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## Non-invasive embryo assessment in IVF

Vergouw, C.G.

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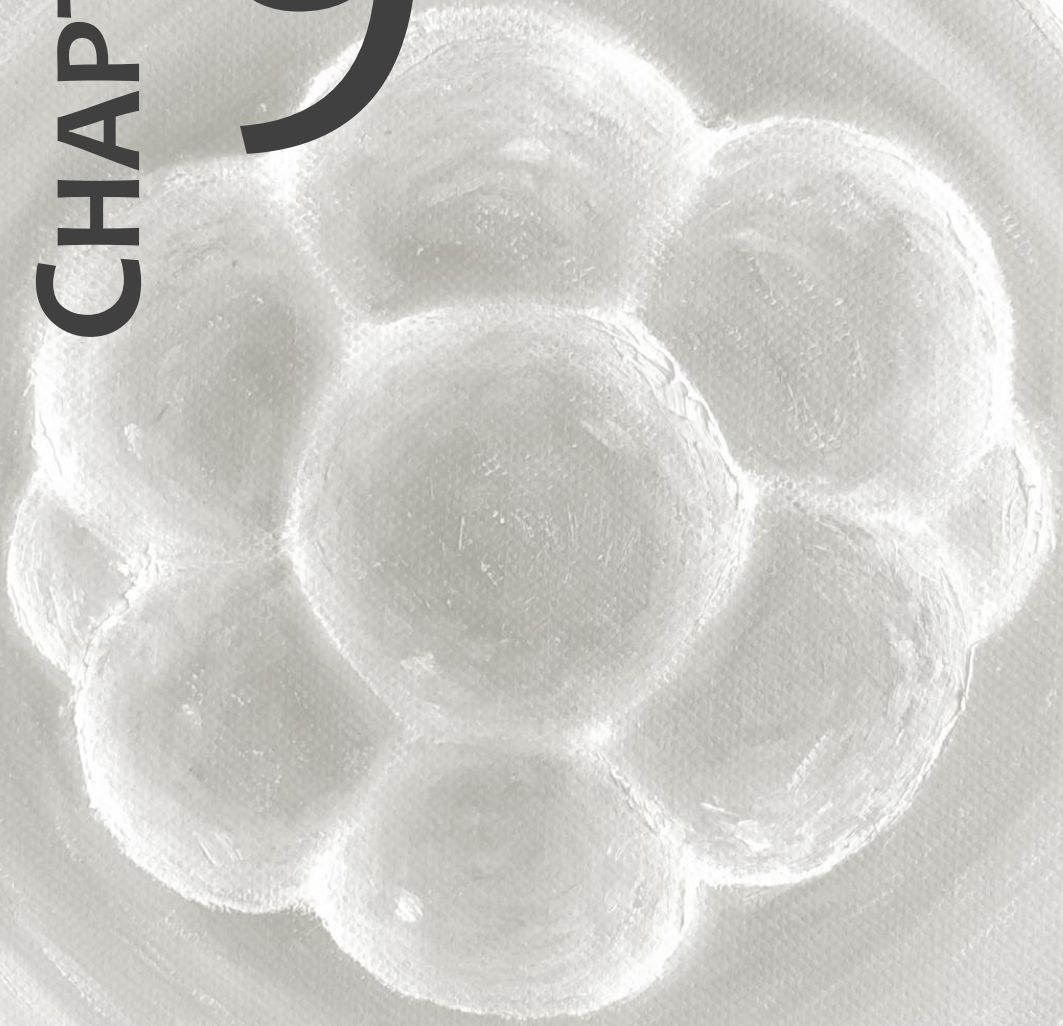
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**CHAPTER**

**9**



# GENERAL DISCUSSION

## GENERAL DISCUSSION

Traditionally, embryo selection for IVF is based on morphological criteria and cleavage rate using light microscopy. Although it requires a considerable amount of expertise, it is by far the most practical, least technically challenging method to assess embryo quality<sup>1</sup>. Relying solely on just a few predetermined static morphology evaluations has lately become a subject of debate among several experts. One of the main concerns is the considerable degree of intra- and inter-observer variability in embryo grading among experienced embryologists<sup>2,3</sup>. This makes visual embryo grading a highly subjective method that may lead to inconsistency within and between IVF clinics. Furthermore, the inability of morphological assessment to accurately predict an embryo's viability has led to the search for new embryo selection tools.

