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Traceability of human sperm samples by direct tagging with polysilicon microbarcodes

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The increasing number of patients undergoing assisted reproductive technology (ART) treatments and of cycles performed in fertility centres has led to some traceability errors. Although the incidence of mismatching errors is extremely low, any error is unacceptable, therefore different strategies have been developed to further minimize these errors, such as manual double witnessing or electronic witnessing systems. More recently, our group developed a direct tagging method consisting of attaching microbarcodes directly to the zona pellucida of human oocytes/embryos. Here, this method is taken a step further by using these microbarcodes to tag human semen samples, demonstrating that the barcodes are not toxic and do not interfere in the selection of motile spermatozoa nor in the cryopreservation of the sperm samples. In addition, when this tagging system was applied to an animal model (rabbit), pregnancy rate and kitten viability were not affected.

KEYWORDS: assisted reproduction, barcodes, spermatozoa, traceability
Introduction

The growing number of patients who resort to assisted reproductive techniques (Centers for Disease Control and Prevention, A. S. for R. M, 2013; Ferraretti et al., 2013; Macaldowie et al., 2013) makes it indispensable to set up a reliable traceability control of the samples derived from the practice of these techniques. Despite the fact that the risk of sample mismatching errors (mix-ups) is extraordinarily small, several mix-ups have been reported in fertility clinics worldwide (Alvarez and Tweed, 2007; Bender, 2006; Devlin, 2009; Parry, 2011; Spriggs, 2003). To prevent such mix-ups, several strategies have been developed in the context of human assisted reproduction technologies (ART). One of them is the manual double-witnessing protocol used in most ART laboratory procedures, first recommended by the Human Fertilization and Embryology Authority in 2003 (Brison et al., 2004), and later by the European Society of Human Reproduction and Embryology (Magli et al., 2008). However, the effectiveness of the manual double-witnessing has been questioned because of the risk of involuntary automaticity (Toft and Mascie-Taylor, 2005). Consequently, a new generation of double-witnessing systems were generated, the electronic witnessing systems, which allow the automation of the process of sample identification during the laboratory process (Adams and Carthey, 2006). These systems are based on the labelling of all labware used for each particular case with barcode adhesive stickers (Matcher™, IMT, UK) or radio frequency identification adhesive labels (IVF Witness™, Research Instruments, UK), which can be identified by special readers connected to a computer.

An important limitation of these current systems is that the label is linked to the container and not directly to the sample. Therefore, the possibility of misidentification persists, as gametes and embryos are moved
from one container to another several times during the course of an ART cycle. For this reason, our group proposed a direct gamete/embryo tagging system in which the tag and the sample would move together throughout the whole ART process. This system, initially developed for oocytes and embryos, is based on the attachment of polysilicon microbarcodes (barcodes, from now on) to the outer surface of the zona pellucida by means of their biofunctionalization with the wheat germ agglutinin lectin (Novo et al., 2013; Penon et al., 2012). The application of this system is simple, safe, highly efficient and allows the identification of human oocytes and embryos during the various steps of an ART cycle (Novo et al., 2014). However, due to the small size and very high numbers of sperm cells in a semen sample, the system cannot be applied to sperm cells using the same approach.

After searching the literature for all the reported mix-up errors, approximately two-thirds were found to be related to sperm misidentification; therefore it was decided to adapt the direct tagging system for semen samples, using nonbiofunctionalized barcodes. In this case, the aim is to label the whole sample, instead of each individual sperm cell. The validation of this new approach was focused on two goals: to rule out any potential detrimental effect of barcodes on sperm viability, and to test the effectiveness of the tagging system during the laboratory procedures typically conducted in a human ART cycle. Moreover, any possible effect of barcodes on the ability of spermatozoa to fertilize was assessed in an animal model by artificially inseminating female rabbits with tagged rabbit spermatozoa.

