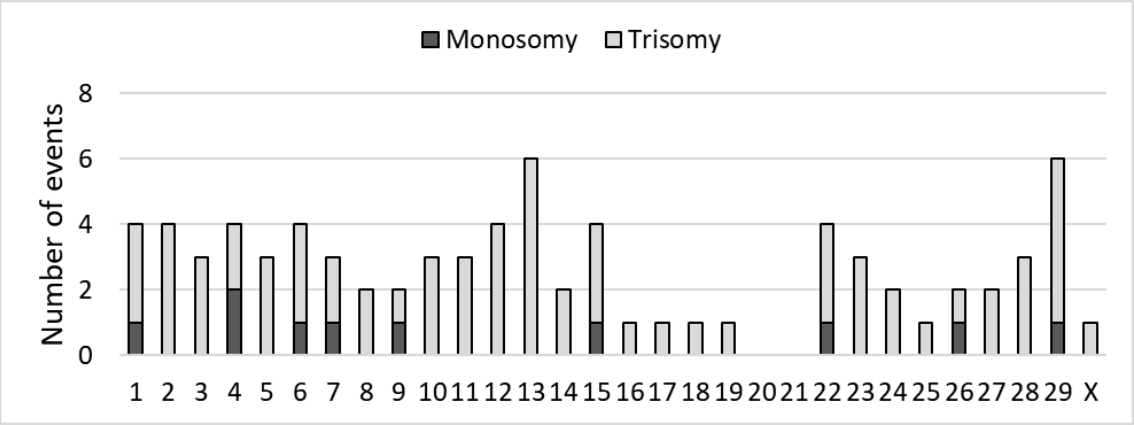


Fig. 6.5 – Number of monosomies and trisomies detected per chromosome. The results were collected from n=56 embryo karyomaps and a total of 3248 chromosomes. No information on the paternal X chromosome was available.

6.4.4 Specific aim 4d: to test the hypothesis that chromosomal aberrations in bovine embryos are more often of maternal rather than paternal origin

Since karyomapping is able to detect the parental origin of chromosome abnormalities, it was possible to test the hypothesis that numerical aberrations are more commonly derived from the maternal germ line. Due to the nature of the sex chromosomes and of karyomapping, however, abnormalities of the X chromosome could not be compared between sexes. Parthenogenetically activated embryos and triploid embryos were also excluded from comparison.

Monosomies were detected with a frequency of 0.24% (n=4/1653) and 0.36% (n=6/1653) for chromosomes of paternal and maternal origin, respectively, and no statistically significant differences were found between these two groups (chi square, χ^2_1=0.40, P=0.52). These results are summarised in figure 6.6.

On the other hand, trisomies appeared over represented in the maternally derived chromosome population where they were observed with a frequency of 2.60% (n=43/1653) as opposed to a frequency of 1.51% (n=25/1653) for the paternally derived chromosome population, resulting in a statistically significant difference (chi-square, χ^2_1=4.7, P=0.03). This analysis is further illustrated in figure 6.7.

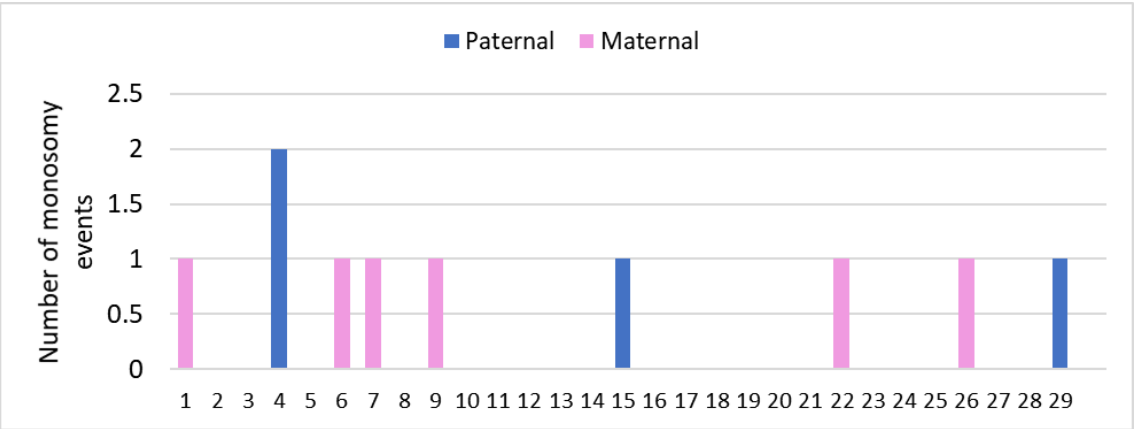


Fig. 6.6 – Number of monosomies per chromosome by parent of origin. No obvious pattern was detected from the analysis of 56 embryos.

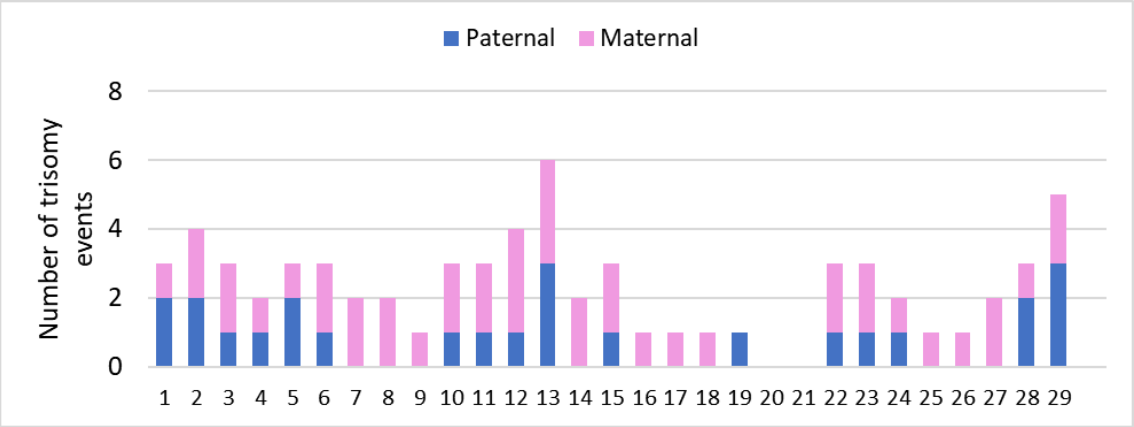


Fig. 6.7 – Number of trisomies per chromosome by parent of origin. Overall, trisomies appeared more common in the maternal chromosome population (chi-square, P<0.05) as detected from the analysis of 56 embryos.

6.4.5 Specific aim 4e: to test the hypothesis that crossover events occur with equal frequency in the maternal and paternal germline in cattle and estimate average recombination distance

On average, 87.7 crossover events were detected per euploid embryo, of which 44.6 were of paternal and 43.1 of maternal germline origin. Chromosomes involved in trisomies were excluded from this analysis due to the impossibility to obtain a crossover count by karyomapping in these cases. While there was no difference in the total number of crossover events between the two germ lines overall (t-test, t_{96}=1.06, P=0.29), significant differences became apparent at a chromosomal level. For example, chromosome 1 displayed more rearrangemnents in the paternal (2.9 ± 0.2 events/chromosome) rather than in the maternal germline (2.3 ± 0.2 events/chromosome) (t-test, t_{102}=2.07, P=0.03). A complete breakdown of this analysis is presented in figure 6.8.

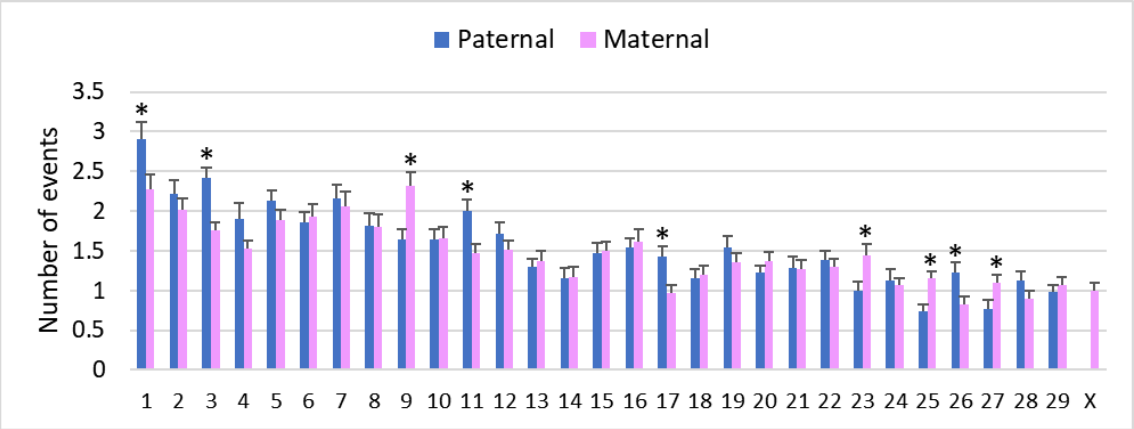


Fig. 6.8 – Average number of crossovers per chromosome and parent of origin. The analysis was completed on a total of 56 embryos. For each chromosome pair, a star (*) indicates that a significant difference was found between the number of events per parent (paired student t-test, $P<0.05$). Data given as mean ± S.E.M.

Assuming that, on average, one crossover event occurs in a chromosome of size 1 Morgan (M), based on the number of crossover events detected in this set of tests the genetic length of the bovine genome was estimated to be 4453 cM (sex averaged length across 29 autosomes plus female chromosome X). By dividing the genetic length by the physical genome length measured in mega base pairs (Mb), the average genome-wide recombination distance per Mb was calculated as 1.67 cM/Mb.

Moreover, from the information presented in figure 6.8 it appeared that chromosome size decreases more rapidly than the number of crossovers per chromosome. To investigate this hypothesis, a linear regression analysis was performed, as portrayed in figure 6.9. It was found that a significant correlation exists between chromosome size and average number of recombination events with smaller chromosomes having smaller distances between events (linear regression, $R^2=0.40$ $F=171.6$, $P=3.26\times10^{-13}$). Interestingly, the maternal chromosome X appeared as an outlier, showing a much greater space between adjacent events than other chromosomes of similar size.

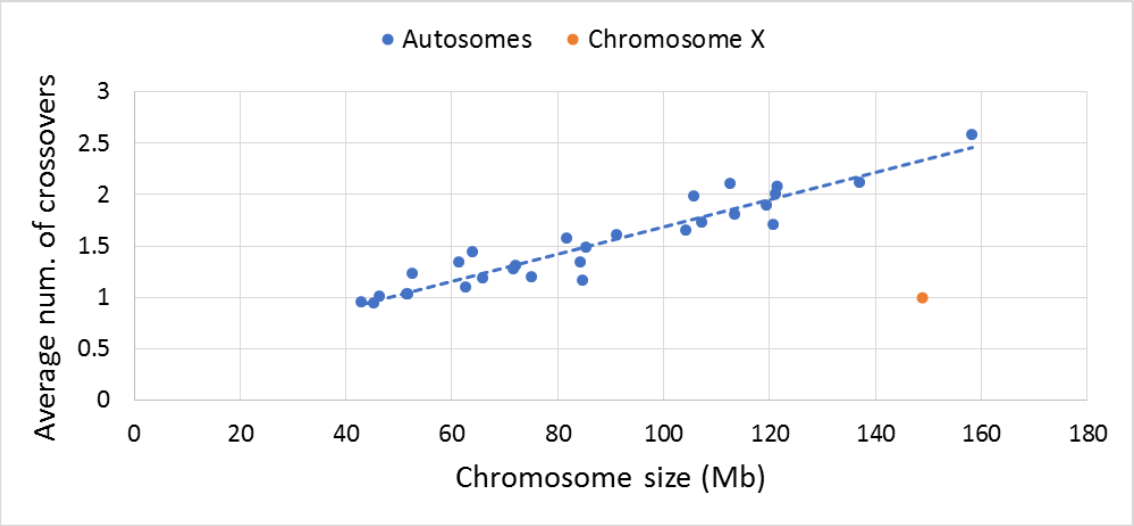


Fig. 6.9 – Average number of crossovers as correlated with chromosome size measured in mega base pairs (Mb). A moderate but significant association was found suggesting that smaller chromosomes allow for less space between crossover events. Chromosome X appeared as an outlier due to its reduced recombination frequency.

Finally, the non-recombination rate, indicating the proportion of chromosomes for which no crossover event could be detected at karyomapping was investigated by chromosome and parent of origin. The non-recombination rate ranged from 0% to 42% (average 15.9 ± 2.5%), and a more detailed analysis is presented in figure 6.10.

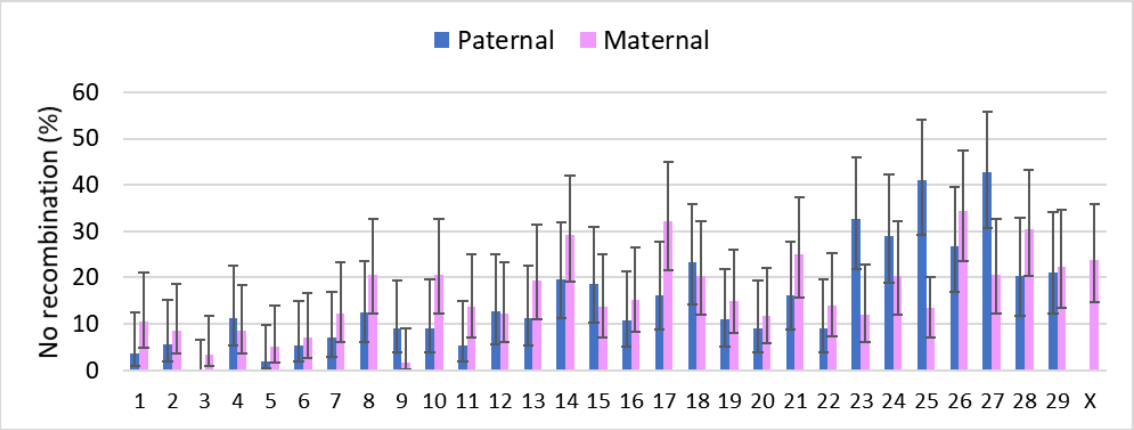


Fig. 6.10 – Non-recombination rate per chromosome and parent of origin. The analysis was completed on a total of 61 embryos and error bars are given as confidence intervals for proportions. Smaller chromosomes appeared to be more often affected by non-recombination. Data given as mean ± C.I. 95% for proportions.

6.5 Discussion

In this chapter, a proof of principle for the application of karyomapping to IVP bovine embryos has been presented, which has led to the birth of several healthy calves. The routine application of PGS has generated some controversy in the literature after a study reported a reduction in live birth rates following transfer of PGS embryos in human IVF (Mastenbroek *et al.* 2007). However, later studies demonstrated a beneficial effect of PGS on implantation rates when TE biopsy was applied as opposed to blastomere biopsy (Kakkali *et al.* 2007; Harton *et al.* 2013; Lee *et al.* 2014). The data available from the human model would suggest that the application of PGS to bovine embryos could lead to an improvement in pregnancy rates while at the same time, allowing for genomic selection prior to implantation.

