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5 Technique to "map" chromosomal mosaicism at the blastocyst stage

6 Tyl H. Taylor, MS[1,2]*,^, Darren K. Griffin, Ph.D.[2], Seth L. Katz, MD[1], Jack L. Crain, MD[1], Lauren Johnson,
7 MD, MSCE[1], Susan Gitlin, Ph.D.[3]

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9 [1] Reproductive Endocrinology Associates of Charlotte

10 Charlotte, North Carolina, USA, 28277

11 * tyltaylor@gmail.com

12 [2] University of Kent

13 School of Biosciences

14 Centre for Interdisciplinary Studies of Reproduction

15 Canterbury, CT2 7NJ, UK

16 [3]Eastern Virginia Medical School

17 Norfolk, Virginia

18 * tyltaylor@gmail.com

19 ^Author's current address

20 Illumina Inc.

21 5200 Illumina Way

22 San Diego, CA 92122

23

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27   **Abstract**

28   **Purpose:** To identify a technique that allows for comprehensive chromosome screening (CCS) of

29   individual cells within the human blastocysts along with the approximation of their location in the

30   trophectoderm relative to the inner cell mass.   This proof of concept study will allow for a greater

31   understanding of chromosomal mosaicism at the blastocyst stage and the mechanisms by which

32   mosaicism arises.

33   **Methods:** One blastocyst was held by a holding pipette and the inner cell mass was removed. While

34   still being held, the blastocyst was further biopsied into quadrants. To separate the individual cells from

35   the biopsied sections, the sections were placed in Calcium/Magnesium free medium with serum for 20

36   minutes. A holding pipette was used to aspirate the sections until individual cells were isolated.

37   Individual cells from each section were placed into PCR tubes and prepped for array comparative

38   genomic hybridization.

39   **Results:** A total of 18 cells were sent for analysis of which 15 (83.3%) amplified and provided a result

40   and three (16.7%) did not.

41   Fifteen cells were isolated from the trophectoderm, 13 (86.7%) provided an aCGH result while two

42   (13.3%) did not amplify. Twelve cells were euploid (46, XY) while one was complex abnormal (44, XY)

43   presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21. A total of three cells were isolated

44   from the ICM, two were euploid (46, XY) and one did not amplify.

45   **Conclusion**: Here we expand on a previously published technique which disassociates biopsied sections

46   of the blastocysts into individual cells. Since the blastocyst sections were biopsied in regard to the

47   position of the ICM, it was possible to reconstruct a virtual image of the blastocyst while presenting with

48   each cell's individual CCS results.

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Introduction

The presence of two or more distinct cell lines, commonly referred to as chromosomal

mosaicism, is one of the potential pitfalls when analyzing embryos by comprehensive chromosome

screening (CCS). The ability to detect mosaicism accurately is determined by the technology used,

number of chromosomes examined and number of cells analyzed (1).   Even if mosaicism is present, the

impact on subsequent development varies depending upon which chromosome is involved and at what

stage the chromosomal abnormality occurs (1).

CCS requires that the cells be pipetted into a PCR tube for analysis rather than fixed on a slide as

previously performed with fluorescence in-situ hybridization (FISH) studies (2). To examine individual

cells, each cell needs to be pipetted individually into a PCR tube, and each tube must undergo the CCS

procedure. This makes the process labor intensive and expensive compared to FISH.

Although multiple studies have examined mosaicism at the blastocyst stage with CCS, these

studies have all involved biopsied sections with multiple cells in each section, perhaps masking the true

extent of mosaicism (3, 4, 5). The examination of individual cells at the blastocyst stage is particularly

important to gain insight into possible origins and mechanisms of mosaicism, such as non-disjunction,

endoreduplication, anaphase lagging, uniparental disomy, and their prevalence during preimplantation

development (1). Indeed, mosaicism could be responsible both for false negative and false positive PGS

diagnoses (6, 7).

In this present study, we expand upon a novel technique by which individual cells of a blastocyst

could be isolated and a virtual image of the blastocyst with CCS results could be created (8).

71   Unfortunately, the previous study did not perform CCS. With this report, we have successfully isolated

72   individual cells from the blastocyst, mapped their location in reference to the ICM, and successfully

73   performed CCS on the individual cells.   This proof of concept study could allow insights into the

74   mechanism through which mosaicism arose in the blastocyst.