**Materials and methods**

The use of human samples, animal care and protocols employed in this study were approved by the Ethics Committee on Animal and Human Research of the Universitat Autònoma de Barcelona and by the Departament d’Agricultura, Ramaderia i Pesca of the Generalitat de Catalunya (protocol numbers 2115, approved on 24/05/2013 and 2299, approved on 13/12/2013).
Human sperm samples

Eight donors from 24 to 34 years old were recruited after a public call for semen donation for this specific study. Informed consent was obtained from all of them. Semen samples were obtained by masturbation after 3 to 5 days of sexual abstinence and ejaculated into a clean sterile container (Deltalab, Spain). Samples were processed within 60 min after ejaculation. Upon arrival, containers were placed over a hotplate (37°C) to allow seminal liquefaction. Afterward, a routine semen analysis (volume, pH, concentration, motility and morphology) was performed according to the World Health Organization’s Laboratory Manual for the Examination and Processing of Human Semen (WHO, 2010).

Barcode fabrication and design

Barcodes were fabricated on 4-inch p-type (100) silicon wafers through silicon microtechnologies used for microelectromechanical systems fabrication, as previously described in detail (Novo et al., 2011).

Barcodes are designed as two-dimensional polysilicon microparticles 10 m in length, 6 m in width and with a thickness of 1 m. They have a start reading marker and carry 8 bits of binary codification (two rows of 4 rectangular bits), which can be easily converted into a decimal number (Figure 1a) and associated with a specific donor (Figure 1b–i). The presence of 8 bits allows 256 different possible combinations (decimal numbers 0 to 255). However, the fabrication of barcodes with more bits or with other shapes and dimensions is available and could exponentially increase the number of possible combinations.

Sample tagging

Sample tagging containers were prepared in advance, by placing 120,000 barcodes of a specific codification, diluted in absolute ethanol, into each sterile container. Once ethanol was evaporated, containers were stored at room temperature from several days to up to 3 months.
For sample tagging, liquefied seminal samples were transferred to tagging containers and homogenously mixed with the barcodes by pipetting up and down (Figure 2).

Figure 1 Barcodes and semen sample identification. Schematic representation of a barcode showing the 8 bits and the start marker (a). Each donor semen sample was tagged with a different barcode (b–i). For each semen sample, a general view at ×600 magnification and a detailed image of the barcode at ×1000 magnification is provided. For each barcode, its binary value, decimal number (in bold) and a schematic representation are shown.
**Motile sperm fraction selection**

Motile sperm fractions were selected by density gradient centrifugation. Briefly, SpermGrad™ (Vitrolife, Sweden) was mixed with SpermRinse™ (Vitrolife, Sweden) to obtain 80% and 40% stock solutions. To layer the gradient, 1 ml of 80% solution was first pipetted into a sterile conical centrifuge tube and then 1 ml of 40% solution was slowly deposited on top. Next, a maximum of 1 ml of the semen sample (depending on the initial volume) was gently layered on the top. Sperm pellet was obtained after 20 min centrifugation at 300g. The pellet was washed twice with 2 ml of equilibrated SpermRinse™ by centrifugation for 7 min at 800g and resuspended in 1 ml of equilibrated SpermRinse™. Finally, the sperm concentration was assessed using a Makler® Counting Chamber (Irvine Scientific, USA). In the tagged samples, barcodes were able to cross the gradient layers and could be found in the pellet together with the sperm cells. To check if more stringent separation conditions could affect the recovery of barcodes, a 90/45% gradient was also tested, but without a semen sample.

**Sperm cryopreservation**

Sperm cryopreservation was performed by adding Sperm CryoProtecTMII (Nidacon, Sweden) drop by drop into the sample in a proportion of 3:1 (v/v, semen sample: cryoprotector) and thoroughly mixing. Then, the sperm suspension was transferred to a 0.5 ml straw (CryoBioSystemIMV, France), equilibrated at 4°C for 45 min, placed in nitrogen vapour for 30 min and finally transferred to and stored in liquid nitrogen.