6.5.1 Reliability of SNP typing and karyomapping from embryo biopsies and its correlation with live borne calves

The results presented in section 6.4.1 strongly suggest that the methodology used to obtain the embryo biopsy had no impact on the overall reliability of the SNP typing. This observation is in agreement with a previous report suggesting that TE biopsy is highly reproducible across different practitioners (Capalbo *et al.* 2015).

Call rates between 75 and 95% can be expected for WGA embryo biopsies at SNP typing (Natesan *et al.* 2014a), a result that once again compares well with the results presented here. Moreover, a high concordance was found between the results of the embryo biopsy and the results obtained from the corresponding calves born after embryo transfer (98.2 ± 1.4%), a result that compares well with similar previous investigation for SNP typing (Sargolzaei *et al.* 2012). Finally, comparisons between embryo biopsy and live born calves highlighted a total ADO rate of 1.8%; however, this should not represent a cause for concern with karyomapping. Because only heterozygous calls are used to establish a phase, ADO issues are virtually eliminated (Handyside *at al.* 2010) whilst complete mismatches were shown to be very rare. Overall, the data presented provides evidence for the robustness of the assay method employed.

6.5.2 Characterisation of chromosomal abnormalities by karyomapping

In this work, a significantly higher number of trisomies than monosomies were detected. In theory, random meiotic segregation errors should cause equal (or at least similar) numbers of monosomies and trisomies as a gamete carrying an extra chromosome copy will be formed at the same time as a gamete carrying a missing chromosome. Moreover, karyomapping is able to detect meiotic but not post-zygotic trisomies, whilst being able to detect both meiotic and post-zygotic monosomies (Griffin & Gould 2017). Therefore, the results contradicted the expectation that karyomapping would detect similar rates of trisomy and monosomy or higher rates of monosomy in bovine embryos.

This might suggest that some of the trisomies recorded were in fact false positives. However, to mitigate this risk, three independent operators were required to agree on the diagnosis before a trisomy was called. An alternative explanation is that embryos carrying meiotic trisomies can tolerate the increased chromosome count for a time and reach the blastocyst stage more often than embryos affected by meiotic monosomies, so that the population examined in this study (blastocyst stage embryos) was indeed partially depleted of monosomies, a hypothesis supported by evidence gathered from studies in humans (Clouston et al 1997; Sandalinas *et al.* 2001; Clouston et al 2002). This would imply that the numerical aberration rates presented here are not necessarily informative of the original number of monosomy and trisomy events occurring in bovine germlines. However, total blastocyst aneuploidy rates appear very similar to the total gamete aneuploidy rates in humans (Fragouli & Wells 2011) suggesting that the meiotic trisomy rates recorded here could be a good estimate of meiotic trisomy rates in the parental germline.

Interestingly, in this data set a case of uniparental disomy was identified, demonstrating the applicability of karyomapping for the discovery of this condition in bovine embryos, the diagnosis of which normally escapes detection with other methodologies like aCGH (Griffin & Gould 2017). However, since uniparental disomy was only discovered once in this set, no further conclusions can be drawn.

Additionally, in this data set n=4 embryo biopsies showed evidence of parthenogenetic activation. Mammalian oocytes are known to have the potential to become activated and progress to cleavage divisions and even form a blastocyst without need for a male gamete contribution (Presicce & Yang 1994); however, the spontaneous activation of an oocyte is a rare occurrence (King *et al.* 1988). Instead, in this study, parthenogenetically activated oocytes were discovered in 4 out of 61 biopsies. It is also interesting to note that three of these parthenotes originated from the same IVF cycle, which used semen from the same sire. A spike in the calcium concentration in an oocyte is sufficient to induce parthenogenetic activation (Fulton & Whittingham 1978). Therefore, it is possible that some oocytes become activated after a fertilisation-induced calcium spark, but problems with sperm decondensation, perhaps due to a bull-specific factor, prevented syngamy and resulted in a parthenogenetically activate oocyte instead, an event that has been described before in bovine embryos fertilised by intracytoplasmic sperm injection (ICSI) (LI *et al.* 1999).

6.5.3 Parental effect on chromosome number aberrations

Aneuploidy is much more common in human oocytes (20% incidence) than in human sperm cells (2% incidence) and a strong correlation has been found between maternal age and oocyte aneuploidy rate (Hassold & Hunt 2001). Similar aneuploidy rates to humans have been discovered in bovine oocytes (30%) (Nicodemo *et al.* 2010) while information is scarce about total aneuploidy levels in bull semen, with one non-comprehensive study suggesting a small incidence of aneuploidy in bull semen (disomy rate of approximately 0.03-0.04% for chromosomes X and 6) (Rybar *et al.* 2010).

In this data set, no sex-related difference was discovered in the prevalence of monosomies, however trisomies were 1.7 times more common in the maternal chromosome population, somewhat confirming the original hypothesis. Remarkably, karyomapping, thanks to its ability to assign the parental origin of aneuploidy, can be used to report on all bull sperm chromosomes at the same time and fill the current gap in the literature. A total aneuploidy rate of 1.75% was estimated for bull sperm in this

study. However, this is likely to be an underestimation due to embryos derived from aneuploid sperm cells failing to reach the blastocyst stage.

6.5.4 Crossover events

The number of crossovers per meiotic event differs significantly between the two sexes in several mammalian species (Barton & Charlesworth 1998; Otto & Lenormand 2002; Lynn *et al* 2005). For example, female mice have higher recombination rates as compared to males (Otto & Lenormand 2002) but crossovers are more frequent in rams rather than in ewes (Maddox *et al.* 2001). Previous studies have failed to identify any difference in the recombination rate of bulls and cows (Barendse et al 1994; Kappes *et al.* 1997; Ihara *et al.* 2004). In agreement with these previous findings, the results presented here failed to detect a clear sex skew. However, a recent study by Ma *et al.* (2015), which also made use of SNP analysis for haplotyping, discovered an increased recombination frequency in bulls and suggested that the difference is most evident at sub telomeric regions. In this work, while no difference between the numbers of crossover events in the paternal and maternal germlines were found overall, single chromosomes appeared more frequently rearranged in one or the other sex. It is therefore conceivable that a larger sample size would eventually have led to similar conclusions as Ma *et al.* (2015).

The genetic length of the bovine genome as estimated in this study by karyomapping for the 29 autosomes plus the maternal X (4453 cM), exceeded the estimate of 3097 cM previously published by Zi-Quing *et al.* (2013). However, Zi-Quing *et al*. did not include the X chromosome in their analysis. It also greatly exceeded the distance calculated by Ma *et al.* (2015) of 2435 cM (sex average). In a similar way, the recombination frequency estimated here by karyomapping (1.67 cM/Mb) was much greater than the recombination frequency previously estimated by other groups, which fell in the range 0.8 – 1.12 cM/Mb (Ihara *et al.* 2004; Zi-Quing *et al.* 2013; Ma *et al.* 2015). This could be explained by a higher number of crossover events detected by karyomapping as compared to other methods. It is also possible that the high number of events recorded in this study was caused by false positives; however, steps were taken to reduce the

number of false positives by requiring crossover events to be supported by at least three consecutive informative SNPs (Kong et al 2010; Ma *et al.* 2015). An alternative explanation is that the discrepancy observed with other results published in literature was caused by a genuinely higher than average recombination frequency in the population studied. In this regard, it is important to highlight that all the embryos investigated in these tests were derived from a total of 20 parents, representing a much smaller population as compared to previous reports (Kong *et al.* 2010; Ma *et al.* 2015).

Interestingly, as shown in figure 6.10, no crossover events could be detected on a number of euploid chromosomes. However, it is known that a minimum of one chiasma per chromosome is required to form during meiosis in order to maintain the structural integrity of the spindle, achieve correct segregation, and avoid aneuploidy (Mather 1938; Jones 1984). Karyomapping can only detect crossover events that generate different haplotypes between the sample and reference however, and therefore a shared crossover (an event happening in the exact or very similar location in both sample and reference) will remain undetectable. Crossover events are not evenly spaced across the genome, but tend to accumulate close to the so-called "recombination hot-spots" (Kauppi *et al.* 2004). This observation would suggest that sample and reference might regularly display shared crossovers, explaining the non-recombination rates presented. Moreover, karyomapping relies on the presence of heterozygous markers to track haploblocks; therefore, the presence of shared homozygosity regions between parents could have masked some events as well. Therefore, these considerations suggest that the genomic distance here calculated by karyomapping could be an underestimation due to some crossover events remaining unaccounted for.

Finally, as evidenced by figure 6.9, chromosome X displayed a smaller number of crossover events than expected for its size as compared to autosomes. This is in agreement with a previous study in humans suggesting a small crossover count for chromosome X (Ottolini *et al.* 2015) and with similar reports in rat and mouse (Jensen-Seaman *et al.* 2004). However, as discussed before, the number of crossovers in the X

could have been underestimated due to the presence of recombination hot spots (Kauppi *et al.* 2004) or due to homozygosity as discussed above.

Additionally, the trend displayed in figure 6.9 suggests that the average distance between crossovers is not consistent across the genome, with smaller chromosomes generally allowing less space between events, which would lead, overall, to a higher recombination frequency in smaller chromosomes. This is in agreement with previous observation that recombination rates across the genome of mammals are not homogeneous (Jensen-Seaman *et al.* 2004). Interestingly, a similar increased recombination rate in smaller chromosomes has been detected in birds and is especially pronounced in their microchromosomes (Hillier *et al.* 2004).

6.5.5 Study limitations, reasons for caution

Intuitively, because chromosome Y is only present in a single copy in the paternal genome, it cannot be used for haploblock-based investigations like karyomapping. Similarly, because sires only have one copy of the chromosome X, the paternal X chromosome will be passed more or less unchanged from father to female offspring, once again preventing haploblock analysis, so that a truly complete analysis of the bovine genome by karyomapping will remain impossible using this technique. Moreover, the relatively small sample size and the comparatively low rate of chromosomal number aberration discovered in this study should make us cautious when attempting to generalise the findings presented.

Additionally, a degree of subjectivity was required in this study when diagnosing trisomies. As described in figure 6.4, trisomies appear as frequent short blocks of alternation between haplotypes; however, the identification of this pattern is left to the operator's judgement. While in most cases the trisomic pattern was obvious and three independent operators agreed on the diagnosis, in three cases (out of 68) a trisomy diagnosis was only agreed upon by two out of three operators.

Finally, it is important to consider that, due to the time required for SNP typing, blastocyst stage embryos must be cryopreserved whilst a karyomapping analysis is completed. The necessity for this extra step adds a cost to the method and embryos run the risk of being lost to cryodamage (Stinshoff *et al.* 2011).

6.6 Conclusions

The work described in this chapter has demonstrated the usefulness of karyomapping for both PGS, genomic selection and for conducting fundamental biology studies on bovine embryos.

The birth of the first five karyomapped calves in the world has opened the way to a methodology of breeding that combines genomic selection and IVP, which is expected to improve rapidly the genetics of cattle. While bovine IVP has historically dominated the commercial landscape as compared to other domestic animal species, successful breeding strategies are readily exported for use in other species. For example, high-density SNP chips for the screening of porcine embryos are already commercially available; therefore, it is possible to adjust karyomapping for use in pigs as well.

7. General Discussion

7.1 Achievement summary

Overall, this thesis was largely successful in achieving its specific aims, and in particular:

1. The developmental competence of porcine oocytes was examined through a series of tests investigating their nuclear maturation, CG migration, GSH content, fertilisation and embryo development rates. The results presented suggest that the extension of the COC at oocyte collection might be a good predicative indicator of the oocyte's developmental competence. Moreover, the findings seem to suggest that current IVM practice is wasteful, through the elimination of oocytes with slightly reduced COC investment, which still have remarkable developmental potentials. The implementation of the selection criteria presented is expected to lead to an increased embryo yield per donor; however, due to the limitations described in Chapter 3, further validation work will likely be required before robust guidelines can be produced.

2. Several methodologies for both single and serial splitting of bovine embryos were compared under the same IVP system by measuring the number of blastocysts produced per starting embryo. The viability of the resulting blastocysts was also investigated by obtaining cell counts. The evidence collected from these investigations supported the hypothesis that single splitting strategies are superior to serial splitting strategies in that they produce higher numbers of better quality blastocysts. In particular, it was found that the splitting of an 8-cell stage embryo in four parts is the strategy most likely to produce the greatest output of good quality embryos. The application of embryo splitting following the methods described in Chapter 4, therefore, is expected to multiply the availability of embryos derived from high merit donors. Moreover, time-lapse investigation of the embryo splits found evidence of the existence of a

developmental clock that tightly regulates early cleavage events, providing support for some of the current theories of early embryonic development.

3. A new imaging modality, SS-OCT, was successfully tested for use in early stage bovine embryos to obtain both structural (static) and functional (dynamic) imaging and investigate the vital status of the sample, although blastocyst stage embryos could be described at a finer resolution than cleavage stage embryos. Moreover, the evidence collected from functional analysis suggests that SS-OCT is able to measure micron-scale movement within living blastocysts and can rapidly discriminate between live and deceased embryos, representing a new application of this optical methodology.

4. The applicability of karyomapping to bovine embryos was demonstrated by the birth of the first five karyomapped cows in the world. The evidence presented in Chapter 6 suggests that SNP typing and karyomapping are robust techniques for the assessment of genomic information from TE biopsies. At the same time, the usefulness of karyomapping for answering fundamental biology questions about aneuploidy and recombination frequency was explored. The incidence of aneuploidy in bovine blastocysts could be measured and the parental origin of the abnormalities tracked. Additionally, the genetic length of the bovine genome could be estimated and the recombination frequency measured at a chromosomal level.

In summary, the focal point of this work has been improving the yield of competent, euploid and high genetic merit IVP embryos available for transfer. Less wasteful selection criteria for IVM have been proposed and two novel techniques: SS-OCT and karyomapping were implemented in bovine IVP for the first time, establishing proof of principles and paving the way for new studies. Moreover, some of the gaps identified in the literature about the behaviour of split embryos were addressed. With the above in mind, the findings presented have the potential to have a positive impact on current IVP practice for both cattle and pigs, and these methods could be extended for use with

other farm animals as well. The wider implications of this research are discussed hereafter.

7.2 An optimised IVP protocol: the wider scope of the project

The results presented in this thesis converge together towards the development of a single, optimised IVP protocol by suggesting methods to either increase embryo production from elite parents or increase the ability to select for the most developmentally competent and high genetic merit embryos for transfer.

