Technique to “map” chromosomal mosaicism at the blastocyst stage

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

[1] Reproductive Endocrinology Associates of Charlotte

Charlotte, North Carolina, USA, 28277

* tyltaylor@gmail.com

[2] University of Kent

School of Biosciences

Centre for Interdisciplinary Studies of Reproduction

Canterbury, CT2 7NJ, UK

[3]Eastern Virginia Medical School

Norfolk, Virginia

* tyltaylor@gmail.com

^Author’s current address

Illumina Inc.

5200 Illumina Way

San Diego, CA 92122

Abstract

Purpose: To identify a technique that allows for comprehensive chromosome screening (CCS) of individual cells within the human blastocysts along with the approximation of their location in the trophectoderm relative to the inner cell mass. This proof of concept study will allow for a greater understanding of chromosomal mosaicism at the blastocyst stage and the mechanisms by which mosaicism arises.

Methods: One blastocyst was held by a holding pipette and the inner cell mass was removed. While still being held, the blastocyst was further biopsied into quadrants. To separate the individual cells from the biopsied sections, the sections were placed in Calcium/Magnesium free medium with serum for 20 minutes. A holding pipette was used to aspirate the sections until individual cells were isolated. Individual cells from each section were placed into PCR tubes and prepped for array comparative genomic hybridization.

Results: A total of 18 cells were sent for analysis of which 15 (83.3%) amplified and provided a result and three (16.7%) did not.

Fifteen cells were isolated from the trophectoderm, 13 (86.7%) provided an aCGH result while two (13.3%) did not amplify. Twelve cells were euploid (46, XY) while one was complex abnormal (44, XY) presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21. A total of three cells were isolated from the ICM, two were euploid (46, XY) and one did not amplify.

Conclusion: Here we expand on a previously published technique which disassociates biopsied sections of the blastocysts into individual cells. Since the blastocyst sections were biopsied in regard to the position of the ICM, it was possible to reconstruct a virtual image of the blastocyst while presenting with each cell's individual CCS results.

Keywords: PGS, mosaicism, CCS, blastocyst, aneuploidy

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).

Unfortunately, the previous study did not perform CCS. With this report, we have successfully isolated individual cells from the blastocyst, mapped their location in reference to the ICM, and successfully performed CCS on the individual cells. This proof of concept study could allow insights into the mechanism through which mosaicism arose in the blastocyst.

Methods

This study was approved by an institutional review board (WIRB #1138244) and utilized blastocysts deemed not viable and destined for discard. The University of Kent Research Ethics Advisory Group also approved this study.

One blastocyst from a 33 year old patient, donated to research, that did not initially have assisted hatching, underwent the following procedure. The whole blastocyst was placed into a 20 µL drop of Calcium/Magnesium (Ca^{2+}/Mg^{2+}) free medium (Cooper/Sage, Trumbull, CT, USA) with 10% serum substitute supplement (SSS; Irvine Scientific, Santa Ana, California, USA) and overlayed by oil (Irvine Scientific, Santa Ana, California, USA). The blastocyst was held with a holding pipette (Origio, Denmark), positioning the ICM at the 9 o'clock position (Figure 1A). A laser was used to create a hole in the trophectoderm at the 3 o'clock position. A biopsy pipette was inserted into the blastocyst and the ICM was removed with gentle suction and isolated (Figure 1B). The ICM was removed from the drop and placed into another drop of Ca^{2+}/Mg^{2+} free with 10% SSS. Using a similar method, Capalbo and colleagues (9) demonstrated a 2% trophectoderm contamination rate when removing the ICM.

The blastocyst underwent four further biopsies, thereby separating the blastocyst into quadrants (Figure 1C and Figure 1D). After each biopsy, the biopsy needle was changed and the biopsied piece was pipetted out of the biopsy drop and into an individual drop of Ca^{2+}/Mg^{2+} free medium + 10% SSS for 20 minutes (Figure 1E). This process was repeated after each section so there was no cross contamination or mislabeling of sections during the procedure. After 20 minutes, a holding

pipette was used to gently aspirate the sections of the blastocysts (Figure 1F). Doing so allowed the sections of the blastocyst to break apart into smaller pieces. Therefore, multiple, individual cells were obtained from each quadrant (Figure 1G).