To thaw, the straw was introduced in a water bath at 37°C for 30 s. After drying the straw surface, the sperm suspension was transferred into a tube containing 5 ml (for motile spermatozoa) or 0.5 ml (for non-selected spermatozoa) of GMOPS(Vitrolife,Sweden). Thawed motile spermatozoa were centrifuged for 10 min at 500g and the pellet resuspended in 0.2 ml GMOPS. Thawed non-selected spermatozoa were
centrifuged in a density gradient (as explained earlier) to obtain the motile phase, and the pellet was resuspended in 0.5 ml GMOPS.

Figure 2 Experimental design. After a routine semen analysis, each semen sample was split in two. One part was tagged with 120,000 barcodes (tagged) whereas the other was used as a control (control). From this point, tagged and control samples were processed in parallel. Each sample was split again into two
Sperm viability and acrosome integrity

Sperm viability was evaluated using the LIVE/DEAD Sperm Viability Kit (Molecular Probes, USA) whereas acrosome integrity was assessed with Alexa Fluor 647-conjugated peanut (Arachis hypogaea) agglutinin (Alexa647-PNA; Sigma, Spain). Briefly, sperm samples diluted in 1 ml of equilibrated SpermRinse™ were incubated at 37°C for 10 min with the two components of the LIVE/DEAD kit, SYBR-14 (a green, membrane permeable stain) at a final concentration of 100 nmol/l and propidium iodide (PI; a red, membrane-impermeable stain) at a final concentration of 12 mmol/l. After incubation, and in order to concentrate the sample, a 7 min centrifugation at 900 g was performed. After removing the supernatant, 8 l of the pellet were used to quantify live/dead spermatozoa, whereas the rest was used for acrosome integrity analysis.

Live/dead spermatozoa were assessed immediately by inverted fluorescence microscopy (Olympus IX71 Microscope) at ×200 magnification. Live spermatozoa were defined as those presenting only green stain (SYBR-14), whereas red spermatozoa were considered dead because PI only enters cells with a damaged plasma membrane.

For sperm acrosome integrity analysis, the pellet was fixed in 200 l of 4% paraformaldehyde (Sigma, Spain) for 5–7 min, spun for 7 min at 900g, washed in phosphate-buffered saline (PBS; Sigma, Spain)
and spun again for 7 min at 900g. Finally, the pellets were spread onto slides and air-dried at room temperature. Labelling was accomplished by adding the acrosome marker Alexa647-PNA (far-red) at a final concentration of 2.5 g/ml during 10 min at 4°C. Slides were then rinsed in PBS and mounted (Mortimer et al., 1987). Sperm acrosome morphology was determined under a fluorescence microscope (Olympus BX60 Microscope) at ×1000 magnification. Acrosomes were considered intact when about 40–70% of the normal oval-shaped sperm head was labelled.

**Artificial insemination with tagged rabbit spermatozoa**

*Oryctolagus cuniculus* females, aged between 8 and 12 months, were used to perform the artificial inseminations. Females were maintained and inseminated at the experimental rabbit farm of the Universitat Autònoma de Barcelona. Ovulation was induced by subcutaneous injection of 25 IU of pregnant mare serum gonadotrophin (Foligon, Intervet Schering Plough Animal Health, Spain) 48 h before artificial insemination. At the time of insemination, a second injection of 0.2 ml gonadorelin (Gestavet Lab Hipra, Spain) was administered. Commercial extended rabbit semen samples (Shelter SL, Spain) were liquefied at 28°C. The individual dose per animal was 0.8 ml, and contained at least 20 million spermatozoa. Tagged samples contained 120,000 barcodes/ml of semen.