75   **Methods**

76   This study was approved by an institutional review board (WIRB #1138244) and utilized

77   blastocysts deemed not viable and destined for discard. The University of Kent Research Ethics Advisory

78   Group also approved this study.

79   One blastocyst from a 33 year old patient, donated to research, that did not initially have

80   assisted hatching, underwent the following procedure. The whole blastocyst was placed into a 20 µL

81   drop of Calcium/Magnesium (Ca^{2+}/Mg^{2+}) free medium (Cooper/Sage, Trumbull, CT, USA) with 10%

82   serum substitute supplement (SSS; Irvine Scientific, Santa Ana, California, USA) and overlayed by oil

83   (Irvine Scientific, Santa Ana, California, USA). The blastocyst was held with a holding pipette (Origio,

84   Denmark), positioning the ICM at the 9 o'clock position (Figure 1A). A laser was used to create a hole in

85   the trophectoderm at the 3 o'clock position. A biopsy pipette was inserted into the blastocyst and the

86   ICM was removed with gentle suction and isolated (Figure 1B). The ICM was removed from the drop

87   and placed into another drop of Ca^{2+}/Mg^{2+} free with 10% SSS. Using a similar method, Capalbo and

88   colleagues (9) demonstrated a 2% trophectoderm contamination rate when removing the ICM.

89   The blastocyst underwent four further biopsies, thereby separating the blastocyst into

90   quadrants (Figure 1C and Figure 1D). After each biopsy, the biopsy needle was changed and the

91   biopsied piece was pipetted out of the biopsy drop and into an individual drop of Ca^{2+}/Mg^{2+} free

92   medium + 10% SSS for 20 minutes (Figure 1E). This process was repeated after each section so there

93   was no cross contamination or mislabeling of sections during the procedure. After 20 minutes, a holding

94   pipette was used to gently aspirate the sections of the blastocysts (Figure 1F). Doing so allowed the

95   sections of the blastocyst to break apart into smaller pieces. Therefore, multiple, individual cells were

96   obtained from each quadrant (Figure 1G).

97   The cells of the blastocyst were identified under a dissecting scope. Cells were rinsed in wash

98   solution and prepped for aCGH. aCGH was performed as previously described (10).

99

100   **Results**

101   A total of 18 cells were sent for aCGH. Of the 15 cells isolated from the trophectoderm, 13

102   (86.7%) provided a result while two (13.3%) did not amplify. Twelve were euploid (46, XY) and one was

103   complex abnormal (44, XY) presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21.   The

104   complex aneuploid cell was located in region "3" which is from the polar trophectoderm adjacent to the

105   ICM (Figure 2).

106   A total of three cells were isolated from the ICM, 2 (66.7%) were euploid and one did not

107   amplify (Figure 2).

108   **Discussion**

109   We herein describe a novel approach that we believe to be the first to combine isolation of

110   individual blastocyst cells with the utilization of CCS. This powerful approach can be used to determine

111   the extent of mosaicism in the human blastocyst. Moreover, by examining the CCS results of individual

112   cells within the blastocyst, the mechanisms of mosaicism can be determined (e.g.; non-disjunction,

113   uniparental disomy, endoreduplication, or anaphase lagging) (1).

114   Multiple studies have attempted to determine mosaicism at the blastocyst stage with mosaicism

115   rates ranging from 16-70% (11, 12, 13). All three of these studies examined mosaicism in two to three

116   sections of the trophectoderm, each containing several cells. Examining these large of sections would

117   not allow the chromosome constitution of individual cells within the blastocyst to be determined and

118   thus, the true rate of mosaicism may be masked by the presence of multiple cells. In order to minimize

119   the impact of multiple cells on the rate of mosaicism, the chromosome results for individual cells must

120   be examined.