The cells of the blastocyst were identified under a dissecting scope. Cells were rinsed in wash solution and prepped for aCGH. aCGH was performed as previously described (10).

Results

A total of 18 cells were sent for aCGH. Of the 15 cells isolated from the trophectoderm, 13 (86.7%) provided a result while two (13.3%) did not amplify. Twelve were euploid (46, XY) and one was complex abnormal (44, XY) presenting with monosomy 7, 10, 11, 13, 19 and trisomy 14, 15, 21. The complex aneuploid cell was located in region "3" which is from the polar trophectoderm adjacent to the ICM (Figure 2).

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

Discussion

We herein describe a novel approach that we believe to be the first to combine isolation of individual blastocyst cells with the utilization of CCS. This powerful approach can be used to determine the extent of mosaicism in the human blastocyst. Moreover, by examining the CCS results of individual cells within the blastocyst, the mechanisms of mosaicism can be determined (e.g.; non-disjunction, uniparental disomy, endoreduplication, or anaphase lagging) (1).

Multiple studies have attempted to determine mosaicism at the blastocyst stage with mosaicism rates ranging from 16-70% (11, 12, 13). All three of these studies examined mosaicism in two to three sections of the trophectoderm, each containing several cells. Examining these large of sections would not allow the chromosome constitution of individual cells within the blastocyst to be determined and thus, the true rate of mosaicism may be masked by the presence of multiple cells. In order to minimize the impact of multiple cells on the rate of mosaicism, the chromosome results for individual cells must be examined.

As previously mentioned, the detection of mosaicism is dependent upon on how many cells are analyzed. All of these aforementioned studies examined mosaicism in these large sections which contained multiple cells. In our study, our blastocyst was mosaic but this mosaicism would not have been detected had we not analyzed individual cells. Eight individual aneuploidies were detected in the trophectoderm. In a background of otherwise euploid cells we would infer that each was an individual post-zygotic error. In the absence of a reciprocal pattern for each (i.e. a corresponding trisomy and monosomy of the same chromosome) we would infer that the +14, +15, +21 aneuploidies arose via independent chromosome gain (perhaps some mechanism involving endoreduplication) and the monosomies -7, -10, -11, -13, -19 by independent chromosome loss (anaphase lag). Utilizing FISH, Delhanty and colleagues (14) and Ioannou and colleagues (15) demonstrated a lack of mitotic non-disjunction (3+1 pattern), suggesting that mitotic non-disjunction is rare as a mechanism for post-zygotic aneuploidy in human development. More recent data utilizing CCS supports the notion that non-disjunction is a rare event, demonstrating that chromosome losses occur at 4x higher rate than chromosome gains (16). We didn't test individual cells and that it's possible we "missed" the corresponding reciprocal aneuploidies. Further studies are certainly warranted to improve upon our technique.

A meiotic error should be present in the entire, or at least a majority, of cells analyzed. In our proof of concept study, only one cell contained aneuploidies while the remaining cells were euploid. This would suggest that the error arose during mitosis and not meiosis. Previous research has demonstrated that approximately 25% of polar bodies are aneuploid (17) while approximately 50% of blastocysts are aneuploid (18, 19). The higher incidence of aneuploidy at the blastocyst stage suggests that a majority of aneuploidy may be mitotic in origin. The approach described in this study will allow us to test the hypothesis that post-zygotic aneuploidy of individual cells is commonplace in the trophoblast during human development but less so in the inner cell mass.