There is a long list of new embryo assessment and selection technologies that have been introduced into IVF clinics the last decade. In this chapter, we first discuss some of these technologies to see to what end their (clinical) use is supported by a scientific foundation. This includes the main focus of this thesis: embryo selection by metabolomic profiling of culture media with near-infrared (NIR) spectroscopy. We further discuss the importance of a validation of new laboratory technologies with regard to the practical application in a clinical setting. Finally, we provide some future perspectives with regard to research on new non-invasive embryo selection technologies.

## GENETIC PROFILING

### PGS

Preimplantation genetic screening (PGS) is and has been used to aid embryo selection in IVF treatments. The main aim of PGS is to identify euploid embryos which allow decisions to be made regarding which embryos are suitable to transfer<sup>4</sup>. For many years, the prevalent method of PGS has mainly been blastomere biopsy of cleavage stage embryos on day 3 of development; and their genetic analysis has mainly been done by fluorescent in situ hybridization (FISH)<sup>4,5</sup>. However, recent randomized controlled trials have shown that PGS using cleavage stage embryos and FISH for several indications of subfertility failed to improve delivery rates<sup>6</sup>. Possible causes for this might be the inability of FISH to analyse all chromosomes and the high incidence of chromosomal mosaicism in cleavage stage embryos<sup>4,6</sup>. Chromosomal mosaicism is the biological phenomenon that not all blastomeres within an embryo have the same chromosomal constitution. Therefore, the blastomere removed might not be

representative of the rest of the embryo and could result in false positive and negative results<sup>5</sup>. Besides, the removal of either a diploid or aneuploid blastomere changes the diploid:aneuploid ratio of blastomeres within an embryo which might lead to a change in viability<sup>7</sup>.

Due to the negative results of cleavage stage biopsy with FISH analysis, there has been a move towards polar body and blastocyst biopsy. Besides, microarrays that are able to detect aneuploidies in all 23 pairs of chromosomes such as single nucleotide polymorphism (SNP) or comparative genome hybridization (CGH) are more recently used in PGS<sup>4,5</sup>. But at present, there is a lack of evidence that supports the superiority of these new genetic tests or biopsy techniques and therefore, large randomized controlled trials are necessary to establish whether one or more of these new methods will lead to increased live birth rates<sup>4,6</sup>.

## ANALYSIS OF CULTURE MEDIUM

Non-invasive measurement of the appearance and/or depletion of culture medium constituents has been an area of interest for many scientists. Suboptimal culture conditions may cause cellular stress and lead subsequently to metabolic transformation which causes a change in viability<sup>8</sup>. Besides, the intrinsic quality of the embryo can also lead to differences in metabolism. Changes in culture medium constituents might thus be used to analyse an embryo's reproductive potential. Most importantly, analysis of culture medium constituents is a non-invasive technique which has little or no risk for the embryo<sup>9</sup>. Historically, there is a wide range of culture medium constituents that has been analysed, focusing mainly on glucose and pyruvate uptake, formation of lactate and ammonium; and amino acid turnover. More recently, metabolomic profiling has become a widely popular area of research. The main focus of this thesis is research of metabolomic profiling of embryo culture medium using near-infrared (NIR) spectroscopy and its relation to embryo viability and its ability to select a single embryo to transfer in IVF (chapters 2-6).

## GLUCOSE AND PYRUVATE

Pyruvate and lactate are the main energy sources of pre-compaction embryos. Glucose is the main energy source of post-compaction stage embryos. Because of the switch from carboxylic acid to glucose metabolism that occurs between pre- and post-compaction stage embryos, a decline in uptake of pyruvate and lactate is expected in pre-compaction stage embryos. For post-compaction stage embryos a decline in glucose due to glycolysis should be indicative for an effective metabolism<sup>1</sup>.