In particular, as discussed in Chapter 3, by applying adequate criteria for oocyte selection before IVM, it would possible to reduce wastage and increase the number of cleavage stage embryos produced. On the one hand, similar benefits could be achieved by simply deciding to culture the entire COC yield, regardless of morphology, postponing selection until after IVF. However, this would likely involve culturing of non-developmentally competent or even damaged oocytes. Damaged cells in culture might die and release factors such as ROS (Golstein & Kroemer 2007), which would have the potential to negatively affect the growth of competent oocytes. Moreover, COCs with compromised morphology are known to form the majority of the oocyte yield (Lin *et al.* 2015), and their inclusion in an IVM/IVF programme would considerably slow down operations withouth the prospect for a much increased embryo yield as compared with the strategy outlined in the present work.

Having established efficient criteria for the selection of oocytes leading to an optimised embryo production, the results presented under Chapter 4 strongly suggest that the embryo yield could be further multiplied by the application of embryo splitting, and in particular by the splitting of 8-cell stage embryos in four parts. The application of this strategy would be particularly desirable when elite parents have been employed for IVP.

Additionally, the combination of embryo splitting and PGS in the form of karyomapping, as outlined in Chapter 6, would be especially beneficial: the availability of embryo splits would increase the number of samples available for PGS, reducing the chances of a

particular genetic background being lost due to an unsuccessful biopsy. Moreover, a single karyomapping test would be informative on all twins simultaneously, improving the cost-effectiveness of testing while providing information on both the ploidy status and the genomic value of each twin set.

Furthermore, the results presented under Chapter 5 suggest that it would be possible to adapt a SS-OCT system for use as a time-lapse device. This development would allow to track the development of the embryos in culture non-invasively while gathering information from multiple optical planes simultaneously, ensuring that only the embryos that demonstrate higher developmental potentials are biopsied and screened, greatly reducing the economic cost associated with rejecting an aneuploid embryo only after karyomapping, by exploiting the known association between morphokinetic parameters and ploidy status (Campbel *et al.* 2013).

7.3 Pathways to impact

The benefits of genomic selection can more readily be realised through IVP as opposed to testing live bornes thanks to the ability to select embryos before the establishing of a pregnancy (Saadi *et al.* 2014), and to the increased selection intensity deriving from the immediate removal of suboptimal embryos from the pool (Merton *et al.* 2013). Therefore, the present work has provided additional supporting evidence for the usefulness of ARTs and PGS in industrial animal breeding (Humblot *et al.* 2010), with a particular focus on the ability of these techniques to maximise the offspring of superior animals (Chapters 3 and 4), and to increase selection intensity (Chapter 6), and the rate of genetic gain (Chapters 3-6).

Additionally, the ready availability of IVP embryos simplifies transportation of high genetic merit stock whilst minimising animal welfare and biosecurity issues (Pontes *et al.* 2010; Kenyon *et al.* 2014). It would also allow for the establishment of banks of genomically evaluated embryos for future distribution or for conservation purposes. The availability of a large number of embryos derived from specific donors could also be used

advantageously to widely disseminate specific genetics. Moreover, PGD technology or the use of sexed semen (Garner 2006) could be used to alter the natural sex ratio to enable the shipment and breeding of predominantly male or female animals according to the market need, thus reducing the birth of unwanted animals, which would benefit the breeding industry and animal welfare (Johnson *et al.* 2005).

Furthermore, advancements in farm animal IVP have the potential to provide for improvements in human IVF as well, preventing the ethical issues associated with direct experimentation in humans (Ménézo & Hérubel 2002; Campbell *et al.* 2003; Kuwayama *et al.* 2005).

Finally, the methodologies presented would allow for a fundamental insight into the process of meiosis. For example, information on the incidence of aneuploidy in farm animals is very limited when compared to the information available for the human model (Fragouli *et al.* 2013). Indeed, should karyomapping become a widely-used method for PGS in IVP, it would not only permit the transfer of the most chromosomally normal embryos, but it would also have the benefit of generating large data sets that will help answer (largely unexplored) fundamental questions related to aneuploidy and recombination in animal models.

7.4 The need for a careful application of the IVP technologies arising from this project: concerns about inbreeding and genetic diversity

Due to sustained levels of inbreeding, the effective population size of dairy cattle breeds (size of an ideal population with identical genetic drift and inbreeding as the population in study) has been shown to be very small; for example, intense selection and AI practices are thought to have reduced the effective population size of Holstein cattle in the USA to just 39 (Weigel 2001; Taberlet *et al.* 2008). This is a worrying figure considering that conservation biologists recommend that the effective population size should not fall below 50 to avoid extinction in the short term, and should ideally exceed

500 to avoid extinction in the long term (Franklin 1980). Moreover, similar scenarios are true for other farm animals such as pigs and sheep (Taberlet *et al.* 2008).

At a farm level, high rates of inbreeding are known to impair growth, lactation, health, fertility, parturition and survival as a consequence of the so-called "inbreeding depression" effect (Smith *et al.* 1998; González-Recio *et al.* 2007; Leroy 2014; Pryce *et al.* 2014b). Additionally, inbreeding is a cause of concern from a conservationist standpoint because it potentially leads to the complete loss of rare breeds and leaves traits vulnerable to genetic drift (Roosen *et al.* 2005) and, therefore, limits the gene pool available for selection, should different traits or combinations of traits ever become necessary in the future (Notter 1999). Moreover, populations with a limited diversity are more susceptible to catastrophic losses caused by highly infectious diseases (Spielman *et al.* 2004), suggesting that high levels of farm animal inbreeding might put future food security in jeopardy.

Whilst the availability of large numbers of twins with high genetic merit might well be beneficial to increasing production for the breeding industry in the short term, the question arises as to whether this would be a sustainable long-term breeding strategy due to the potential increase of inbreeding within herds caused by the presence of large numbers of closely related, or even identical animals. Moreover, the short generational intervals driven by the practices here described (IVP) risk increasing the annual rate of inbreeding as well (Lillehammer *et al.* 2011). Furthermore, the wide application of genomic selection could result in the unintended fixation in a population of homozygous chromosomal regions flanking the QTLs which are actively selected for (Sonesson & Woolliams 2010). Considered together, these observations suggest that the implementation of the IVP protocol discussed above could lead to a further reduction in the genetic diversity of the current farm animal breeds if applied without appropriate strategy planning.

Several methods to control inbreeding have been discussed in the literature. These include restriction on family size strategies which advocate inbreeding avoidance by

limiting the number of sires and dams used and preventing crosses between siblings or half-siblings (Goddard & Smith 1990), creation of separate sublines with subsequent crossing of lines in response to rising inbreeding (Smith & Quinton 1993), and the development of specialised and often computer assisted mating allocation strategies to control inbreeding and maintain acceptable rates of genetic gain in the short and long term (Klieve *et al.* 1994; Meuwissen & Sonesson 1998; Weigel *et al.* 2000; Weigel *et al.* 2001; Pryce *et al.* 2012). The implementation of these or similar strategies and a continuous monitoring of genetic diversity should form an integral part of any breeding programme aiming to apply the methodologies described in the present work.

Interestingly, these same IVP technologies could also be actively used for conservation purposes. For example, embryos produced by IVP from oocytes recovered from the endangered wild bovid gaur (*Bos gaurus*) resulted in a live birth when transferred to a Holstein cow, demonstrating the applicability of these methods for the preservation of a rare cattle species (Johnston *et al.* 1994). Similar accomplishments were achieved in a number of other species (Pope 2000; Ptak *et al.* 2002; Loskutoff 2003; Amstislavsky *et al.* 2004; Ullah *et al.* 2006; Andrabi & Maxwell 2007). Moreover, cryopreservation could be used to archive gametes or embryos from specific breeds, allowing the resurrection of specific traits in case of adverse events (Hiemstra *et al.* 2006; Santos *et al.* 2010b).

7.5 Future studies arising from this thesis

While the evidence presented here has contributed to filling some of the current gaps in the literature, the observations made over the course of this thesis have also highlighted new, potential avenues worthy of further exploration, for example:

1. Establishing an efficient IVM system is of capital importance for successful porcine IVP. Future research could focus on methodologies to preserve the developmental potential of the oocytes over long transport. For example, a variety of transportation temperatures and media could be tested. Moreover, efforts could be made towards the design of optimal and completely defined culture media, which would improve reproducibility and biosecurity. It would

also be particularly beneficial to focus efforts on peripubertal animals, the use of which would further reduce generational intervals.

2. While a number of methodologies for the splitting of bovine embryos have been investigated in Chapter 4, not all possible splitting strategies have been tested. For example, the splitting of 4-cell stage embryos was not included in this thesis and several other serial splitting protocols could be designed in an attempt to improve on the results presented. Finally, the ability to perform embryo transfers following embryo splitting would greatly increase the confidence in the conclusions so far reached by complementing the work carried out *in vitro* with robust field data. Furthermore, similar embryo splitting studies could be performed in other important farm animals such as pigs and sheep.

3. We have only just started exploring the potentials of OCT in embryology. As such, it would be interesting to observe embryos during their entire development through an SS-OCT time-lapse system in an attempt to establish more stringent morphokinetic criteria than the ones currently available for the selection of developmentally competent embryos. Interestingly, SS-OCT system with a higher resolution should become available in the near future, which would be expected to allow for a detailed structural characterisation of cleavage stage embryos and to produce accurate description of cell counts and ICM parameters within a blastocyst in a completely non-invasive way. Moreover, OCT could be used to discriminate immediately between living and deceased embryos following cryopreservation to reduce or eliminate the need for extended observation of thawed embryos before transfer. Similar systems, if made portable, could greatly assist embryo transfer practitioners on site, by confirming that only live thawed embryos have been transferred into the intended recipient.

4. Currently, due to the time constraints imposed by WGA and array analysis, the applicability of karyomapping is limited by the need to cryopreserve a blastocyst whilst a diagnosis is obtained. In the future, the ability of embryo splits to survive

cryopreservation should be investigated to confirm whether these two techniques could coexist in their current form.

5. Additionally, other non-invasive methods could be employed for the screening of embryo splits. For example, the analysis of spent culture medium as a method to predict an embryo's sex and viability could be employed. Moreover, the ability to obtain cell counts non-invasively by OCT, combined with predictions based on metabolic profiling through analysis of spent medium would be expected to produce highly dependable viability predictions, which would be very valuable in clinical programmes aiming for single embryo transfer in human patients.

7.6 Personal perspectives and concluding remarks

This PhD has been quite a journey, one I am glad I undertook. Over the last three years, I had the opportunity to investigate what I found most interesting, to work independently, to formulate my own thoughts and hypotheses and to learn a lot. I definitively fell like a changed person from when I left Italy to come and study in the UK.

Perhaps, the aspect I cherish the most about my research is that it is very much applied to real world needs. The question of how to best to feed humanity in the coming years touches everybody and the applied nature of the areas investigated in this work is well testified by the ample industry support we received as a research group.

We now live in a world where genetic engineering has been made highly accessible thanks to the revolution brought to us by the CRISPR/Cas9 system. As briefly discussed in the introduction, genetic engineering technologies could be used to create transgenic animals capable of producing important biomolecules in their milk or supplement the human diet with specific nutrients, whilst transgenic pigs are promising candidates for xenotransplantation. Moreover, whilst commercial interest in nuclear transfer technologies seems to be quiescent at present, improvements have been made allowing for far greater cloning efficiency than in the early days. The great costs associated with

cloning prevent this technique from being commercially viable at present, but it might well become accessible in the near future. It is therefore possible that these approaches will be integrated in animal production, should they ever be met with public acceptance. Importantly, their implementation will require the manipulation of embryos *in vitro*. Given its potential, it is certainly not surprising that IVP is currently such an active field of research and there is little doubt it will stay so in the coming years.

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9. Appendix I – IVP media composition

9.1. Bovine IVP Media

9.1.1. IVM medium – modified TCM 199

Component	Quantity	Stock concentration	Storage
TCM199 (M4530, Sigma)	4.375 ml	1 x	4 °C up to 4 months
FBS (10270-098, Gibco)	500 µl	1 x	-20°C up to 4 months
PG600 (Intervet)	125 µl	400 IU/ml PMSG and 200 IU/ml hCG	-20°C up to 2 months
Pen/strep (Gibco)	25 µl	200 x	-20°C up to 2 months
Na-pyruvate	10 µl	100 mM	-20°C up to 2 months

Shelf-life: use on the same day

9.1.2. IVM handling medium – HEPES modified TCM 199

Component	Quantity	Stock concentration	Storage
TCM199 HEPES modification (M7528, Sigma)	4.5 ml	1 x	4 °C up to 4 months
FBS (10270-098, Gibco)	500 µl	1 x	-20°C up to 4 months
Pen/strep (Gibco)	25 µl	200 x	-20°C up to 2 months
Na-pyruvate	10 µl	100 mM	-20°C up to 2 months

Shelf-life: use on the same day

9.1.3. Glucose-free TALP base medium

Component	Quantity	Stock concentration	Storage
NaCl	0.3333 g	Powder	-
KCl	0.0120 g	Powder	-
MgCl2 6(H2O)	0.0050 g	Powder	-
NaH2PO4	0.0023 g	Powder	-
NaHCO3	0.1050 g	Powder	-
CaCl2 2(H2O)	0.0150 g	Powder	-
Water (W1503, Sigma)	50 ml	-	-
Na-lactate syrup (L4263, Sigma)	90 µl	60% w/v	4 °C up to 6 months

Shelf-life: 4 °C up to 1 month

9.1.4. PHE base solutions

Component	Quantity	Diluent
Penicillamine	0.0030 g	10 ml PBS
Hypotaurine	0.0110 g	10 ml PBS
Epinephrine solution • Na-lactate syrup (L4263, Sigma) • Na metabisulfite • Epinephrine	 132 µl 0.0400 g 0.0018 g	 40 ml water pH 4.0 (adjust pH before adding epinephrine)

Shelf-life: use on the same day

9.1.5. PHE stock

Component	Quantity	Stock concentration
Penicillamine solution	2.5 ml	See PHE base solutions
Hypotaurine solution	2.5 ml	See PHE base solutions
Epinephrine solution	2 ml	See PHE base solutions
PBS	4 ml	1 x

Shelf-life: -20 °C up to 2 months

9.1.6. IVF medium - Glucose-free TALP complete medium

Component	Quantity	Stock concentration	Storage
Glucose-free TALP base medium	9.44 ml	1x	4 °C up to 2 months
BSA (A8806, Sigma)	0.0600 g	Powder	4 °C up to 6 months
Na-pyruvate	10 µl	100 mM	-20°C up to 2 months
Heparin	100 µl	1 mg/ml	-20°C up to 2 months
PHE stock	440 µl	See PHE stock	-20°C up to 2 months
Pen/strep (Gibco)	25 µl	200 x	-20°C up to 2 months

Shelf-life: use on the same day

9.1.7. Handling medium - HEPES modified, glucose-free TALP

Component	Quantity	Stock concentration	Storage
NaCl	0.2910 g	Powder	-
KCl	0.0115 g	Powder	-
MgCl2 6(H2O)	0.0040 g	Powder	-
NaH2PO4	0.0020 g	Powder	-
NaHCO3	0.1050 g	Powder	-
CaCl2 2(H2O)	0.0150 g	Powder	-
Na-lactate syrup (L4263, Sigma)	90 µl	60% w/v	4 °C up to 6 months
HEPES	0.1190 g	Powder	-
Water (W1503, Sigma)	50 ml	-	-