**Experimental design**

Two different types of analysis were performed with the human semen samples; the first, related to the integrity of sperm plasma membrane and acrosome in the barcodetagged samples (sperm quality); and the second, related to barcode retention rate (% of remaining barcodes in the sperm fraction analysed) and tagging efficiency (when at least one barcode could be clearly read under the inverted microscope, and therefore the semen sample was successfully identified; identification rate). The experimental design is summarized in Figure 2. Each sample was processed individually and within 60 min after ejaculation.
Once the sample was liquefied and the standard semen analysis performed, half of the sample was transferred to a container without barcodes (control) and the other half to a tagging container holding 120,000 barcodes (tagged). From this point, both samples (control and tagged) were processed in parallel. Control and tagged samples were split into two different volume fractions again, depending on the initial semen volume; one part of the sample was directly cryopreserved, whereas the other was processed with density gradients for the selection of the motile sperm fraction. Sperm counting and quality analyses were performed in the motile fraction for control and tagged samples, and barcode retention and identification was only assessed in tagged samples. The motile fraction was then frozen and thawed, and the same analyses were performed for control and tagged samples.

Finally, the portion of sample that was directly frozen was thawed, processed with density gradients for the selection of the motile fraction, and analysed for quantity and quality of spermatozoa, in addition to barcode retention and identification in the case of the tagged samples. Identification of tagged samples was performed by examining one microlitre of the sample under the inverted microscope at ×200 magnification and recording the number of readable barcodes (Figure 1b–i).

On the other hand, to discard any possible undesirable effects of barcodes during fertilization, commercial rabbit semen samples were tagged and used to artificially inseminate female rabbits. Fifteen does were used in two different experiments. In the first one, seven of the females were inseminated using tagged spermatozoa while the other eight were inseminated with control spermatozoa. In the second experiment, the females were reversed, that is, the ones inseminated with control spermatozoa in the first experiment were inseminated with tagged spermatozoa in the second one and vice-versa. The pregnancy rate and the number and viability of kittens per female were evaluated during 24 h following parturition.
**Statistical analysis**

Sperm counts, sperm viability and acrosome integrity were compared between tagged and control samples using the MannWhitney test. A paired $t$-test was performed to confirm the homogeneity of the rabbit samples. Fisher exact test (with Katz approximation) was used to compare fertility rate and kitten survival obtained after artificial insemination. A $P$-value $< 0.05$ was considered statistically significant.

**Results**

All semen samples analysed showed normal values of pH, volume, concentration, motility and morphology, according to the World Health Organization *laboratory manual for the examination and processing of human semen* (WHO, 2010; Table 1).

**Effect of barcodes on the quality of the semen samples**

All semen samples were processed and evaluated as indicated in Figure 2, and the number of spermatozoa recovered after each step is shown in Table 2. No significant differences were found between control and tagged semen samples in the percentage of spermatozoa recovered after the selection of the motile fraction from the fresh samples. A portion of the motile spermatozoa was then frozen (the number differed among donors depending of the initial volume of the semen sample) and, after thawing, no significant differences were observed between control and tagged semen samples in the percentage of spermatozoa recovered. Finally, when the samples were directly cryopreserved and the motile
Table 1  Semen analysis (volume, morphology, pH, motility and concentration) performed according to the World Health Organization laboratory manual (WHO, 2010).

<table>
<thead>
<tr>
<th>Semen donor</th>
<th>Initial volume (ml)</th>
<th>Morphology</th>
<th>pH</th>
<th>Motility % (a+b)</th>
<th>Concentration (millions/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5</td>
<td>normal</td>
<td>8.5</td>
<td>75.0</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>normal</td>
<td>8.5</td>
<td>55.0</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>normal</td>
<td>8.0</td>
<td>58.4</td>
<td>190</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>normal</td>
<td>8.0</td>
<td>53.7</td>
<td>78</td>
</tr>
<tr>
<td>5</td>
<td>3.7</td>
<td>normal</td>
<td>8.0</td>
<td>54.6</td>
<td>130</td>
</tr>
<tr>
<td>6</td>
<td>3.5</td>
<td>normal</td>
<td>8.0</td>
<td>63.3</td>
<td>120</td>
</tr>
<tr>
<td>7</td>
<td>3.0</td>
<td>normal</td>
<td>8.5</td>
<td>63.9</td>
<td>210</td>
</tr>
<tr>
<td>8</td>
<td>3.3</td>
<td>normal</td>
<td>8.0</td>
<td>61.6</td>
<td>55</td>
</tr>
</tbody>
</table>