Results from studies using pyruvate uptake as a predictor for embryo development have varied greatly. Hardy et al.<sup>10</sup> and Gott et al.<sup>11</sup> reported lower pyruvate consumption in embryos that arrested in development compared to those that developed to the blastocyst stage. Conaghan et al.<sup>12</sup>, however, reported that a lower pyruvate uptake was observed by 2 to 8 cell embryos that implanted. These measurements were performed before embryonic genome activation and might therefore reflect differences inherited from the oocyte instead of a representation of the physiology of the embryo in a later stage<sup>8</sup>. Turner et al.<sup>13</sup> reported a wide variation in pyruvate uptake values between individual embryos, but embryos that implanted showed a significantly reduced variation. Gardner et al.<sup>14</sup> reported that embryos that developed into the blastocyst stage had significantly higher use of pyruvate.

There are several reports that show that glucose uptake cannot be used as a marker of viability<sup>10,11,15</sup>. Although it can be argued that the conclusions drawn in these studies are limited by the fact that simple culture media were used, lacking pyruvate, lactate, amino acids and vitamins. This might have caused significant stress on the embryos<sup>8</sup>. Gardner et al.<sup>14</sup> did find a correlation between glucose uptake of embryos and blastocyst formation: glucose consumption was twice as high in day 4 embryos that developed into blastocysts. Besides, good quality blastocysts consumed more glucose than poor quality blastocysts. In 2011, Gardner et al.<sup>16</sup> further demonstrated that glucose consumption on both day 4 and day 5 of embryos which implanted was significantly higher compared to embryos that failed to implant. Differences were also reported between female and male embryos in glucose uptake. Female embryos consume significantly more glucose in the post-compaction stage than their male counterparts<sup>16</sup>.

Results of several studies look very promising: pyruvate and glucose consumption might be tools of embryo viability assessment. However, at this moment only retrospective analyses have been done and there are no commercially available ready to use tools.

#### AMINO ACID TURNOVER

Assessing embryo viability by measurement of amino acid turnover using high-performance liquid chromatography (HPLC) has mainly been studied by the group of Leese<sup>17-19</sup>. Houghton et al.<sup>17</sup> showed that blastocyst formation could be predicted by the way donated day 2 embryos modified the amino acid contents of culture media. Day 2 embryos that developed into blastocysts consumed significantly less arginine and glutamine; and produced significantly less amounts of alanine and threonine compared to day 2 embryos that arrested in development<sup>17,18</sup>. Brison et al.<sup>19</sup> followed in 2004 with

a publication of a clinical study in which evidence was shown that the turnover of asparagine, glycine and leucine of day 2 embryos was significantly correlated with clinical pregnancy and live birth rates, independently from other known predictors. The data in this study however, was retrospectively analysed, because the results of the amino acid analyses were not yet available at the day of transfer. Besides, a mixture of single and double embryo transfer (SET and DET) cycles were analysed, resulting in considerable noise as it was not known which embryo had implanted in cases a singleton was born after DET<sup>18</sup>. Further research in this field showed a positive relation between amino acid turnover and the amount of DNA damage with the lowest DNA damage for embryos with a low (quiet) amino acid turn over<sup>20</sup>. Picton et al.<sup>21</sup> showed that uniformly aneuploid embryos have a different amino acid turnover compared to uniformly euploid embryos. In this study, six chromosomes (13, 18, 19, 21, X and/or Y) were analysed with FISH. Embryos containing a mixture of genetically normal and abnormal cells did not show a significantly different amino acid turnover compared to uniformly euploid or aneuploid embryos<sup>21</sup>. The ability of amino acid profiling by HPLC to select the most viable embryo within a cohort of embryos from one patient has thus far not been tested in randomized controlled trials.