Shelf-life: 4 °C up to 1 month

9.1.8. SOFaaci base medium

Component	Quantity	Stock concentration	Storage
NaCl	0.3145 g	Powder	-
KCl	0.0267 g	Powder	-
KH2PO4	0.0081 g	Powder	-
MgSO4	0.0091 g	Powder	-
CaCl2 2(H2O)	0.0131 g	Powder	-
NaHCO3	0.1050 g	Powder	-
Trisodium citrate	0.0050 g	Powder	-
Myo inositol	0.0250 g	Powder	-
Na-lactate syrup (L4263, Sigma)	30 µl	60% w/v	4 °C up to 6 months
MEM (M7145, Sigma)	0.5 ml	100 x	4 °C up to 4 months
BME (B6766, Sigma)	1.5 ml	50 x	4 °C up to 4 months
L-glutamine	50 µl	200 mM	-20°C up to 2 months
Water (W1503, Sigma)	50 ml	-	-

Shelf-life: 4 °C up to 1 month

9.1.9. IVC medium - SOFaaci complete medium

Component	Quantity	Stock concentration	Storage
SOFaaci base medium	9.73 ml	See SOFaaci base medium	4 °C up to 2 months
BSA (A8806, Sigma)	0.0500 g	Powder	4 °C up to 6 months
FBS, (10270-098, Gibco)	250 µl	1 x	-20°C up to 4 months
Na-pyruvate	20 µl	100 mM	-20°C up to 2 months
Pen/strep (Gibco)	25 µl	200 x	-20°C up to 2 months

Shelf-life: use on the same day

9.2. Porcine IVP Media

9.2.1. Handling medium – TL-HEPES-PVA medium

Component	Quantity	Stock concentration	Storage
NaCl	0.3331 g	Powder	-
KCl	0.0119 g	Powder	-
MgCl2 6(H2O)	0.0051 g	Powder	-
NaH2PO4	0.0020 g	Powder	-
NaHCO3	0.0084 g	Powder	-
CaCl2 2(H2O)	0.0150 g	Powder	-
Na-lactate syrup (L4263, Sigma)	90 µl	60% w/v	4 °C up to 6 months
Na-pyruvate	100 µl	100 mM	-20°C up to 2 months
Glucose	0.0450 g	Powder	-
Pen/strep (Gibco)	250 µl	200 x	-20°C up to 2 months
Polyvinyl alcohol	0.0050 g	Powder	-
HEPES	0.1190 g	Powder	-
Water (W1503, Sigma)	50 ml	-	-

Shelf-life: 4 °C up to 1 month

9.2.2. NCSU-23 base medium

Component	Quantity	Stock concentration	Storage
NaCl	0.1271 g	Powder	-
KCl	0.0071 g	Powder	-
KH2PO4	0.0032 g	Powder	-
MgSO4	0.0059 g	Powder	-
CaCl2 2(H2O)	0.0050 g	Powder	-
NaHCO3	0.0421 g	Powder	-
Glucose	0.0200 g	Powder	-
L-glutamine	0.0029 g	Powder	-20°C up to 2 months
Taurine	0.0175 g	Powder	-20°C up to 2 months
Hypotaurine	0.0109 g	Powder	-20°C up to 2 months
ITS (I3146, Sigma)	200 µl	100 x	4 °C up to 6 months
Pen/strep (Gibco)	400 µl	200 x	-20°C up to 2 months
Water (W1503, Sigma)	20 ml	-	-

Shelf-life: 4 °C up to 1 month

9.2.3. IVM medium – modified NCSU-23 medium

Component	Quantity	Stock concentration	Storage
NCSU-23 base medium	8.7 ml	See NCSU-23 base medium	4 °C up to 2 months
Porcine follicular fluid	1 ml	100%	-20°C up to 4 months
MEM (M7145, Sigma)	0.1 ml	100 x	4 °C up to 4 months
BME (B6766, Sigma)	0.2 ml	50 x	4 °C up to 4 months

Shelf-life: use on the same day

9.2.4. Sperm wash

Component	Quantity	Stock concentration	Storage
NaCl	0.2700 g	Powder	-
BSA (A8806, Sigma)	0.0600 g	Powder	-
Kanamycin	0.0006 g	Powder	-
Water (W1503, Sigma)	30 ml	-	-

Shelf-life: 4 °C up to 1 month
pH: Adjust to pH 7.4

9.2.5. IVF medium – mTRIS medium

Component	Quantity	Stock concentration	Storage
NaCl	0.3400 g	Powder	-
KCl	0.0111 g	Powder	-
CaCl2 2(H2o)	0.1210 g	Powder	-
TRIS	0.0901 g	Powder	-
Glucose	0.0270 g	Powder	-
Na-pyruvate	100 µl	100 mM	-20°C up to 2 months
BSA (A8806, Sigma)	0.0010 g	Powder	4 °C up to 6 months
Kanamycin	0.0555 g	Powder	-
Water (W1503, Sigma)	50 ml	-	-

Shelf-life: 4 °C up to 1 month

9.2.6. IVC medium – modified NCSU-23 medium

Component	Quantity	Stock concentration	Storage
NCSU-23 base medium	8.7 ml	See NCSU-23 base medium	4 °C up to 2 months
BSA (A8806, Sigma)	0.0160 g	Powder	4 °C up to 6 months
L-Cysteine	33 µl	100 mM	-20°C up to 4 months
MEM (M7145, Sigma)	0.1 ml	100 x	4 °C up to 4 months
BME (B6766, Sigma)	0.2 ml	50 x	4 °C up to 4 months

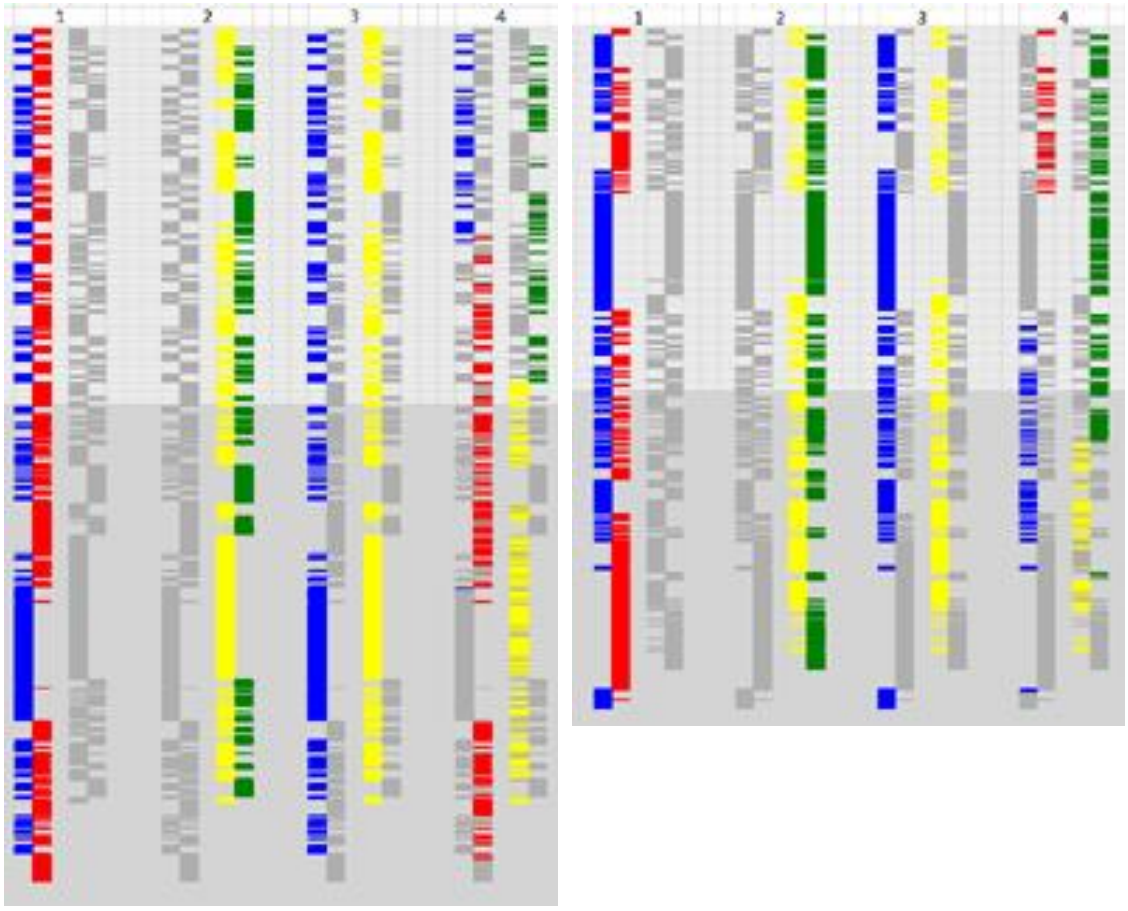
Shelf-life: use on the same day

10. Appendix II – Full karyomap of Cookie Four embryo biopsy

Sample number	Sample ID	Call Rate	Proportion of heterozygous loci
1	Sire (Cinderdoor, SEMEX)	98%	39%
2	Dam (Crossfell Uno Cookie, Paragon Veterinary Group)	98%	40%
3	Reference sibling embryo	94%	34%
4	Cookie Four embryo biopsy	88%	29%

Table 10.1 – Sample information, Cookie Four karyomap.

Chromosome 1 **Chromosome 2**



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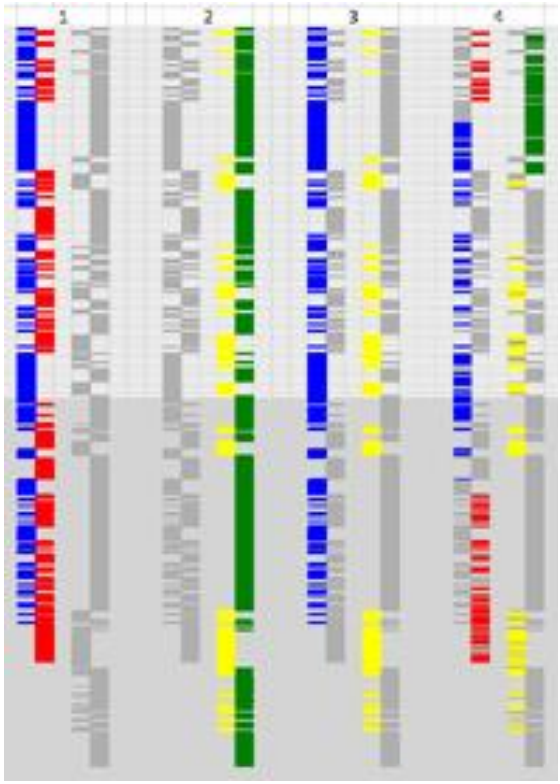

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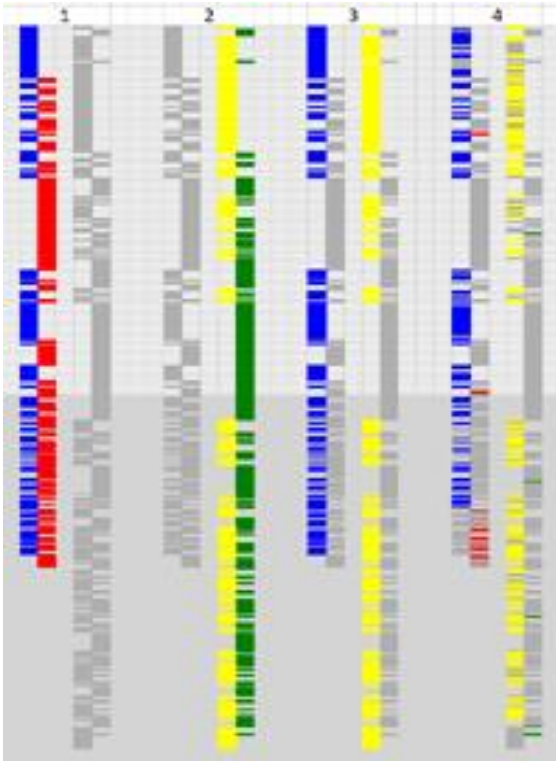

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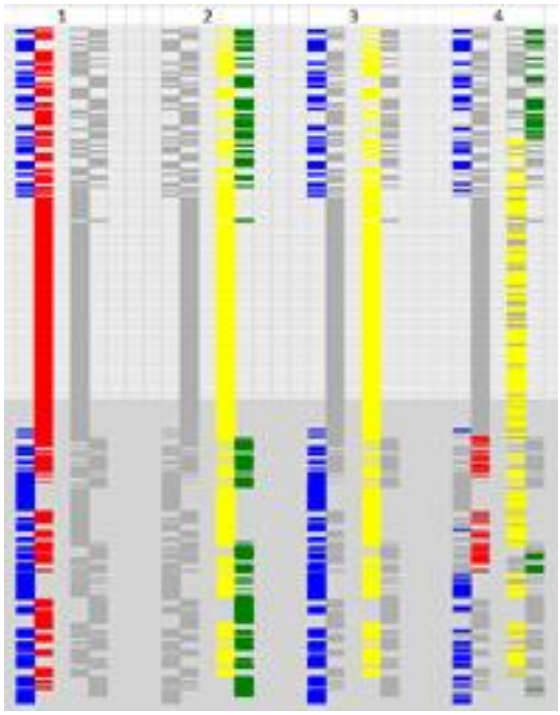

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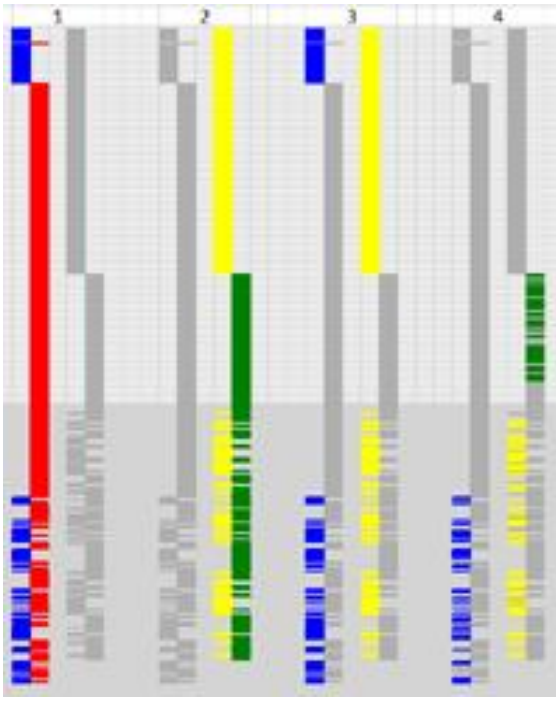

