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Technique to “map” chromosomal mosaicism at the blastocyst stage

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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 trophoctoderm 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 trophoctoderm, 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.

49

50 **Keywords:** PGS, mosaicism, CCS, blastocyst, aneuploidy

51 **Introduction**

52 The presence of two or more distinct cell lines, commonly referred to as chromosomal
53 mosaicism, is one of the potential pitfalls when analyzing embryos by comprehensive chromosome
54 screening (CCS). The ability to detect mosaicism accurately is determined by the technology used,
55 number of chromosomes examined and number of cells analyzed (1). Even if mosaicism is present, the
56 impact on subsequent development varies depending upon which chromosome is involved and at what
57 stage the chromosomal abnormality occurs (1).

58 CCS requires that the cells be pipetted into a PCR tube for analysis rather than fixed on a slide as
59 previously performed with fluorescence in-situ hybridization (FISH) studies (2). To examine individual
60 cells, each cell needs to be pipetted individually into a PCR tube, and each tube must undergo the CCS
61 procedure. This makes the process labor intensive and expensive compared to FISH.

62 Although multiple studies have examined mosaicism at the blastocyst stage with CCS, these
63 studies have all involved biopsied sections with multiple cells in each section, perhaps masking the true
64 extent of mosaicism (3, 4, 5). The examination of individual cells at the blastocyst stage is particularly
65 important to gain insight into possible origins and mechanisms of mosaicism, such as non-disjunction,
66 endoreduplication, anaphase lagging, uniparental disomy, and their prevalence during preimplantation
67 development (1). Indeed, mosaicism could be responsible both for false negative and false positive PGS
68 diagnoses (6, 7).

69 In this present study, we expand upon a novel technique by which individual cells of a blastocyst
70 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 μ L
81 drop of Calcium/Magnesium ($\text{Ca}^{2+}/\text{Mg}^{2+}$) free medium (Cooper/Sage, Trumbull, CT, USA) with 10%
82 serum substitute supplement (SSS; Irvine Scientific, Santa Ana, California, USA) and overlaid 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ 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 $\text{Ca}^{2+}/\text{Mg}^{2+}$ 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 Ca²⁺/Mg²⁺. 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.

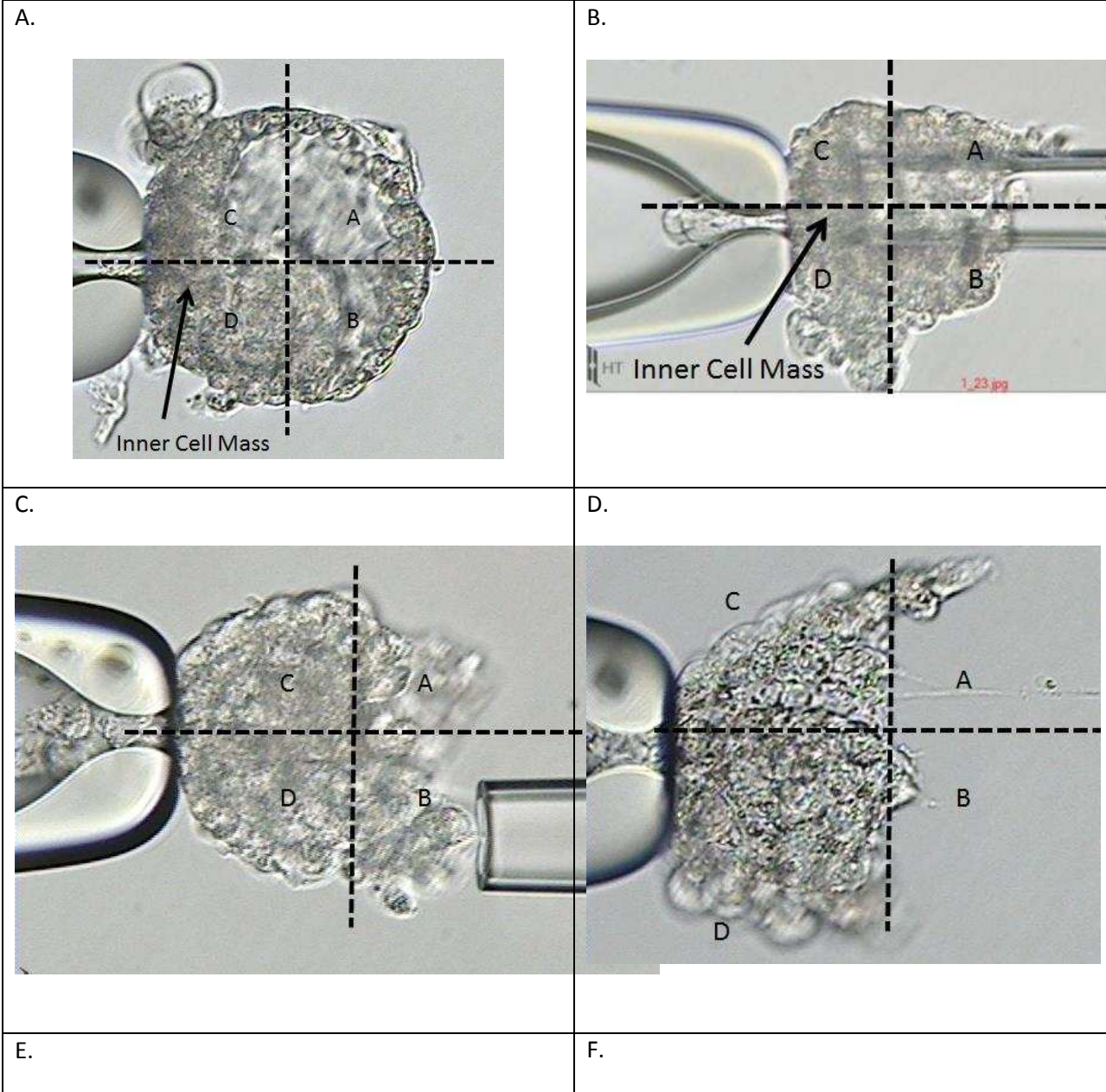
176 Given our success with this proof of concept study, larger studies are certainly warranted,
177 despite the cost of CCS. Even increasing the number of blastocysts to 10 in our study would utilize
178 approximately 200-250 CCS tests and patients may present with different rates of mosaicism thereby
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.