#### METABOLOMIC PROFILING BY NEAR-IR (NIR) SPECTROSCOPY

Seli et al.<sup>22</sup> were one of the first to report on a study where day 3 spent culture media samples of individually cultured embryos with known pregnancy outcome were analysed using Raman and NIR spectroscopy. Spectral profiles were compared between implanted embryos and embryos that failed to implant. Spectral regions that discriminated between implanted and non-implanted embryos were quantified by multi-linear regression algorithms and subsequently expressed as a viability score. Authors showed that embryos that resulted in implantation and live birth had significantly higher mean viability scores compared to embryos that did not implant. Sensitivity and specificity of the test were 83.3% and 75% respectively.

Studies with larger sample sizes in clinics that routinely perform SET were subsequently performed to validate the original proof of concept<sup>23-29</sup>. Two of these studies are described in chapters 2 en 3 of this thesis. All studies uniformly reported significantly higher mean viability scores for embryos that resulted in an ongoing pregnancy and/or live birth, compared to embryos with a negative pregnancy outcome. This was seen regardless of day of transfer and was independent of embryo morphology. Furthermore, the combined morphology and viability score proved to be more accurate in predicting pregnancy results<sup>28</sup> and the viability score was a better predictor of pregnancy outcome than blastocyst morphology<sup>27</sup>. Ahlström et al.<sup>27</sup> even describe a



cross-validation of the technique, where an algorithm developed in one clinic from day 5 spent embryo culture media could predict the implantation potential of a blastocyst in a completely different clinic. The principle of a high viability score that correlates with a high implantation potential proved to be true for frozen-thawed embryos as well (see chapter 4 of this thesis).

The inability of this method to identify specific metabolites was a frequently heard criticism of the NIR technique. For a better understanding of the implantation process, this is of course very true. However, if the method is able to select the most viable embryo within a cohort of embryos from one patient more accurately than standard morphology, this argument becomes less important.

After the retrospective proof of principle studies, in which spent embryo culture medium had to be snap frozen and sent to a single laboratory for analysis, the time was there to see if metabolomic profiling using NIR spectroscopy as an adjunct to morphology would prove a robust test when performed at IVF sites. Therefore, two randomized controlled trials were independently started, one for day 3 SET and one for day 2 and day 5 SET. The use of metabolomic profiling in addition to standard morphological selection with live birth rate as primary endpoint was studied in these RCTs.

Our double blind randomised controlled trial showed that day 3 embryo selection by metabolomic profiling of culture medium with NIR spectroscopy as an addition to morphology was not able to improve ongoing pregnancy and live birth rates compared to embryo selection by morphology alone (chapter 5 of this thesis). Hardarson et al.<sup>30</sup> also showed with data from an interim analysis of the other randomised controlled trial with a similar study design but for day 2 and day 5 SET, that this technique failed to increase live birth rates. The method has been removed from the market after these reports. We also conducted a meta-analysis with IPD, in which we confirmed that adding the NIR spectroscopy technology to morphology in its current state does not improve the chance of a live birth (chapter 6).

A surprising finding in our double blind randomized controlled trial (chapter 5) was that in ~75% of the transfers in the treatment group (embryo selection by morphology plus the viability score), the embryo with the best morphology did not have the highest viability score. This was especially remarkable because the live birth rate in the treatment group (30.8%) was not significantly different from the live birth rate in the control group (31.3%), where the embryos were selected by morphology alone. This probably means that within a group of good quality embryos, there is more than one embryo able to result in an ongoing pregnancy. This may slightly undermine the necessity of embryo selection in SET, since some patients seem to have more than one



embryo available within a cohort that may lead to a live birth. However, it is still crucial to identify these embryos for this group of patients, because they may come back for a second child.

## BACK TO MORPHOLOGY

Back to basics, back to morphology? The Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology recently published the results of a consensus workshop in which common criteria and terminology for the grading of oocytes, zygotes and embryos were established<sup>31</sup>. According to Harper et al.<sup>5</sup>, this highlights the importance of going back to basics and examine oocyte and embryo morphology. It is of course important to have uniform definitions of what is a good, moderate or bad quality oocyte, zygote or embryo, but this does not solve a big problem of using morphology: the subjectivity of the whole morphological assessment method.