121   As previously mentioned, the detection of mosaicism is dependent upon on how many cells are

122   analyzed. All of these aforementioned studies examined mosaicism in these large sections which

123   contained multiple cells. In our study, our blastocyst was mosaic but this mosaicism would not have

124   been detected had we not analyzed individual cells. Eight individual aneuploidies were detected in the

125   trophectoderm. In a background of otherwise euploid cells we would infer that each was an individual

126   post-zygotic error. In the absence of a reciprocal pattern for each (i.e. a corresponding trisomy and

127   monosomy of the same chromosome) we would infer that the +14, +15, +21 aneuploidies arose via

128   independent chromosome gain (perhaps some mechanism involving endoreduplication) and the

129   monosomies -7, -10, -11, -13, -19 by independent chromosome loss (anaphase lag). Utilizing FISH,

130   Delhanty and colleagues (14) and Ioannou and colleagues (15) demonstrated a lack of mitotic non-

131   disjunction (3+1 pattern), suggesting that mitotic non-disjunction is rare as a mechanism for post-zygotic

132   aneuploidy in human development.   More recent data utilizing CCS supports the notion that non-

133   disjunction is a rare event, demonstrating that chromosome losses occur at 4x higher rate than

134   chromosome gains (16). We didn't test individual cells and that it's possible we "missed" the

135   corresponding reciprocal aneuploidies. Further studies are certainly warranted to improve upon our

136   technique.

137   A meiotic error should be present in the entire, or at least a majority, of cells analyzed. In our

138   proof of concept study, only one cell contained aneuploidies while the remaining cells were euploid.

139   This would suggest that the error arose during mitosis and not meiosis. Previous research has

140   demonstrated that approximately 25% of polar bodies are aneuploid (17) while approximately 50% of

141   blastocysts are aneuploid (18, 19). The higher incidence of aneuploidy at the blastocyst stage suggests

142   that a majority of aneuploidy may be mitotic in origin. The approach described in this study will allow us

143   to test the hypothesis that post-zygotic aneuploidy of individual cells is commonplace in the trophoblast

144   during human development but less so in the inner cell mass.

145   Unfortunately, in our study we were only able to detect one aneuploidy cell. It cannot be

146   overlooked that our one aneuploid cell could be due to an error in the CCS test. Capalbo and colleagues

147   (20) demonstrated that aCGH overcalls aneuploidy. However, Capalbo and colleagues (20) also

148   demonstrated that on a per chromosome basis the accuracy of aCGH is >98%. Another source of error

149   could be due to "noise" within the plot of the CCS result. Some NGS protocols minimize "noise" and

150   produce cleaner CCS plots, reducing the chance of misdiagnosis. NGS was not used in this study because

151   it had not been validated on single cells when this study occurred, whereas aCGH had (21). Moreover,

152   Fiorentino and colleagues (22) reran 192 aCGH samples with NGS and found 191 (99.5%) were

153   concordant. Nonetheless, future studies should utilize NGS to reduce the chances of misdiagnosis. Due

154   to the high concordance of NGS to aCGH, the accuracy of aCGH on a per chromosome basis, and the fact

155   that our study had eight different chromosomes from one cell diagnosed as aneuploidy, suggests that

156   this aneuploid diagnosis is indeed biological and not an artifact.

157   Ozawa and Hansen (23) were able to desegregate individual bovine blastocysts by exposure to

158   trypsin and pipetting the blastocysts through a small glass pulled pipette. Similarly, we utilized a holding

159   pipette designed for holding the oocyte or embryo during micromanipulation procedures. This pipette

160   had a very small bore size and assisted in the separation of cells from the trophectoderm. Our

161   technique could also prove valuable for human embryonic stem cells (hESC). Often times these cells are

162   in clumps and clusters and the isolation of single hESC may be desired for hESC culture. Prowse *et al*.

163   (24) performed a similar process by which clumps of hESC were washed with Ca^{2+}/Mg^{2+}. After the wash,

164   they added trypsin to help in the dissociation of cells. Similarly, Hasegawa and colleagues (25) also

165   disassociated clumps of hESC into individual cells utilizing trypsin. We did not add trypsin to our cells

166   and it is unknown if this would have aided in our separation. In these studies, trypsin was used on hESC

167   whereas our study dealt with trophectoderm cells and trypsin may not separate trophectoderm cells as

168   easily as hESC cells. We utilized Ca/Mg free media because it was readily available and has been used in

169   conjunction with CCS tests and embryo biopsy for years and its influence on CCS results would be

170   minimal (26). Another problem is the difficulty in the visualization of the cells after isolation. One

171   suggestion could be the addition of a hypotonic solution to the isolated cells, thereby allowing them to

172   swell and become more easily distinguishable under a microscope (27). Another technique referred to

173   as optical tweezing allows for the control of small particles and possibly could be used to isolate

174   individual cells (28,29). However, this technique would require an expensive piece of equipment and

175   training, neither of which our technique requires.