Figure 3  Sperm viability and acrosome integrity in control and tagged samples. Mean percentages of viability and acrosome integrity in fresh motile fractions (MF), motile fractions frozen and thawed (MF+C), and motile fractions recovered after freezing and thawing of non-selected semen samples (C+MF).
in control (black columns) and tagged semen samples (grey columns). Error bars indicate standard deviations.

fraction was selected after thawing, the sperm counts were again equivalent between the control and tagged samples.

The presence of the barcodes did not alter the percentage of spermatozoa with an undamaged membrane or an intact acrosome, either in fresh or cryopreserved samples (Figure 3). Specifically, the mean sperm viability was equivalent between control and tagged fractions and higher than 47% in all the fractions analysed (Figure 3). The mean percentage of spermatozoa with an intact acrosome was around 20% and equivalent between all control and tagged fractions (Figure 3).

**Efficiency of the tagging system**

The evaluation of barcode retention at different points of the semen processing revealed that barcodes remained associated with the sample during the whole process. A decrease in the number of barcodes present among the spermatozoa was observed in all the fractions evaluated (data not shown). But, independently of the processing performed, all samples could be identified at any step when only one microlitre was analysed under an inverted microscope (Figure 1b–i), indicating that identification of the semen samples can be accomplished during their entire processing.

Additionally, after spinning a 90/45% gradient without a semen sample, barcodes were found in the pellet (data not shown).

**Rabbit artificial insemination using tagged semen samples**

A total of 30 artificial inseminations were performed, 15 with tagged spermatozoa and 15 with control spermatozoa. The results are shown in Table 3. The pregnancy rate for the does inseminated with control
semen was 86.7%, with an average of 9.2 live kittens per litter. On the other hand, insemination with tagged semen resulted in a pregnancy rate of 80.0% and an average of 9.6 live kittens per litter. No significant differences in pregnancy rate, number of kittens at birth, number of kittens alive at 24 h, or live kittens per litter were observed between control and tagged semen samples. As expected, the gestation length for both does inseminated with control or tagged samples was 32 (±1) days.

Discussion

Different systems have been developed to minimize the risk of mismatching errors during ART procedures. The more recent is the direct tagging of oocytes and embryos with functionalized barcodes (Novo et al., 2013, 2014). Although individual cell tagging is not feasible with sperm samples, due to the smaller size and higher numbers of spermatozoa when compared with oocytes and embryos, this study demonstrated that the barcode system can be adapted and successfully applied to the labelling of the sperm samples without any detrimental effect on sperm number, quality and their ability to produce pregnancies and live offspring. The new approach consists of the addition of plain polysilicon barcodes to the semen ejaculate in a quantity that ensures that, after sample processing, a minimum of one barcode per microlitre is present in the sample to successfully identify it by an automatic barcode reading system developed for this purpose.