Unfortunately, in our study we were only able to detect one aneuploidy cell. It cannot be overlooked that our one aneuploid cell could be due to an error in the CCS test. Capalbo and colleagues (20) demonstrated that aCGH overcalls aneuploidy. However, Capalbo and colleagues (20) also demonstrated that on a per chromosome basis the accuracy of aCGH is >98%. Another source of error could be due to "noise" within the plot of the CCS result. Some NGS protocols minimize "noise" and produce cleaner CCS plots, reducing the chance of misdiagnosis. NGS was not used in this study because it had not been validated on single cells when this study occurred, whereas aCGH had (21). Moreover, Fiorentino and colleagues (22) reran 192 aCGH samples with NGS and found 191 (99.5%) were concordant. Nonetheless, future studies should utilize NGS to reduce the chances of misdiagnosis. Due to the high concordance of NGS to aCGH, the accuracy of aCGH on a per chromosome basis, and the fact that our study had eight different chromosomes from one cell diagnosed as aneuploidy, suggests that this aneuploid diagnosis is indeed biological and not an artifact.

Ozawa and Hansen (23) were able to desegregate individual bovine blastocysts by exposure to trypsin and pipetting the blastocysts through a small glass pulled pipette. Similarly, we utilized a holding pipette designed for holding the oocyte or embryo during micromanipulation procedures. This pipette

had a very small bore size and assisted in the separation of cells from the trophectoderm. Our technique could also prove valuable for human embryonic stem cells (hESC). Often times these cells are in clumps and clusters and the isolation of single hESC may be desired for hESC culture. Prowse *et al*. (24) performed a similar process by which clumps of hESC were washed with Ca^{2+}/Mg^{2+}. After the wash, they added trypsin to help in the dissociation of cells. Similarly, Hasegawa and colleagues (25) also disassociated clumps of hESC into individual cells utilizing trypsin. We did not add trypsin to our cells and it is unknown if this would have aided in our separation. In these studies, trypsin was used on hESC whereas our study dealt with trophectoderm cells and trypsin may not separate trophectoderm cells as easily as hESC cells. We utilized Ca/Mg free media because it was readily available and has been used in conjunction with CCS tests and embryo biopsy for years and its influence on CCS results would be minimal (26). Another problem is the difficulty in the visualization of the cells after isolation. One suggestion could be the addition of a hypotonic solution to the isolated cells, thereby allowing them to swell and become more easily distinguishable under a microscope (27). Another technique referred to as optical tweezing allows for the control of small particles and possibly could be used to isolate individual cells (28,29). However, this technique would require an expensive piece of equipment and training, neither of which our technique requires.