176   Given our success with this proof of concept study, larger studies are certainly warranted,

177   despite the cost of CCS. Even increasing the number of blastocysts to 10 in our study would utilize

178   approximately 200-250 CCS tests and patients may present with different rates of mosaicism thereby

179   making a well-designed, high powered study difficult and costly. Our findings stress the need to perform

180   a similar study on a greater number of embryos with the ultimate aim of both improving diagnosis for

181   PGS families and better understanding the nature of our own early development.

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Figure 1: (A) The whole blastocyst with the quadrants and inner cell mass (ICM) marked prior to biopsy. (B) Blastocyst undergoing ICM removal, the quadrants are marked. (C) The blastocyst during the biopsy of the "B" quadrant. The "A" quadrant has already been biopsied. (D) The blastocyst after the biopsy of ICM, quadrant "A", and quadrant "B". (E) Quadrant "B" of the blastocyst prior to separation into single cells. (F) Quadrant "B" being pipetted through the holding pipette. (G) Individual cells of Quadrant "B" prior to placement into the PCR tube.
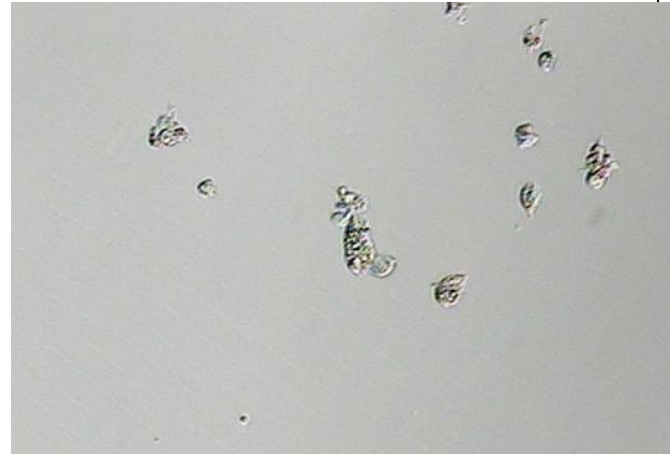

A.

B.

C.

D.

E.

F.