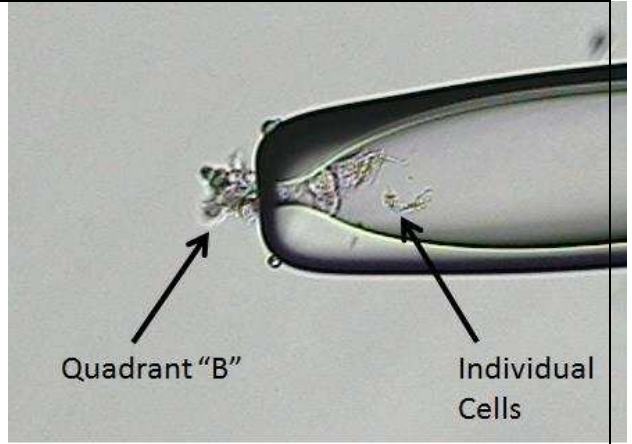
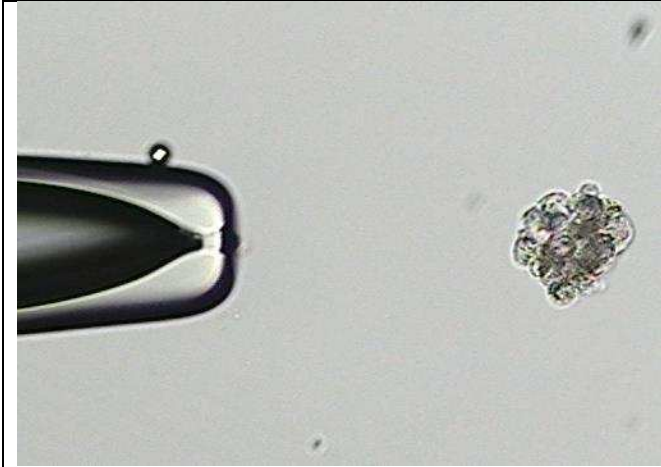
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Figure 1: (A) The whole blastocyst with the quadrants and inner cell mass (ICM) marked prior to biopsy. (B) Blastocyst undergoing ICM removal, the quadrants are marked. (C) The blastocyst during the biopsy of the "B" quadrant. The "A" quadrant has 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.





G.

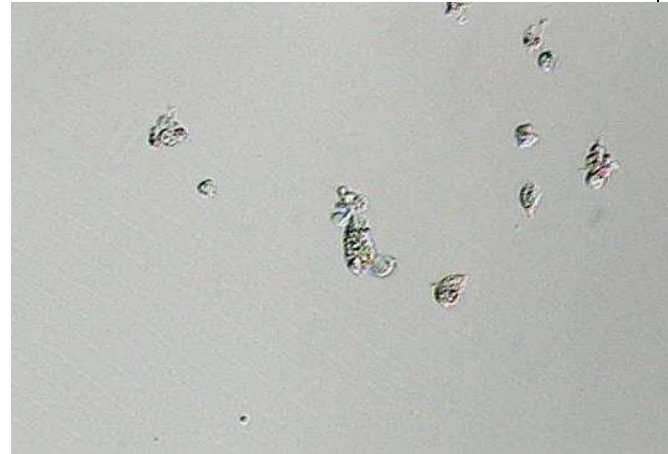


Figure 2: Reconstructed trophoctoderm and inner cell mass (ICM) with the location and the CCS results of individual cells within the blastocyst.