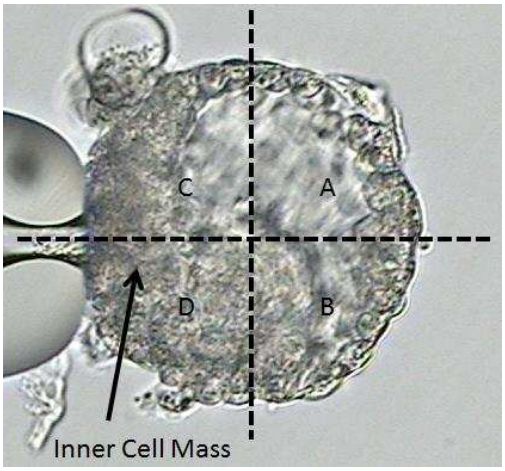
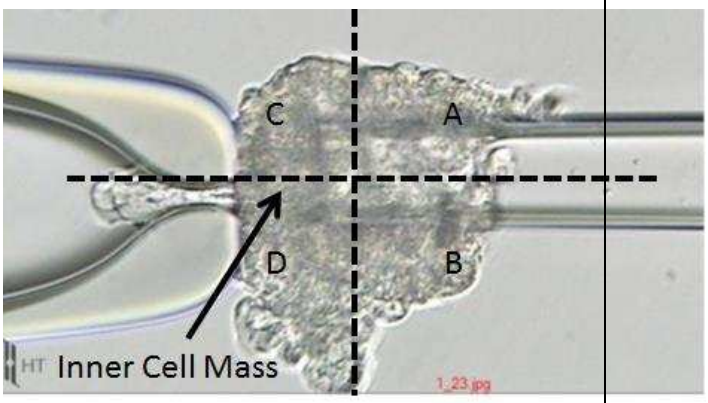
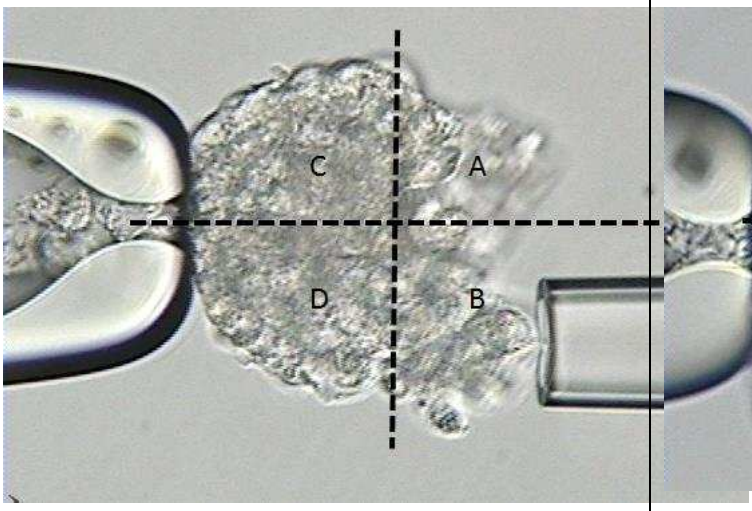
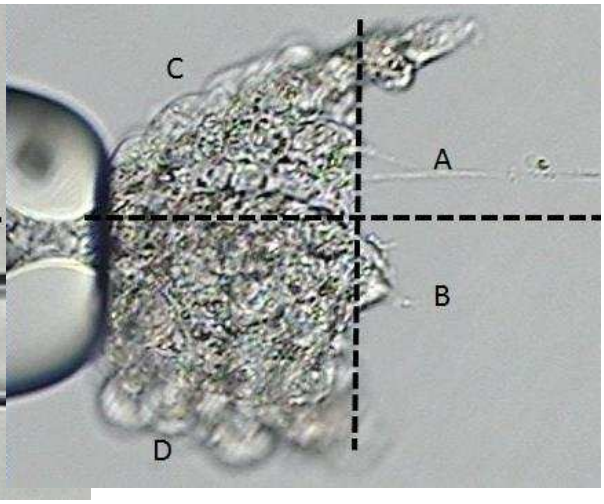
Given our success with this proof of concept study, larger studies are certainly warranted, despite the cost of CCS. Even increasing the number of blastocysts to 10 in our study would utilize approximately 200-250 CCS tests and patients may present with different rates of mosaicism thereby making a well-designed, high powered study difficult and costly. Our findings stress the need to perform a similar study on a greater number of embryos with the ultimate aim of both improving diagnosis for PGS families and better understanding the nature of our own early development.