### BVI, BSI AND MO

We tried to address the problem of the subjectivity of morphological assessment in a study in which we objectified familiar parameters of embryo morphology using multilevel imaging of single-transferred embryos (Figure 1). Computer-assisted multilevel morphometric analysis has been used before to quantify the degree of fragmentation<sup>32,33</sup>. Paternot et al.<sup>34</sup> also studied a computer-assisted scoring system using multilevel digital images of embryos. They showed that the computer-assisted scoring system provided a more accurate and mathematical way to assess embryo morphology, possibly resulting in a reduction of the intra- and inter-observer variability<sup>34</sup>. We analysed whether this novel method of measuring instead of estimating important embryo quality parameters could refine the way of selecting the most viable embryo within a cohort of good quality embryos (chapter 7). We introduced three new variables: the blastomere volume index (BVI), the blastomere symmetry index (BSI) and the mean ovality (MO). The BVI is the ratio between the total blastomeric volume of an embryo and the cytoplasmic volume of



**Figure 1.** Sequence of seven digital images of one embryo.

an oocyte, calculated by dividing the total volume of all blastomeres within an embryo by the mean cytoplasmic volume of an oocyte. The BSI is the volume of the smallest blastomere divided by the volume of the largest blastomere within an embryo. The MO is the sum of the relative difference between the X-axis and Y-axis of a blastomere diameter of all blastomeres within the embryo divided by the number of blastomeres of the embryo. Unfortunately, multiple logistic regression analyses showed that the BVI, BSI and MO are not associated with ongoing implantation. Therefore, we did not pursue this line of research.

#### TIME-LAPSE MICROSCOPY

Another relatively novel embryo assessment strategy that uses morphology in a different way is time-lapse monitoring. Time-lapse monitoring uses cameras incorporated in the incubation chamber. It is used to study embryo development and kinetics in a non-invasive manner. Embryos are cultured directly on an imaging device that takes images of developing embryos at specific time intervals. These images can be processed into video sequences from which embryo morphology and kinetic data can be extracted. The advantage of time-lapse imaging over traditional morphology assessment is firstly that more constant culture conditions can be maintained. Embryos do not have to be removed from the incubator anymore, because the images are analysed on a computer screen. Detrimental effects of changes in temperature and culture medium pH are thus limited. Second, the high frequency of imaging provides a more complete picture of the biological process which may lead to a more accurate assessment of embryo morphology<sup>35</sup>.

Payne et al.<sup>36</sup> were one of the first to report the use of time-lapse cinematography in clinically-focused experiments with human embryos. Oocytes after ICSI were recorded for 17-20 h at one minute intervals. They were the first to describe in detail the sequence and timing of events after fertilization, including the extrusion of the second polar body, the appearance of pro-nuclei (PN) and the occurrence of cytoplasmic waves of granulation within the ooplasm. Payne et al.<sup>36</sup> also described that the good quality embryos on day 3 were originated from oocytes that had a more synchronous timing of events after injection.

Lemmen et al.<sup>37</sup> published a study in 2008 in which 102 IVF/ICSI 2 PN zygotes from a clinical setting were imaged every 5 min for 20-24 h. They showed that early disappearance of PN and early first cleavage were related to a higher number of blastomeres on day two. Synchrony in appearance of nuclei after the first cell division was significantly associated with better pregnancy rates.

Wong et al.<sup>38</sup> followed in 2010 with a time-lapse microscopy study of 242 frozen-thawed 2 PN IVF embryos in which they identified key parameters that could accurately predict blastocyst formation at the four cell stage. P1, the duration of the first mitosis (within  $14.3 \pm 6$  min); P2, the time between the first and second mitosis (within  $11.1 \pm 2.2$  h) and P3, the time between the second and third mitosis (within  $1.0 \pm 1.6$  h) proved to be predictors of blastocyst formation with a sensitivity and specificity of 94% and 93% respectively. The strength of this study lays in the analysis of the gene expression profiles. The imaging phenotypes were linked to underlying gene expression and molecular health of the embryos.