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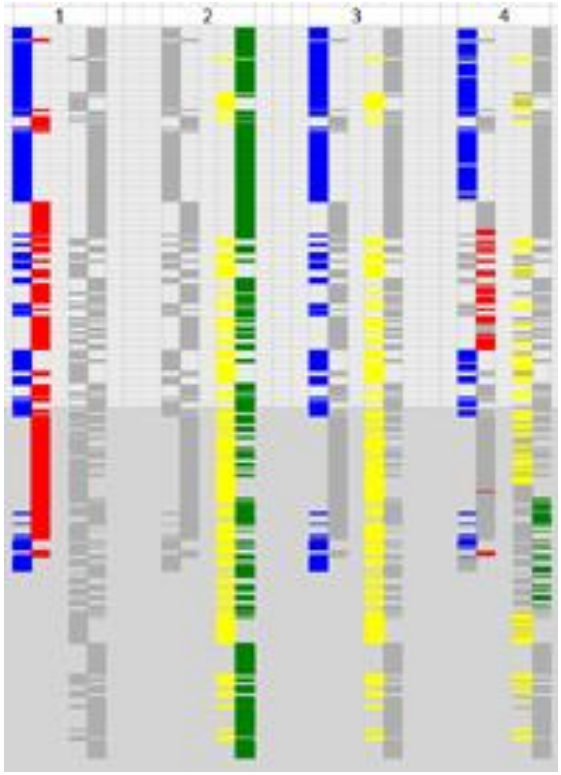

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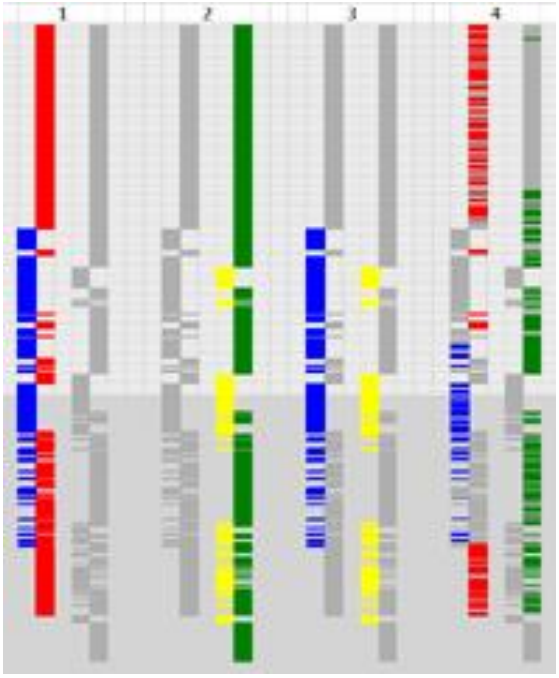

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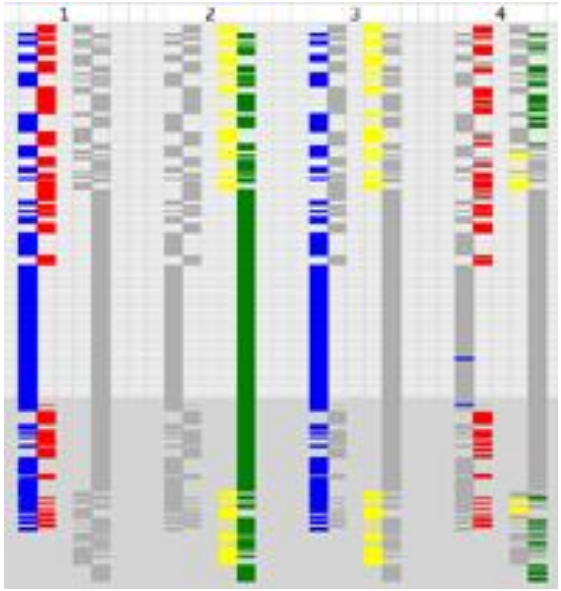

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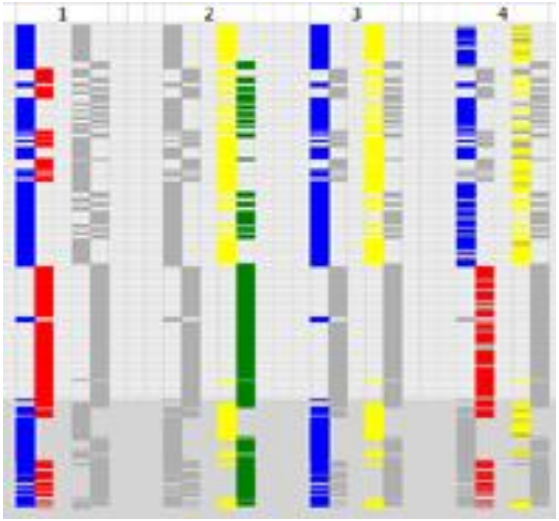

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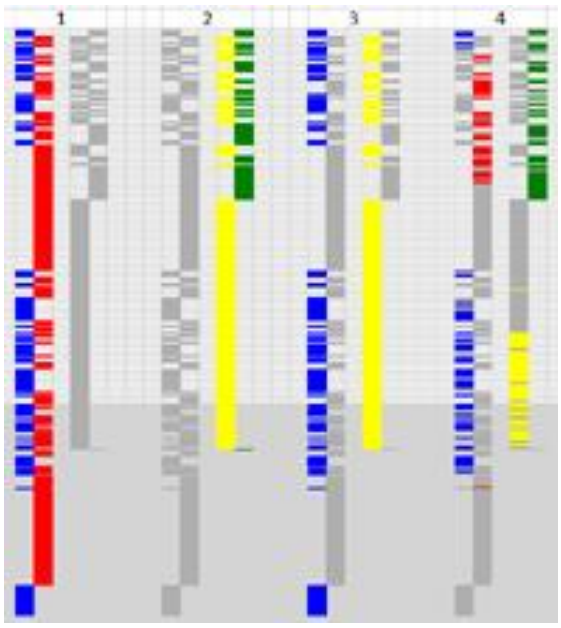

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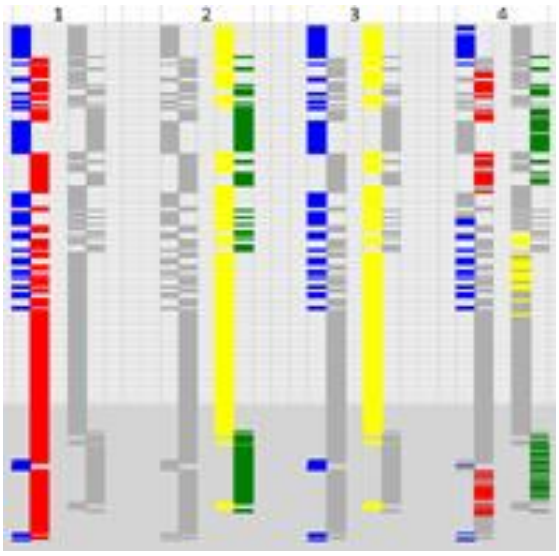

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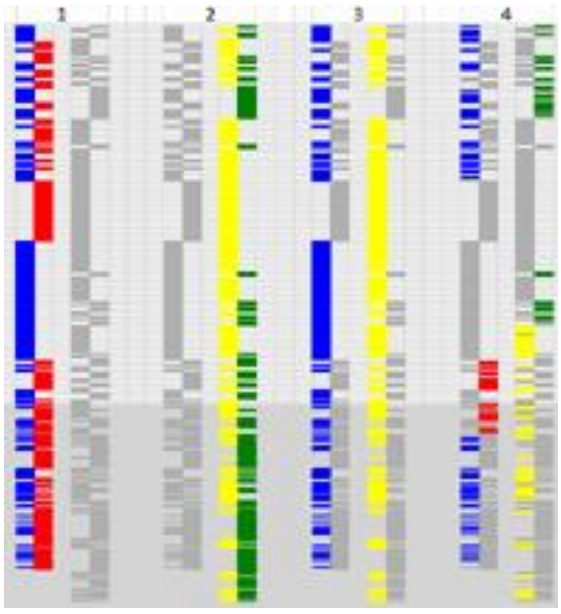

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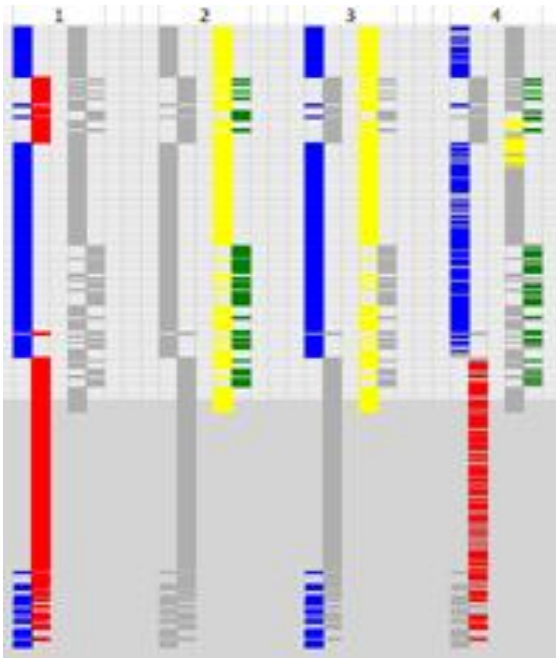

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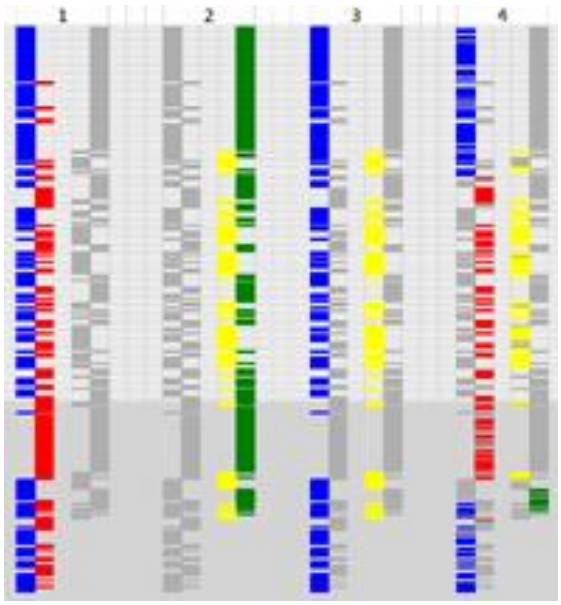

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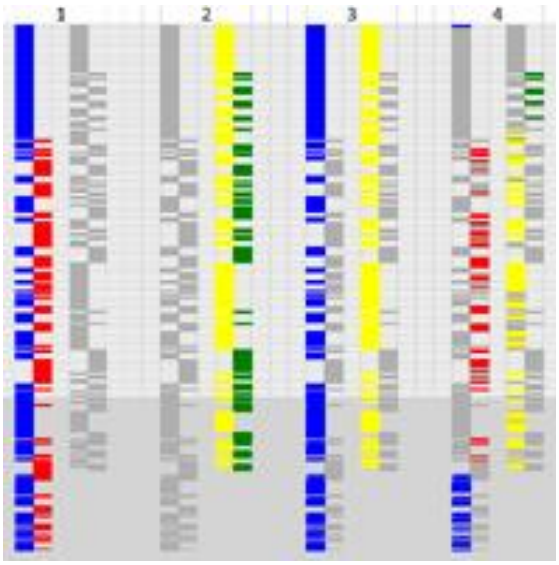

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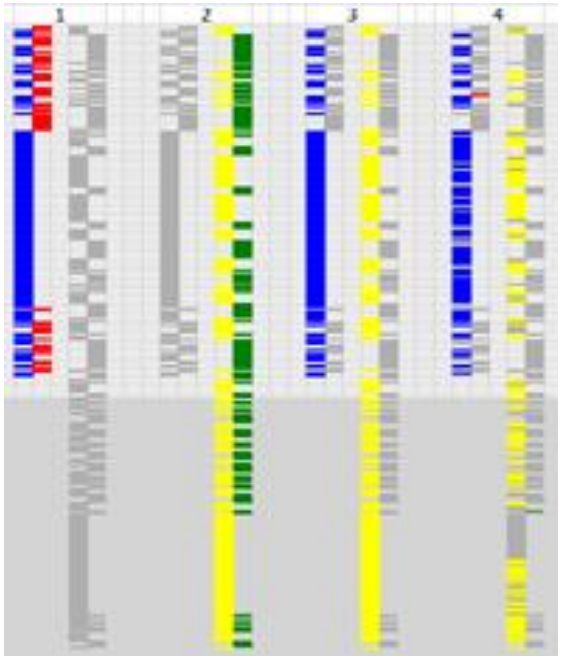

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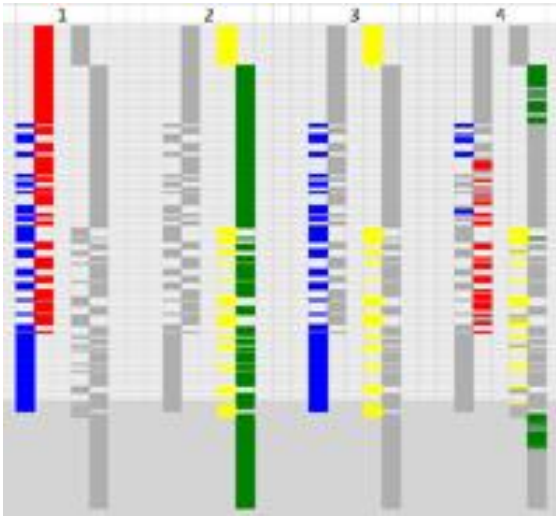

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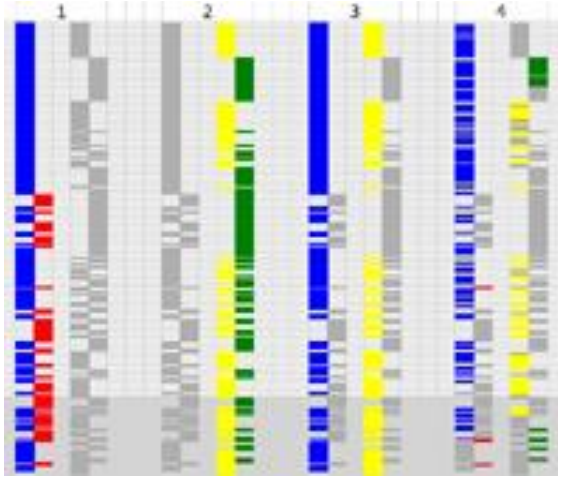