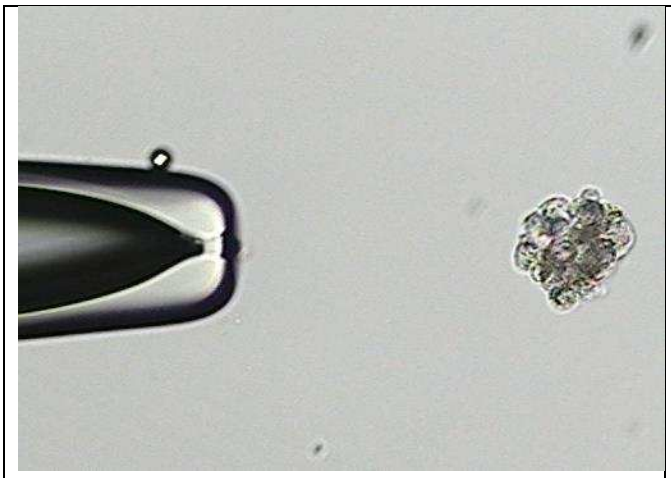
References

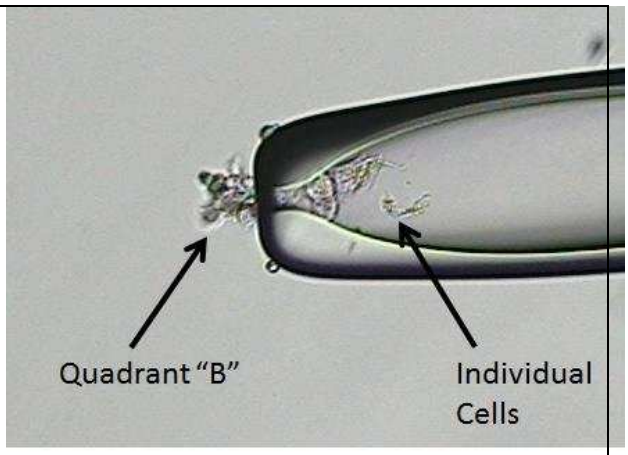
1. Taylor TH, Gitlin SA, Patrick JL, Crain JL, Wilson JM, Griffin DK. The origin, mechanisms, incidence and clinical consequences of chromosomal mosaicism in humans. Hum Reprod Update 2014;20:571-81.
2. Magli MC, Jones GM, Gras L, Gianaroli L, Korman I, Trounson AO. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. Hum Reprod 2000;15:1781-6.
3. Capalbo A, Wright G, Elliott T, Ubaldi FM, Rienzi L, Nagy ZP. Fish reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocyst shows high accuracy of diagnosis and no major impact of mosaicism at the blastocyst stage. Hum Reprod 2013;28:2298-307.
4. Liu J, Wang W, Sun X, Liu L, Jin H, Li M, Witz C, Williams D, Griffith J, Skorupski J, Haddad G, Gill J. DNA microarray reveals that high proportions of human blastocyst from women of advanced maternal age are aneuploid and mosaic. Biol Reprod 2012;87:148.
5. Fragouli E and Wells D. Aneuploidy in the human blastocyst. Cytogenetic Genome Res 2011;133:149-59.
6. Haddad G, He W, Gill J, Witz C, Wang C, Kaskar K, Wang W. Mosaic pregnancy after transfer of a “euploid” blastocyst screened by DNA microarray. J Ovarian Res 2013;6:70.
7. Wener MD, Leondires MP, Schoolcraft WB, Miller BT, Copperman AB, Robins ED, Arrendondo F, Hickman TN, Gutmann J, Schillings WJ, Levy B, Taylor D, Treff NR, Scott RT Jr. Clinically recognizable error rate after the transfer of comprehensive chromosomal screened euploid embryos is low. Fertil Steril 2014;102:1613-8.
8. Taylor TH, Griffin DK, Katz SL, Crain JL, Johnson L, Gitlin SA. Technique to isolate individual cells of the human blastocyst and reconstruct a virtual image of their location. J Clin Embryo. 2016 Accepted.
9. Capalbo A, Wright G, Elliott T, Ubaldi FM, Rienzi L, Nagy ZP. Fish reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocyst shows high accuracy of diagnosis and no major impact of mosaicism at the blastocyst stage. Hum Reprod 2013;28:2298-307.
10. Harton GL, Munne S, Surrey M, Grifo J, Kaplan B, McCulloh DH, *et al.* Diminished effect of maternal age on implantation after preimplantation genetic diagnosis with array comparative genomic hybridization. Fertil Steril 2013;100:1695-703.
11. Liu J, Wang W, Sun X, Liu L, Jin H, Li M, Witz C, Williams D, Griffith J, Skorupski J, Haddad G, Gill J. DNA microarray reveals that high proportions of human blastocyst from women of advanced maternal age are aneuploid and mosaic. Biol Reprod 2012;87:148.
12. Fragouli E and Wells D. Aneuploidy in the human blastocyst. Cytogenetic Genome Res 2011;133:149-59.
13. Northrop LE, Treff NR, Levy B, Scott RT Jr. SNP microarray-based 24 chromosome aneuploidy screening demonstrates that cleavage-stage FISH poorly predicts aneuploidy in embryos that develop to morphologically normal blastocysts. Mol Hum Reprod 2010;16:590-600.
14. Delhanty JD, Harper JC, Ao A, Handyside AH, Winston RM. Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. Hum Genet 1997;99:755-60.