Meseguer et al.<sup>39</sup> used a commercial time-lapse incubator designed for clinical use and studied 247 ICSI embryos transferred on day 3 from first cleavage onwards. They confirmed independently two (P2 and P3) of the three parameters introduced by Wong et al.<sup>39</sup> as predictors of implantation. Besides, they showed that the timing of subsequent cleavages up to the fifth cell stage of viable embryos proved to have a more uniform division pattern compared to embryos that did not implant.

Kirkegaard et al.<sup>40</sup> evaluated the safety of a commercially available time-lapse incubator by culturing sibling oocytes after randomization in either a conventional incubator or a commercially available time-lapse incubator. There were no differences found between the incubators in proportion of four cell embryos on day two, proportion of eight cell embryos on day three, proportion of blastocysts on day five, clinical pregnancy rates and implantation rates.

Time-lapse microscopy is currently one of the hot topics in IVF. Embryo development can be monitored continuously, possibly leading to novel morphological embryo viability markers. Whether these new parameters are actually tools to select the most viable embryo within a cohort of embryos from one patient remains subject to further studies.

## NO EMBRYO SELECTION AT ALL

Recently, it has been suggested that the improvement of embryo selection methods might become less relevant in the future<sup>41</sup>. The increasing success of frozen-thawed embryo transfer cycles and the first reports of randomized controlled trials (RCTs) that compared elective fresh versus elective frozen-thawed embryo transfers<sup>42,43</sup> might lead to a complete revolution in IVF: the avoidance of fresh embryo transfers in favour of cryopreservation of all available embryos.

Both Aflatoonian et al.<sup>42</sup> and Shapiro et al.<sup>43</sup> showed in RCTs higher clinical pregnancy rates in the freeze-all group compared to the fresh embryo transfer group. However,

these studies have some limitations because no live birth rates or cost-effectiveness analyses were presented. At present, there is a great need for large, multicenter, randomized trials in which clinical outcomes (preferably live birth rates) and cost-effectiveness of elective frozen-thawed single embryo transfer from freeze-all cycles versus elective fresh single embryo transfer are evaluated<sup>43</sup>.

The costs of extra transfer cycles in the freeze-all strategy should counterbalance against the costs of extra hyperstimulation cycles, selection methodologies and the possible avoidance of multiple pregnancies<sup>41</sup>. Regarding the multiple pregnancies, it is of course important to perform single embryo transfer in freeze all cycles and not be tempted to transfer multiple embryos in order to increase pregnancy rates. Besides, the increasing amount of embryo freezing and thawing and hence the increasing demand for laboratory staff and storage capacity should not be forgotten in the cost-effectiveness analysis.

Another important factor to explore is the acceptance of couples, but also of health care providers of this new strategy<sup>44</sup>. Especially a possible increased time to pregnancy and a possible increased number of embryo transfers might be mentally challenging for the patients. Unsuccessful treatment cycles lead to an increase in depression with significant interaction effects between time and treatment outcome<sup>45</sup>. Besides, the 'wait at home' interlude between embryo transfer and pregnancy test has been indicated as the most stressful aspect of IVF treatment<sup>46</sup>. Therefore, patients might have more trouble to mentally cope with an all-freeze IVF treatment, if it turns out that freeze-all cycles lead to a higher number of embryo transfers.

One of the most persuading factors to (possibly) shift to freeze-all cycles might be the reported results of better obstetric and perinatal outcomes after frozen-thawed embryo transfers compared to fresh embryo transfers. A recent systematic review and meta-analysis comparing obstetric and perinatal outcomes of fresh and frozen-thawed embryo transfers showed lower risks of antepartum haemorrhage, preterm birth (<37 weeks), small for gestational age (SGA) babies, perinatal mortality and low birthweight babies (<2500 g) for women with a singleton pregnancy after a frozen-thawed embryo transfer<sup>47</sup>. Our own data (chapter 8 of this thesis) also showed significantly higher mean birthweights, more large for gestational age (LGA) babies and less SGA babies in the frozen-thawed embryo transfer group compared to the fresh embryo transfer group. These promising data of observational studies should also be considered in the validation process of freeze-all cycles.