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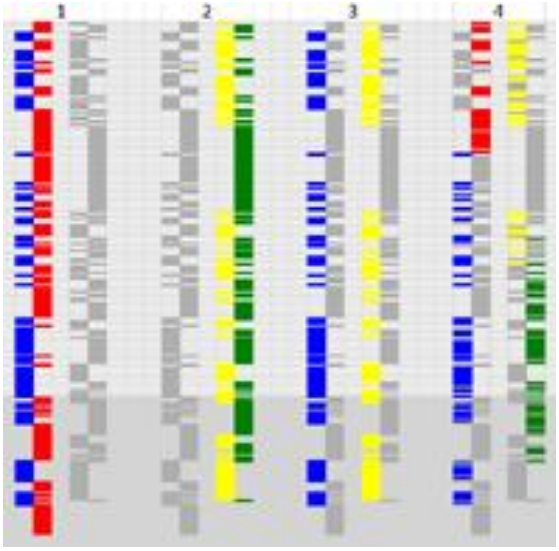

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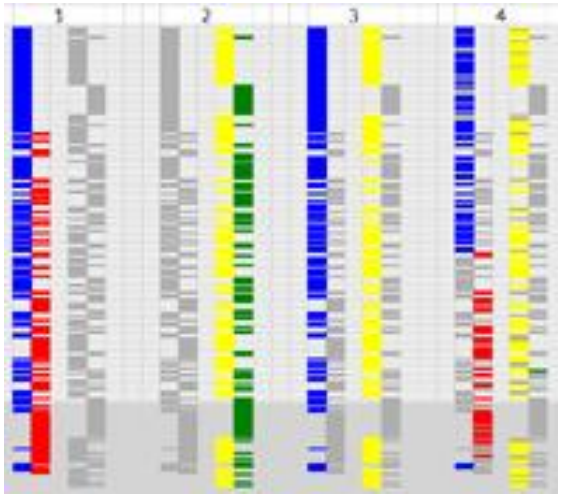

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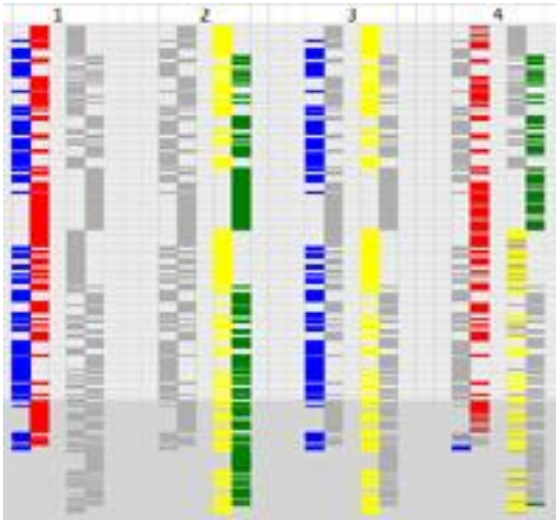

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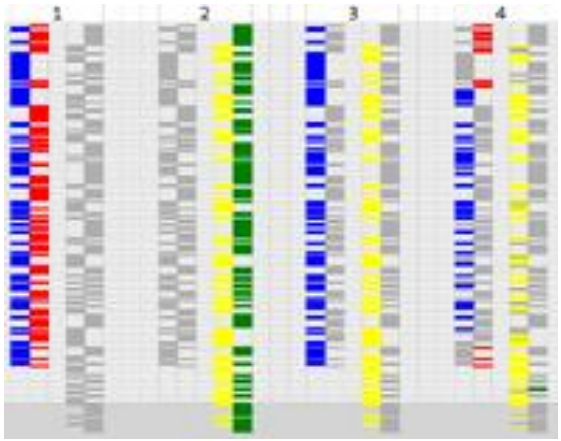

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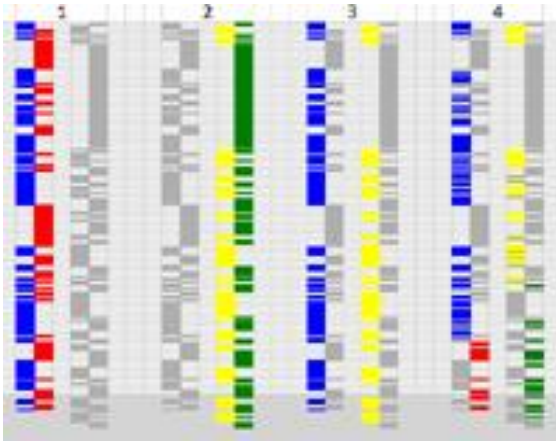

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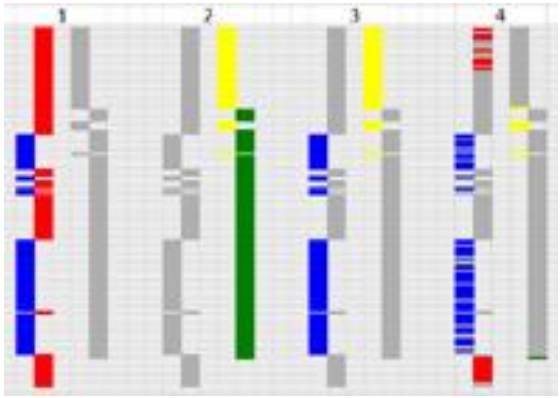

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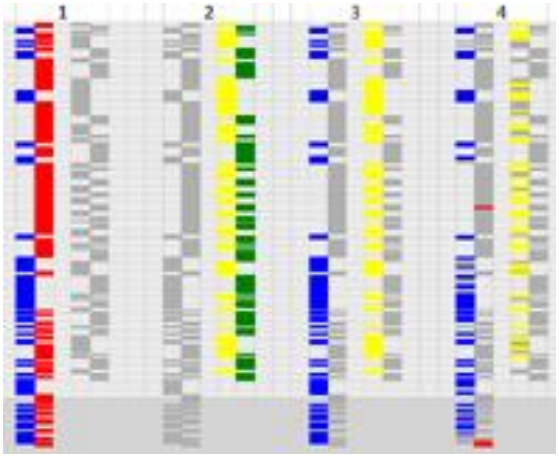

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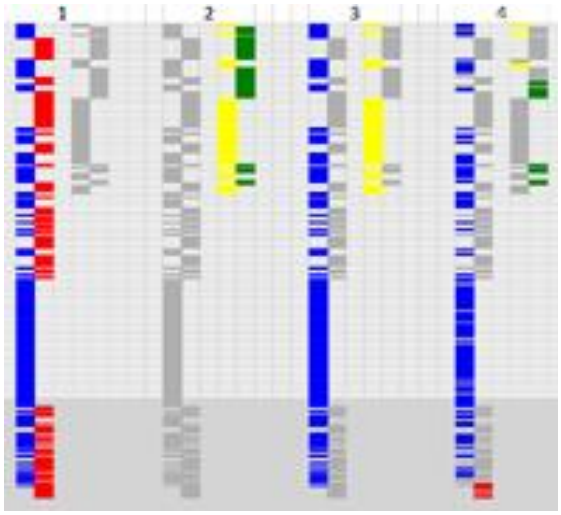
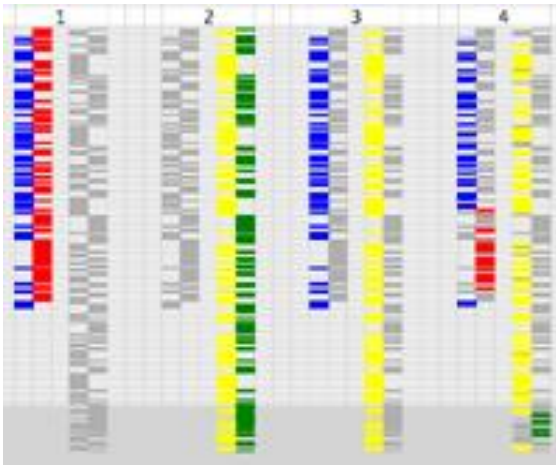


Chromosome 28



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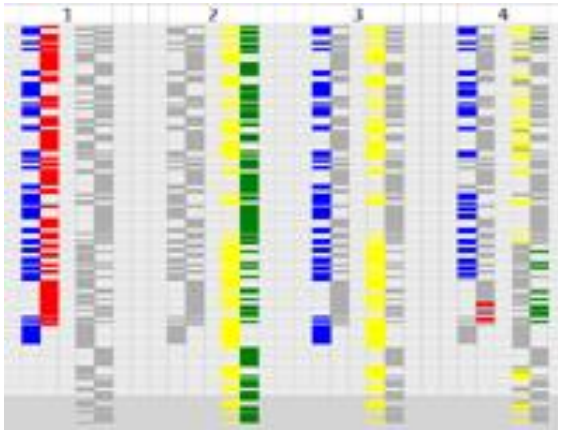

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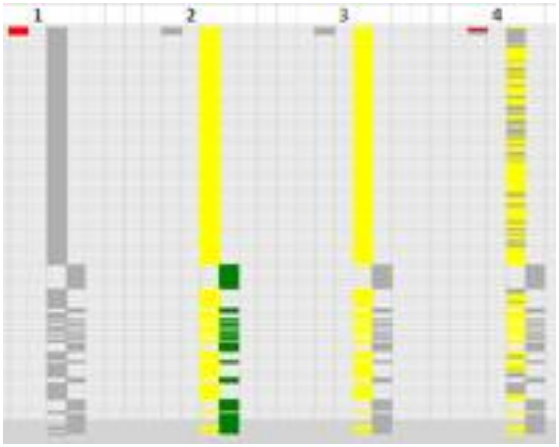

11. Publications and activities arising from this work

Publications

Silvestri, G., Caujolle, S., Cernat, R., Marques, M., Bradu *et al.* (2017) Speckle variance OCT for depth resolved assessment of the viability of bovine embryos. *Biomed Optic Exp* **8:** 5139-5150.

Manuscripts in preparation

Silvestri, G., Turner, K., Smith, C., Black, D.; Sinclair, D., *et al.* Karyomapping in cattle breeding to optimise the delivery of superior genetics and improve food production: Introducing the first live born calves.

Silvestri, G., Turner, K., Silcock, J., Griffin, D. Effects of Different Embryo Splitting Strategies on the Viability and Morphokinetics of Bovine Embryos Produced *in vitro*.

Presentations

Oral presentation, 2CCOCT, University of Kent, Canterbury, UK, September 2017, Caujolle, S., Cernat, R., **Silvestri, G.,** Marques, M., Bradu, A., *et al.* Assessing Embryo Development using Swept Source Optical Coherence Tomography

Oral presentation, ESDAR, Bern, Switzerland, August 2017, Turner, K., Griffin, D., **Silvestri, G.,** Sinclair, K., Smith, C., *et al.* Introducing karyomapping to the cattle breeding industry: use of a comprehensive preimplantation genetic test to optimise the delivery of superior genetics.

Oral presentation, Postgraduate Symposium, University of Kent, Canterbury, UK, July 2017, Optimising food production through the use of IVF in farm animal breeding.

Oral presentation, CISoR mini-symposium, CCCU University, Discovery Park, Sandwich, UK, April 2016, Karyomapping and Cloning: the future of cattle IVP?

Oral presentation, Postgraduate Induction Symposium, University of Kent, Canterbury, UK, September 2015, Embryo splitting for the production of cloned farm animals.

Oral presentation, Postgraduate Research Festival, University of Kent, Canterbury, UK, June 2015, Flying Pigs: From the Laboratory to Your Digestive System – Awarded prize for best presentation.

Poster presentation, Postgraduate Research Festival, University of Kent, Canterbury, UK, June 2015, A New, Two Step Medium for Porcine Embryo Culture.

Poster presentation, Postgraduate Symposium, University of Kent, Canterbury, UK, July 2016, Application of Serial Embryo Splitting in Bovine *in vitro* Production.

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Biomedical Optics EXPRESS

Speckle variance OCT for depth resolved assessment of the viability of bovine embryos

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Abstract: The morphology of embryos produced by *in vitro* fertilization (IVF) is commonly used to estimate their viability. However, imaging by standard microscopy is subjective and unable to assess the embryo on a cellular scale after compaction. Optical coherence tomography is an imaging technique that can produce a depth-resolved profile of a sample and can be coupled with speckle variance (SV) to detect motion on a micron scale. In this study, day 7 post-IVF bovine embryos were observed either short-term (10 minutes) or long-term (over 18 hours) and analyzed by swept source OCT and SV to resolve their depth profile and characterize micron-scale movements potentially associated with viability. The percentage of *en face* images showing movement at any given time was calculated as a method to detect the vital status of the embryo. This method could be used to measure the levels of damage sustained by an embryo, for example after cryopreservation, in a rapid and non-invasive way.

OCIS codes: (110.4500) Optical coherence tomography; (170.1420) Biology; (110.4153) Motion estimation and optical flow; (170.3880) Medical and biological imaging; (030.6140) Speckle.

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Biomedical Optics EXPRESS

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1. Introduction

After fertilization, mammalian zygotes undertake a series of rapid mitotic divisions known as cleavage events which bring them from the original 1 cell to 16-32 cells. In cattle, this phase has a duration of approximately 4 days, after which the embryo undergoes the process of compaction during which it appears as a tight cluster of cells with hardly any distinguishable inter-cellular borders. Between day 6 and 7 after fertilization, as the embryo continues to grow, a liquid filled cavity develops, termed as blastocoel, whilst the embryo is referred to as

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the blastocyst. By the time the embryo reaches the blastocyst stage, its cells have formed two distinct populations: a thin outer layer known as trophectoderm and a compact cluster known as the inner cell mass [1]. A normal bovine blastocyst is presented in Fig. 1.

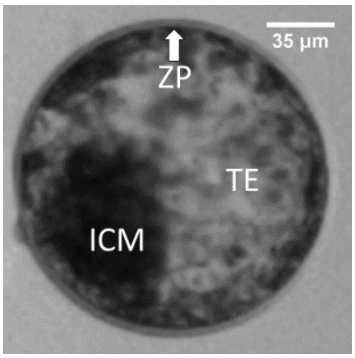

Fig. 1. A typical bovine blastocyst produced *in vitro* using the same method described under section 2.1. ICM: inner cell mass, TE: trophectoderm, ZP: zona pellucida. The embryo can be imagined as a liquid filled sphere. The ICM forms a discrete unit and is attached to the sphere's internal surface, while the surface itself is formed by the thin TE cell layer. Additionally, an outer proteic shell, the ZP (arrow), encapsulates the whole embryo. The image was acquired by a Nikon Eclipse TE200 inverted modulation contrast microscope at x200 total magnification and by using an RI DC2 camera and its dedicated software RI Viewer.

In order to establish a pregnancy, bovine blastocysts produced by *in vitro* fertilization (IVF) are transferred into recipient animals, but the eligibility for transfer requires assessment of the viability of each candidate embryo. Evaluation of the embryo morphology, as a method to estimate its viability, has found wide use in human IVF [2] and the principle that the transfer of embryos of better morphology leads to higher pregnancy rates has long been validated in cattle [3]. One of the most substantial differences between human and bovine embryos, however, is that the latter are made opaque by the accumulation of lipid droplets in their cytoplasm, which makes the embryo harder to assess though conventional methods [4]. Commonly, bovine blastocysts produced by IVF are screened morphologically at x50 to x100 magnification using a stereomicroscope [5]. Whilst this level of investigation is simple and non-invasive, it is also highly subjective, gives little indication of intracellular activity [4], and is unable to quantify accurately the percentage of fragmentation (amount of sub-cellular, non-viable material) in the embryo which is known to affect its viability [6].