Quadrant "B"
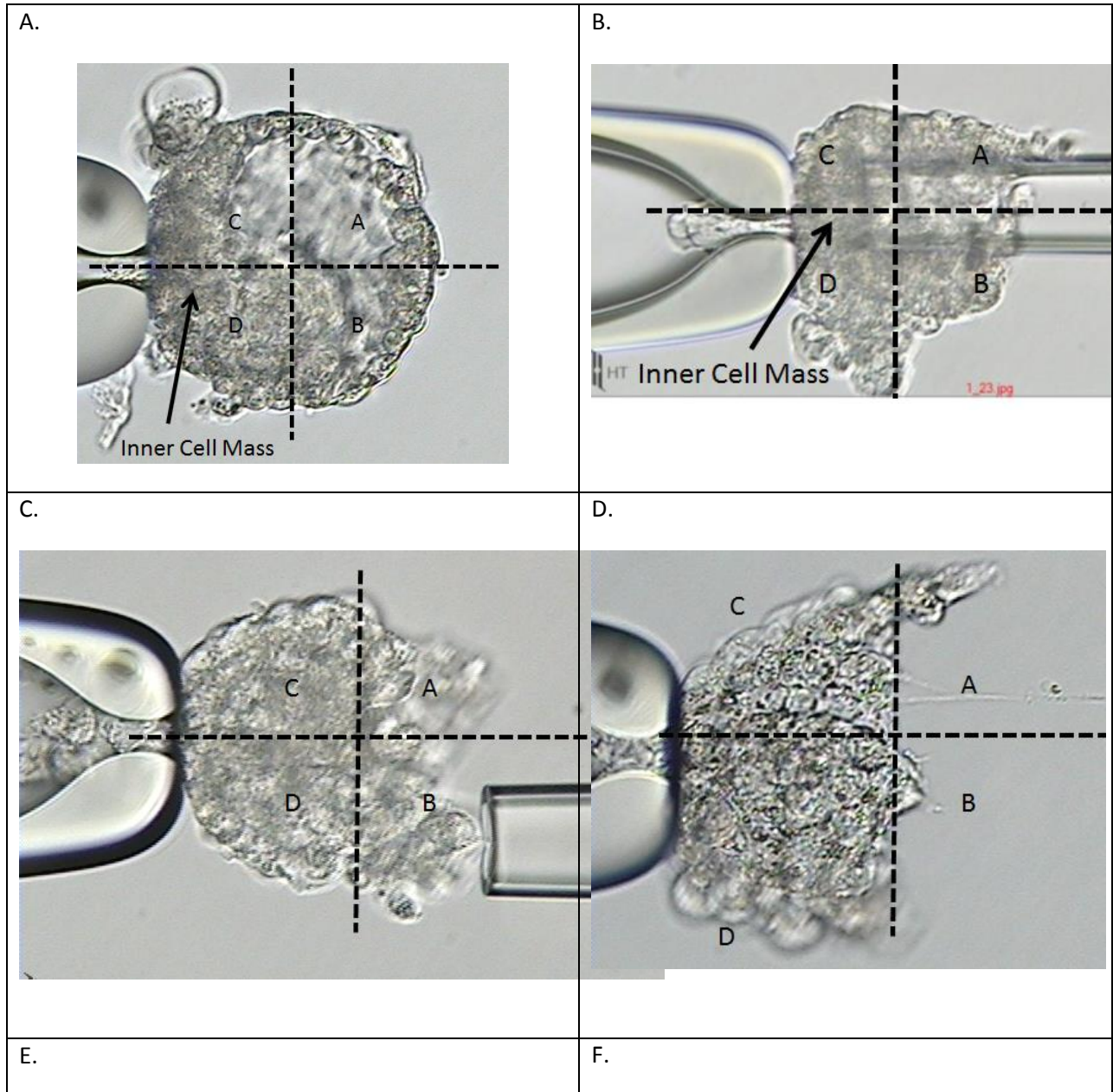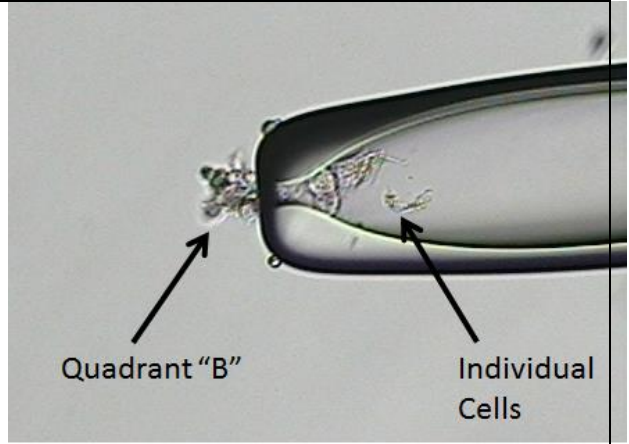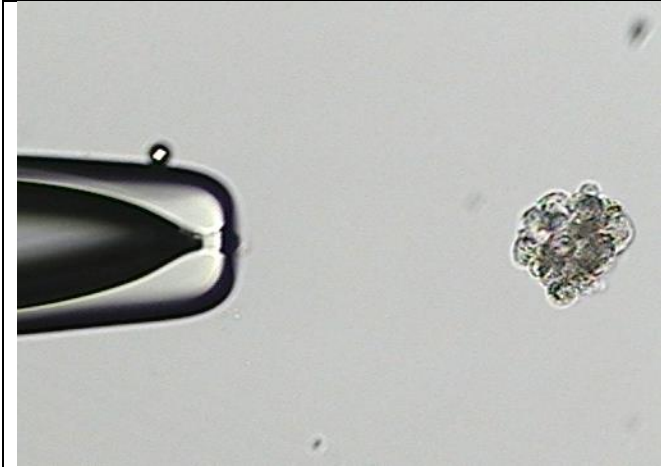
Individual Cells

G.

Figure 2: Reconstructed trophectoderm and inner cell mass (ICM) with the location and the CCS results of individual cells within the blastocyst.