15. Ioannou D, Fonseka KG, Meershoek EJ, Thornhill AR, Abogrein A, Ellis M, Griffin DK. Twenty-four chromosome FISH in human IVF embryos reveals patterns of post-zygotic chromosome segregation and nuclear organization. Chromosome Res 2012;20:447-60.
16. McCoy RC, Demko ZP, Ryan A, Banjevic M, Hill M, Sigurjonsson S, Rabinowitz M, Petrov DA. Evidence of selection against complex mitotic-origin aneuploidy during preimplantation development. PLOS Genet 2015;22: doi:10.1371/journal.pgen.1005601
17. Salvaggio CN, Forman EJ, Garnsey HM, Treff NR, Scott RT Jr. Polar body based aneuploidy screening is poorly predictive of embryo ploidy and reproductive potential. J Assist Reprod Genet 2014;31:1221-6.
18. Taylor TH, Patrick JL, Gitlin SA, Wilson JM, Crain JL, Griffin DK. Comparison of aneuploidy, pregnancy and live birth rates between day 5 and day 6 blastocysts. Reprod Biomed Online 2014;29:305-10.
19. Taylor TH, Patrick JL, Gitlin SA, Crain JL, Wilson JM, Griffin DK. Blastocyst euploidy and implantation rates in a young (<35 years) and old (≥35 years) presumed fertile and infertile patients population. Fertil Steril 2014;102:1318-23.
20. Capalbo A, Treff NR, Cimadomo D, Tao X, Upham K, Ubaldi FM, Rienzi L, Scott RT Jr. Comparison of array comparative genomic hybridization and quantitative real-time PCR-based aneuploidy screening of blastocyst biopsies. Eur J Hum Genet 2015;23:901-6.
21. Gutierrez-Mateo C, Colls P, Sanchez-Garcia J, Escudero T, Prates R, Ketterson K, Wells D, Munne S. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. Fertil Steril 2011:95;953-8.
22. Fiorentino F, Bono S, Biricik A, Nuccitelli A, Cotroneo E, Cottone G, Kokocinski F, Michel CE, Minasi MG, Greco E. Application of next-generation sequencing technology for comprehensive aneuploidy screening of blastocysts in clinical preimplantation genetic screening cycles. Hum Reprod 2014:29;2802-13.
23. Ozawa M and Hansen PJ**.** A novel method for purification of inner cell mass and trophectoderm cells from blastocysts using magnetic activated cell sorting. Fert Stert 2011: 95;799-802.
24. Prowse A, Wolvetang E, Gray P. A rapid, cost-effective method for counting human embryonic stem cells numbers as clumps. BioTechniques 2009:47;599-606.
25. Haseqawa K, Fujika T, Nakamura Y, Nakatsuji N, Suemori H. A method for the selection of human embryonic stem cell sublines with high replating efficiency after single-cell dissociation. Stem Cells 2006:24;2649-60.
26. Orris JJ, Taylor TH, Gilchrist JW, Hallowell SV, Glassner MJ, Wininger JD. The utility of embryo banking in order to increase the number of embryos available for preimplantation genetic screening in advanced maternal age patients. J Assist Reprod Genet 2010:27;729-33.
27. Drey LL, Graber MC, Bieschke J. Counting unstained, confluent cells by modified bright-field microscopy. BioTechniques 2013:55:28-33.
28. Grier DG. A revolution in optical manipulation. Nature 2003:424;810-6.
29. Prada I, Amin L, Furlan R, Legname G, Verderio C, Cojoc D. A new approach to follow a single extracellular vesicle-cell interaction using optical tweezers. BioTechniques 2016:60;35-41.

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.

<table>
<tr><td>A.
 
</td><td>B.
 
</td></tr>
<tr><td>C.
 
</td><td>D.
 
</td></tr>
<tr><td>E.</td><td>F.</td></tr>
</table>





G.

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