To support the morphological assessment of embryos and in an attempt to provide a better prediction of viability, time-lapse systems have been introduced [7]. Although widespread, the use of these systems has failed to produce obvious benefits due to the lack of a stringent correlation between the morphokinetic parameters measured and clinical pregnancy outcomes [8]. Time-lapse systems also present the disadvantages of a long time required to complete an assessment and of a poor depth of view. The second problem is made worse in post-compaction embryos like blastocysts and in bovine embryos generally due to their unfavourable lipid distribution [4]. An example of these limitations is given in Fig. 2, showing that protracted time-lapse observation is likely required to detect any changes in embryo morphology while at the same time, information on the different optical planes of the embryo is likely lost.

Therefore, there is scope for the development and application of new imaging modalities able to resolve the embryo structure in full depth and provide for a rapid and non-subjective assessment of viability.

Optical Coherence Tomography (OCT) is a non-invasive optical method developed in the 1990s that has historically found most applications in ophthalmology [9]. It can create structural images of biological tissues with high axial and transverse resolution providing cross-sectional 2D maps in the (x,z) or (y,z) planes (B-scans) and *en face* 2D maps in the

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(x,y) plane (C-scan), where x and y coordinates are measured along the lateral directions of the sample and z coordinate is measured along its depth.

Moreover, recent advancements have also allowed for the acquisition of functional images, one example being Optical Coherence Tomography Angiography (OCTA) which is used to differentiate moving blood cells from stationary features. The detection is possible due to algorithms like Speckle Variance (SV) analysis which can quantify the changes in the speckle pattern of a sample associated with movement [10–15].

OCT has already found some limited application in developmental biology in model organisms like *Xenopus laevis* [16], and *Rattus norvegicus* [17]. Recently, high-resolution intracellular imaging on live mouse and pig oocytes and embryos has been reported [18]. In this context, the fundamental advantages of OCT are its ability to image embryos without labels, and its use of low power light sources as compared to confocal systems. The use of low power optical beams reduces the chance of damaging the embryo during observation [18, 19]. However, no study so far has applied the motion detection principles of OCTA to early stage embryos, like blastocysts, to assess their viability.

In this study, an OCT system was used to produce a full depth structural characterization of a day 7 post IVF blastocyst and display its 3-D models. Moreover, to test whether kinetic differences could be measured between live and dead embryos, micron-scale movements were measured with the SV method to enable quantitative analysis of embryo viability over time.

Fig. 2. Images of a day 7 bovine blastocyst at different time points after insemination. The images were acquired by phase contrast microscopy at x200 magnification using a time-lapse system PrimoVision Evo (Vitrolife). Completing a viability assessment is likely to require protracted observation as the general morphology of the embryo changes very little over time. Scale bar = 50 µm.

2. Materials and methods

2.1 Preparation of bovine embryos

Bovine blastocysts were produced *in vitro* following a previously described method [20]. Briefly, oocytes were collected from abattoir material and *in vitro* matured, then fertilized with frozen/thawed sperm (Semex). The resulting embryos were then cultured in Synthetic Ovarian Fluid (SOF) medium droplets until they reached the blastocyst stage 7 days post-IVF. Five of the resulting blastocysts were monitored by OCT over short-term observation of several minutes whereas one embryo was monitored over long-term observation (exceeding 18 hours).

2.2 Set-up

The OCT system used is schematically presented in Fig. 3. A swept source at 1310 nm center wavelength (Axsun Technologies), 100 kHz sweep rate, 12 mm coherence length, 106 nm FWHM bandwidth in the range (1256.6 nm–1362.8 nm) [21] is used. The source is connected to a 2 × 2 fiber coupler (C1) with an 80:20 ratio which splits 80% of the power towards the reference arm and 20% of the reference power towards the sample arm. In the sample arm, light is collimated through lens L1 and directed to a pair of galvanometer scanners which, via the imaging lens L2, scan the beam laterally in the (x,y) plane. Light from the reference and

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sample arms are combined in the 50:50 fiber coupler C2. The output ports from C2 are routed to a balanced photo-detector (Thorlabs Model PDB460C, DC 200 MHz). A 12-bit waveform digitizer (AlazarTech ATS9350 - 500 MS/s) digitizes the output signal which is processed using an "in-house" acquisition software written in LabVIEW (National Instruments). This software calculates 500 *en face* images from 500 depths and delivers a real-time compound display of two cross section OCT images, 9 *en face* OCT images and a summed voxel projection (SVP) image, all made possible due to the use of Master-Slave interferometry protocol [21–23]. The combined rendering of the 12 such images is updated every 0.8 s. The depth interval between the 9 selected depth positions is chosen to cover the axial range of the full embryo. Images are produced from 200x200 lateral pixels. The axial resolution was measured to be approximately 15 µm in air and the transverse resolution approximately 4.2 µm.

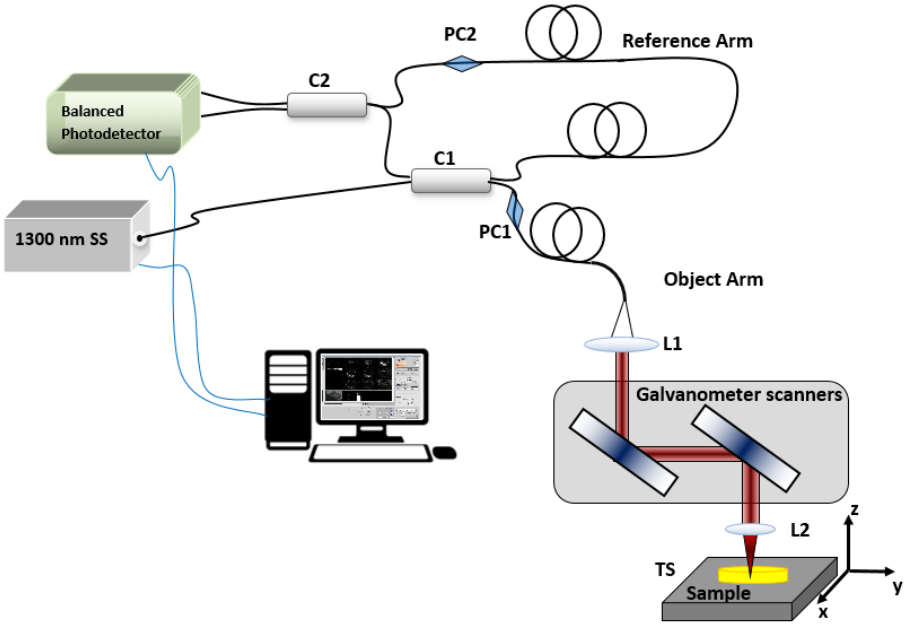

Fig. 3. OCT set-up. SS: swept source; C1, C2: directional optical couplers, PC1, PC2: polarization controllers, L1, L2: lenses, TS: x,y,z translation stage to position the sample.

2.3 Image acquisition and speckle variance (SV) analysis

The embryos are placed on a Petri dish. Then the dish is adjusted laterally and vertically, using the translation stage, TS, by monitoring the SVP image. Initially, the differential distance between the 9 *en face* images in the display is adjusted approximately based on an estimated embryo thickness. While monitoring the two cross section OCT images, the reference arm length is adjusted in order to position the cross sections of the embryos in their central region.

To characterize the structure and the progressive loss of viability of the embryos, cross section images and *en face* images were acquired over short and long-term observation. For short-term observation, 5 embryos were observed for 10 minutes and a full set of data was acquired every minute. For long-term observation, the embryo was observed until it ceased all motion (18 hours), acquiring a set of data every minute for 10 min followed by 20 min of rest; the no-motion measurement was confirmed by another observation performed after a further 8 hours. The SV analysis was performed as described before [10–14] on successive *en face* images at 1 min intervals. The Speckle Variance for each lateral pixel (j,k) is defined as:

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$$SV(i,j,k)=\frac{1}{N}\sum_{i=1}^{N}\left(I(i,j,k)-\frac{1}{N}\sum_{i=1}^{N}I(i,j,k)\right)^2 \quad (1)$$

In (1), I is the strength of the OCT signal at each pixel, N is the total number of *en face* images considered for the calculation which in our case is N = 2 and the index i is the *en face* image concerned, respectively. The calculation was over j and k, i.e. over 200 by 200 pixels, leading to a 2D motion map of similar size. The lateral size of *en face* images is 230 µm by 230 µm. The SV values represent the variance calculated over two successive images with a time lapse equal to 1 min. The larger the variability, the larger the SV. A SV value equal to 0 means that images are identical and no motion is detected. To compensate the effects of Brownian motion, image sets from a dead embryo were also acquired and used to establish a minimum speckle variance value (threshold) for each pixel. In the final display of motion maps, all pixel values whose SV was below this threshold were assigned zero values. Moreover, to produce a quantitative assessment of motion, a parameter "quantity of movement" was calculated as the summation of all pixel values from the motion map.

3. Results

Overall, 5 live bovine blastocysts were observed for 10 minutes (short-term observation) and 1 bovine blastocyst was observed for over 18 hours (long-term observation). During the assessment, 12 structural images were displayed in real time in the form of SVP, 2 x cross sectional images and 9 x *en face* images. In Fig. 4, an example of the acquisition display is given.

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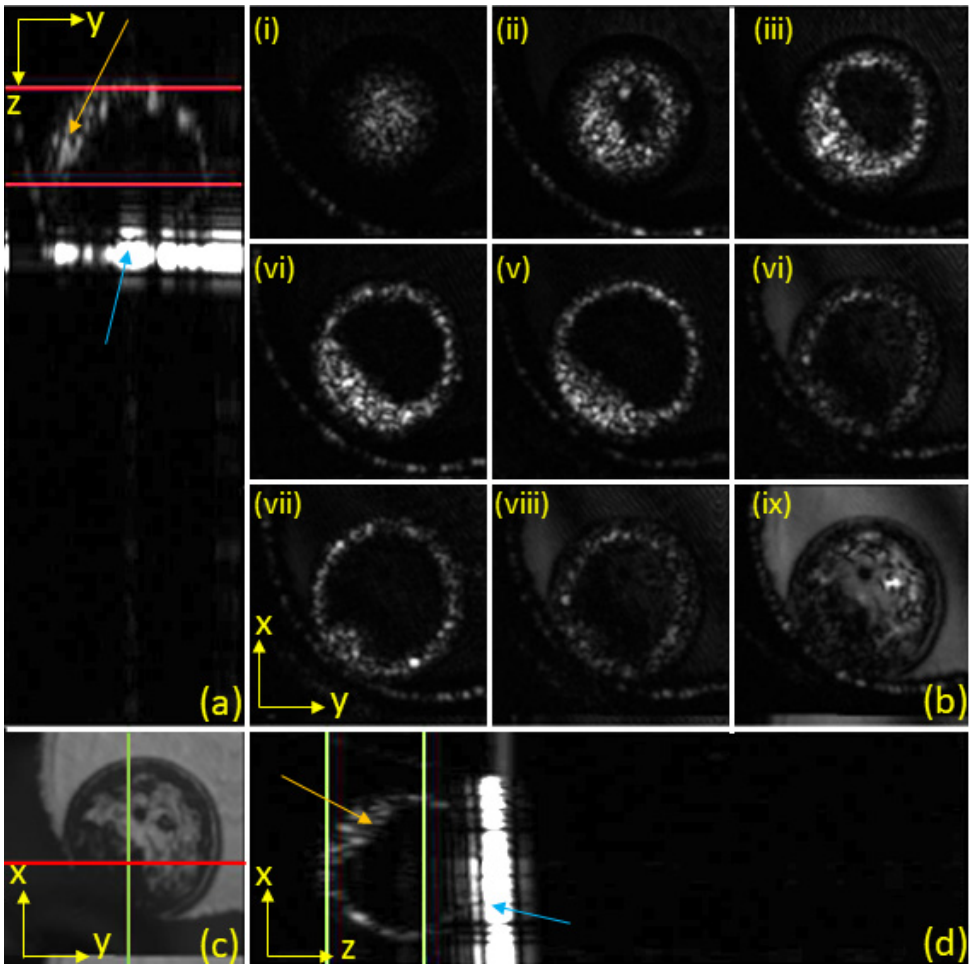

Fig. 4. Screen-shot of the "in-house" Master/Slave software used for image guidance. Three categories of images are displayed simultaneously and in real time in each raster: 2x cross section images, 9x *en face* images and a SVP. (a) Cross section along the red line (plane (z,y)) in the SVP image, (b) (i-ix) *en face* images, (c) SVP and (d) cross section image along the green line (plane (x,z)) in the SVP image. To cover the whole depth of the embryo, 9 *en face* images (i – ix) are shown over a total depth of 225 µm, separated by a depth interval of 25 µm (measured in air). All images are represented on a linear scale. Blue arrows indicate the glass plate and the orange arrow indicates the embryo shape. The lateral size of images is 230 µm and the axial range of the cross sections is 1 mm (measured in air).

3.1 Blastocyst 3D reconstruction and structural imaging

En face images were used to create a 3D volume of the embryo as shown in Fig. 5 and could be used to identify two separate structural entities forming a blastocyst, namely a ring of outer cells (trophectoderm) and a discrete mass of cells in the inside (inner cell mass). The volume allowed by the "in-house" acquisition software written in LabVIEW software extends over a voxel size of 200x200x500 pixels.

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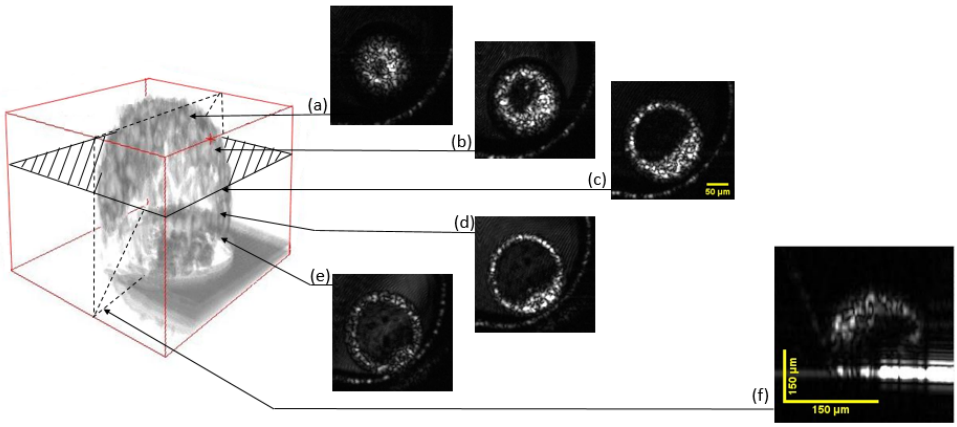

Fig. 5. 3D display of a day 7-post *in vitro* fertilization bovine embryo. *En face* images at different depths from the top embryo surface (a) 25µm, (b) 75µm, (c) 150µm, (d) 225µm and (e) 300µm (all measured in air) at the beginning of the experiment, scale bar = 50µm. (f) cross section view over 500 µm in depth (measured in air), scale bar = 150 µm x 150 µm.