## A PLEA FOR PROPER VALIDATION OF NEW EMBRYO SELECTION TECHNOLOGIES

Techniques that have shown very promising results in retrospective analyses are often marketed for clinical use before clinical proof of their effectiveness in RCTs has been published. From a commercial point of view, this is often driven by the need of making money, usually under pressure of investors. Clinicians often wish to improve pregnancy outcomes. The results from PGS RCTs have shown us that even lower pregnancy and live birth rates were reported and although the metabolomic profiling by NIR spectroscopy RCTs did not show worse live birth rate outcomes, it is never cost-effective to use a technique together with standard morphological assessment that results in similar live birth rates than the use of standard morphology alone.

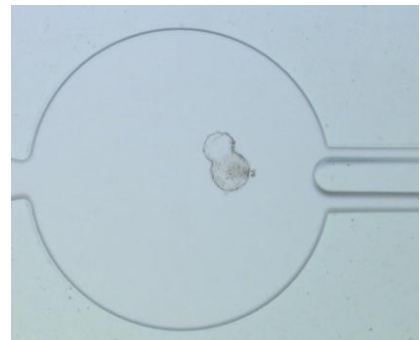
We should learn from past mistakes and prevent premature introduction of new embryo assessment and selection technologies in routine IVF, such as time-lapse microscopy, freeze-all cycles, PGS with new biopsy and array technologies and embryo culture media analysis. New technologies should be evaluated for effectiveness, safety and cost-effectiveness<sup>5</sup>. An important question to ask is: do couples for whom the new (embryo selection) technology is used have a better chance of having a baby than couples who do not<sup>48</sup>? Proper designed and adequately powered studies, preferably RCTs, are essential to analyse whether it is the actual technology causing the effect or other factors. An RCT is in its design aiming at an unbiased evaluation of the technology and outcomes (live birth rates), which are compared to a control group without treatment<sup>49</sup>. If appropriate, follow up of children should be performed to analyse if the risks are within acceptable range<sup>5</sup>. This should also be done when vital changes are made in the culture system of embryos in routine IVF, such as a change of culture medium. In chapter 8 of this thesis, there are results presented of such a follow up of children. Besides, an economic evaluation by means of a cost-effectiveness analysis should make clear whether the costs of the new technology are not too high compared to the benefits.

Research on evaluation of healthcare interventions needs to be sensitive to consumer opinion<sup>44</sup>. In other words, the perception of couples should also be taken into account, especially when new technologies require more effort from the patient. Furthermore, appropriate high quality information to the patient is vital in the process of changes and the acceptance of new technologies.

## FUTURE RESEARCH

In the near future, our research activities will further focus on non-invasive embryo selection. We believe that embryo selection will remain important in the near future, and therefore it will be important to find new embryo selection tools that are more accurate in predicting an embryo's implantation potential than standard morphology (the current gold standard). This way, we might shorten the time to pregnancy for couples. Accurate embryo selection tools might be used to increase pregnancy outcomes and lower multiple pregnancy rates. Even in a freeze-all policy it might help to select which embryos to freeze to lower the work load and costs of the laboratory; and to select which embryo has the most potential to become a viable pregnancy so it can be thawed first.

Our main focus in the near future will be time-lapse microscopy. We are joining the research activities around the Early Embryo Viability Assessment (Eeva™) technology. In order to provide an evidence based introduction of this technology in routine IVF, we start with a multi-center study to analyse the possible increase of pregnancies that can be expected when Eeva™ predictions with morphology grading are used to select a single embryo for transfer. The next step in the validation process will be an RCT in which the efficacy of Eeva™ will be studied by comparing pregnancy outcomes between the group of patients whose embryo was selected by Eeva™ plus morphology and the group of patients whose embryo was selected by standard morphology only. Further steps to validate the Eeva™ technology, such as cost-effectiveness analyses, are not planned at this moment but should be considered later in the validation process as well. The other major line of research is the development of a multiplex microfluidic platform for the pre-implantation culture of individual embryos and their on-line assessment using an integrated multi-parametric approach: morphological criteria and oxygen consumption (Figure 2). This platform is developed in cooperation with the technical University of Twente.



**Figure 2.** Microfluidics chamber with hatching blastocyst.

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