By varying the differential distance between the depths, the axial range was adjusted as such, that the embryo covers 300 depths. Therefore, the size of the volume displayed in Fig. 5 is 200x200x300. Moreover, the shape of the inner cell mass could be clearly distinguished in the 3D reconstruction potentially providing information on its compactness, size and distribution. As described in the introduction (and also in Fig. 2) a blastocyst stage embryo does not necessarily display large morphological changes over a time period of a few hours when observed by standard microscopy, but it can sometimes display a pulsating behavior whereby the embryo collapses on itself and then re-expands to its full size. In this analysis, the blastocyst appeared to maintain its normal structure over the first 12 h; however, by the 13th hour it started to collapse on itself. From this point on, a faint halo of the same size as the original embryo could be detected by OCT indicating that the blastocyst's zona pellucida maintained its shape and position while the rest of the embryo collapsed. Interestingly, the complete collapse of the blastocyst's inner cavity was observed between 15 and 16 h of culture and this detail would have been impossible to notice with standard microscopy. After this point, however, the embryo failed to re-expand, an observation consistent with an embryo approaching the end of its life. A selection of images acquired over long-term observation is given in Fig. 6(A), showing the first 18 h of culture and an additional image acquired 8 h after complete cessation of embryo motion.

3.2 Micron-scale movement detection by SV and quantity of movement calculation

The SV algorithm was applied to obtain motion maps at each embryo depth to highlight the portions of the embryos in motion during each 1 min interval considered. The analysis could also indicate when the embryo ceased all motion (Fig. 6(B)).

For the 5 embryos selected for the short-term observations, motion maps were calculated using *en face* images from a depth approximately in the middle of each embryo.

Figure 7 shows the maximum speckle variance registered in such maps. Usefully, the maximum value calculated for the dead control embryo was found to be at least 5 times smaller than the average maximum value of any live embryo.

For the next evaluations, a threshold was calculated to differentiate the live embryos from the dead one. This was obtained as the maximum SV extracted from the motion maps evaluated over the whole embryo volume.

In Fig. 8, quantity of movement was calculated at 5 different depths (as indicated) in the same embryo over long-term observation and it was possible to pinpoint at which depth the

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embryo maintained movement over time. The calculation included only those pixels out of the 200x200, where the SV exceeded the threshold evaluated above.

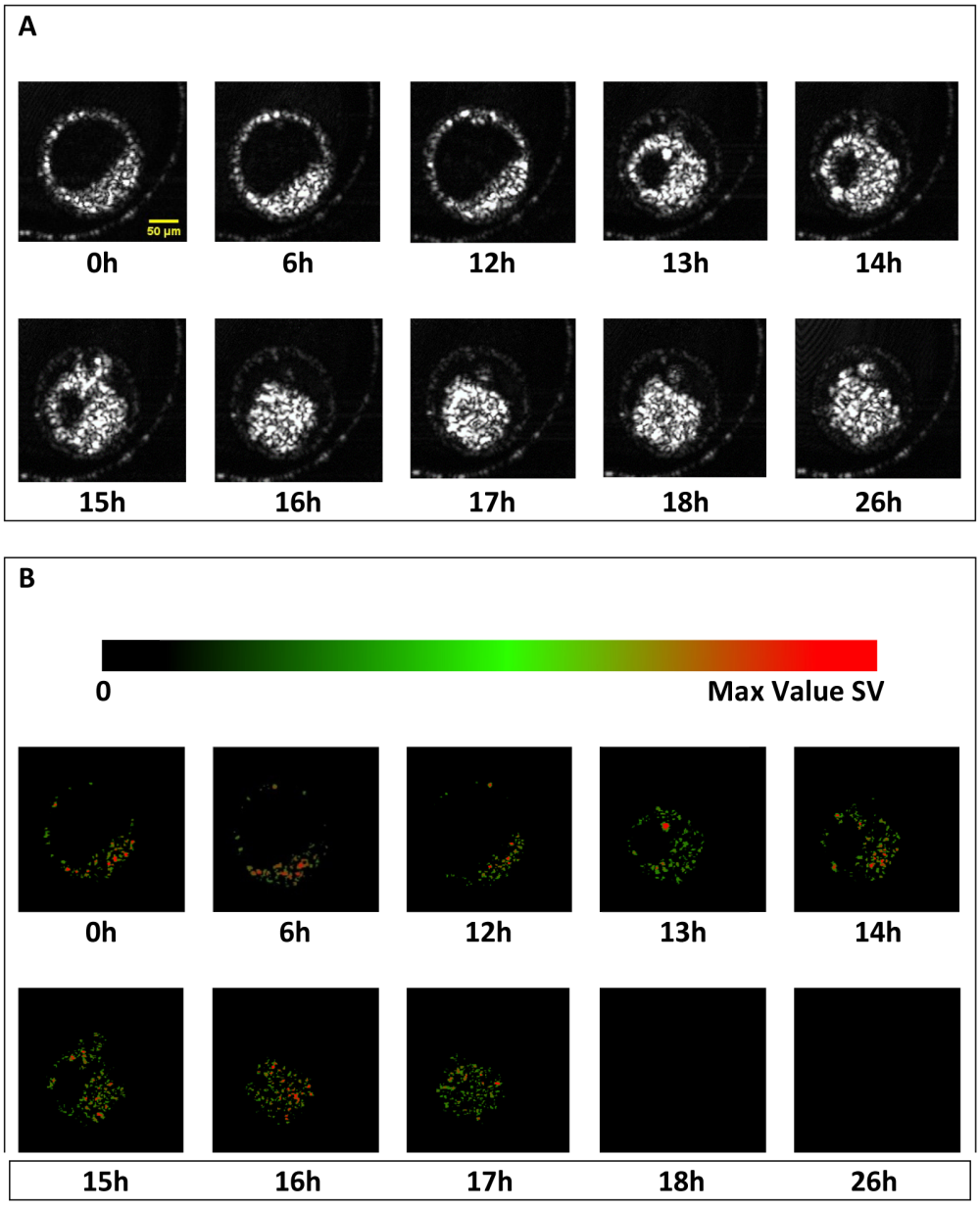

Fig. 6. *En face* images (A) of the embryo over 26 h (actively monitored over the first 18 h) and their SV *en face* display/Motion Map (B) at a fixed depth (150 µm from the top embryo surface measured in air). Scale bar = 50µm. SV value are displayed Red/Green scale. Red represents higher value.

An alternative representation of this information is given in Fig. 9, showing the percentage of *en face* images from the same embryo in which movement could be detected at any given time. The calculation was performed over 300 *en face* images obtained from different depths covering the embryo thickness (i.e. using the same adjustment of axial range as in Fig. 5).

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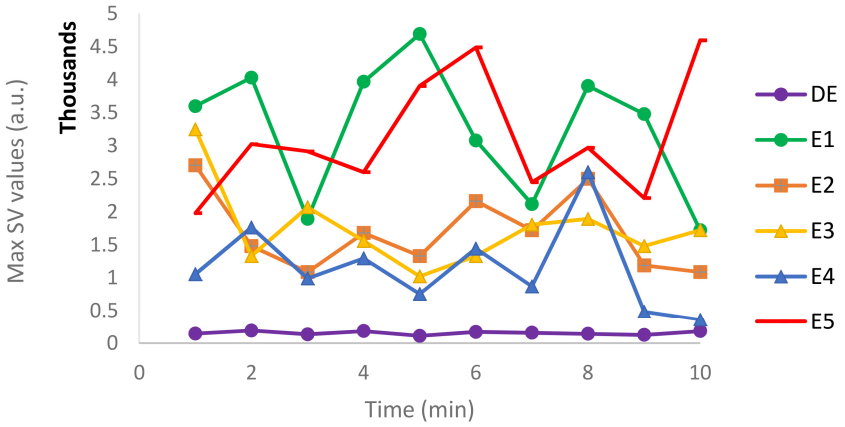

Fig. 7. Maximum value of the Speckle Variance for different embryos over 10 minutes. Embryo 1 – Embryo 5 (E1-E5): Curves representing the quantity of movement for live embryos, DE: Curve for dead embryo.

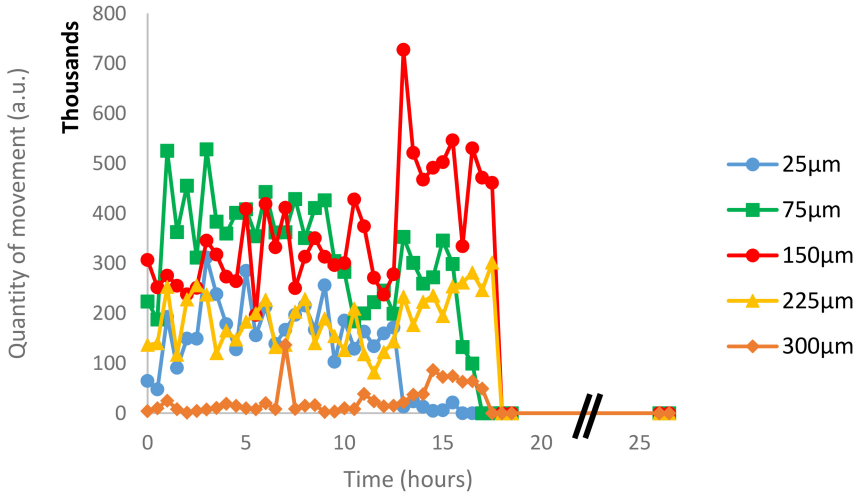

Fig. 8. Quantity of movement (for the embryo 5 in Fig. 6) at superficial (25 µm and 300 µm, middle (75 µm and 225 µm) and central depth (150 µm) over long-term observation.

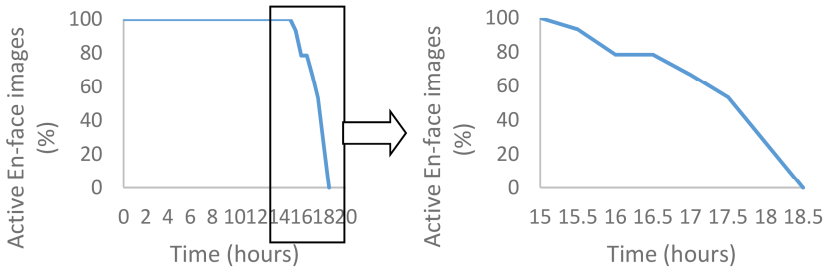

Fig. 9. Percentage of number of *en face* images displaying SV values above threshold at any given time for an embryo (number 5 in Fig. 7) over long-term observation. The downward trend is expanded on the right hand to better display the decreasing motion of the embryo over time.

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4. Discussion

To date, a limited number of reports have focused on applying functional OCT imaging to study early mammalian embryos [18, 24, 25]. However, to our best knowledge, this is the first time OCT and SV have been applied to a day 7 post-IVF mammalian embryo to resolve its 3D structure and quantify micron-scale movement.

As seen in Fig. 5, OCT can be successfully used to identify the key structures within the bovine blastocyst. Additionally, motion maps can be produced (Fig. 6(B)) to detect motion in the areas of the blastocyst which are populated with cells.

Over short-term observation, all live embryos consistently displayed higher SV values (Fig. 7) when compared with dead controls suggesting that a live embryo is likely to possess levels of intracellular activity detectable by OCT.

However, the interpretation of the motion measurement became more complex during long-term observation. As shown in Fig. 6(A), the embryo had collapsed on itself after 12 h of culture. This is an expected behavior for a blastocyst approaching the end of its life. As shown in Fig. 8, this was associated with a surge in movement around the median portion of the embryo at a depth of 150 µm. One possible explanation for this behavior is that the SV measurement is sensitive at the same time to both genuine intracellular movement and to mechanical movement of the blastocyst's tissues caused by the deflation of its internal cavity and that the summation of these two movements caused the spark detected. Indeed, while the embryo could safely be declared dead after 18 h of culture, the actual cessation of biochemical activity within it could have happened at an earlier time point between 15 and 18 hours. On one hand, non-blank motion maps were obtained between 17 and 18 hours of culture after the complete disappearance of the embryo's blastocoel when mechanical motion seemingly came to an end, suggesting that the embryo more likely ceased all biochemical activity around the 18th hour mark. On the other hand, this might not be the only possible interpretation as spontaneous decaying processes, not necessarily linked with intra-cellular activity, could have been detected instead. However, these type of movements were not present in the dead controls. Therefore, based on the comments above, whilst the decaying trend in Fig. 9 expresses the attenuation of motion inside the embryo, it cannot be used to predict the exact time of the embryo's death.

As portrayed in Fig. 8, the embryo did not behave consistently across its depth from a kinetic point of view over long-term functional observation. This finding is not necessarily surprising since it is known that cells or cell groups in a blastocyst can become fragmented or even die [30]. This is also known to happen in embryos that are cryopreserved. Indeed, the cryopreservation of an embryo in liquid nitrogen is a standard procedure, however not all embryos survive the freeze thawing process and those that do are likely to suffer from various levels of damage [31]. Cryodamage assessment on a per embryo basis is very challenging due to the invasive nature of the tests available. Very few papers have used OCT to investigate cryodamage in embryos [32, 33] and none of them studied blastocyst stage embryos which are widely cryopreserved in both human and animal IVF systems. The SV analysis here described could become a useful tool to quantify the level of damage sustained by cryopreserved embryos within minutes of thawing and without a need for extended culture. The results of this assessment would be easy to interpret since they would simply take the form of percentages of *en face* images in motion, as shown in Fig. 8.

5. Conclusions

We anticipate that in future studies, OCT, due to its potential submicron axial resolution, could be used to obtain precise measurements of the shape and volume of a blastocyst's inner cell mass, since these parameters have been shown to correlate well with implantation rates when measured by conventional microscopy [26]. In a similar way, OCT can be used to measure non-invasively the volume of a blastocyst's trophectoderm to obtain another metric

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of embryo quality: the trophectoderm/inner cell mass ratio [27], an assessment that currently implies the destruction of the embryo with standard methods [28, 29].

The assessment of the percentage of *en face* images in active motion could also be expanded to take into account that, due to the spherical nature of the embryo, not all the *en face* images contain the same number of cells so that a proportion of actively moving embryo volume could be calculated instead. This refinement can be conducted to accurately measure the percentage of fragmentation (intended as non-viable material) present in an embryo, a metric closely associated with viability which is currently estimated subjectively [6].

In conclusion, OCT and SV analysis hold promise to become useful tools for the rapid identification of embryos with poor viability for example after freeze/thawing. The proper adjustment of the embryo prior to measurements based on the *en face* direct view of the Master Slave OCT method further facilitates the procedure.

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Disclosures

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