To demonstrate the effectiveness of the barcode-based tagging system to label semen samples, the study tracked eight
<table>
<thead>
<tr>
<th></th>
<th>Control semen</th>
<th>Tagged semen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy rate</td>
<td>86.7</td>
<td>80.0</td>
</tr>
<tr>
<td>Kittens at birth</td>
<td>120</td>
<td>115</td>
</tr>
<tr>
<td>Live kittens at 24h</td>
<td>116 (96.7)</td>
<td>107 (93.0)</td>
</tr>
<tr>
<td>Live kittens per litter</td>
<td>9.2 ± 2.5</td>
<td>9.6 ± 2.4</td>
</tr>
</tbody>
</table>
Table 3: Pregnancy rate and kitten viability in does artificially inseminated with control or tagged semen.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sperm count</th>
<th>Motile sperm</th>
<th>Sperm thawed motile</th>
<th>Cryopreserved sperm</th>
<th>Sperm motile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial sperm</td>
<td>fraction (%)</td>
<td>cryopreserved</td>
<td>fraction (%)</td>
<td>fraction (%)</td>
<td>fraction (%)</td>
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<td></td>
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different samples throughout the different processes that samples can undergo in a sperm bank or a fertility centre. Regardless of the processing applied, it was found that at least 1 barcode per microlitre of sample in all cases, and all semen samples could be successfully identified at any point of the process. Therefore, identification does not depend on the quality of the semen sample, but on the volume analysed at the end of a process. What is more, no differences in sperm counts were found between control and tagged samples for any of the three fractions produced, demonstrating that barcodes did not disturb density gradient selection or cryopreservation. On the other hand, sperm viability and acrosome integrity were similar between control and tagged samples, indicating that the presence of the barcodes mixed among the spermatozoa did not induce plasma membrane damage or increase the percentage of spermatozoa that undergoes acrosome reaction. Surprisingly, acrosome integrity was low in all the samples examined (both control and tagged) and, although the cause is not clear, it has been attributed to the protocol applied or the scoring performed.

The innocuous effect of polysilicon barcodes has been previously demonstrated in mouse and human embryos tagged with WGA-biofunctionalized barcodes. In these studies, the development rates of tagged embryos were similar to those of control embryos, both in vitro and in vivo (Novo et al., 2013, 2014). On the other hand, polysilicon microparticles have been shown to be non-cytotoxic even when internalized into the cytoplasm of human macrophages and mouse embryos (Fernández-Rosas et al., 2010).

Studies on the effect of micro/nanoparticles in sperm samples are scarce (Moretti et al., 2013; Wiwanitkit et al., 2009). In the study of Moretti et al. (2013), human sperm samples were incubated in the presence of gold and silver nanoparticles (50–65 nm in diameter) at different concentrations and times. The authors reported a decrease in both sperm viability and motility at concentrations over 125 mol/l for gold and silver nanoparticles, and the presence of gold but not silver nanoparticles inside the nucleus of the spermatozoa. On the other hand, Wiwanitkit et al. (2009) related the presence of nanoparticles in the nucleus with sperm fragmentation. Cytotoxicity of nanoparticles has been associated with their ability to penetrate cells and
organelles, disrupting their normal function (Buzea et al., 2007), but nanoparticles could also interfere with membrane receptors or with cell signalling pathways involved in motility maintenance (Moretti et al., 2013). Polysilicon barcodes are much larger than spermatozoa, making their internalization inside the cell or the nucleus impossible.

In addition to demonstrating the lack of effects of barcodes on sperm viability and quality, the study aimed to prove that tagged sperm samples were able to produce pregnancies and offspring. To accomplish this aim, artificial insemination was performed in an animal model, rabbits being chosen because embryo and foeto-placental development are similar to those of humans (Fischer et al., 2012). In addition, artificial insemination is routinely performed in rabbit farming and rabbit semen is commercially available. The pregnancy rate of does artificially inseminated either with control or tagged semen in the present study was similar to that described in other studies (Khalifa, 1994; Rebollar et al., 2012). No differences in gestation length, in the number of kittens at birth per female or in the number of live kittens per litter were observed. Thus, the presence of the barcodes among the sperm cells does not seem to impair fertilization or the potential of the resulting embryos to implant and fully develop. Indeed, a previous work conducted in mice demonstrated that barcodes did not hamper the gestation process when tagged embryos were transferred to pseudopregnant female mice (Novo et al., 2013). No data is available on potential harmful or discomforting effects in humans, but these two studies in animals do not show any evidence for this.

As stated by Borman and Racowsky (2012): ‘In the field of assisted reproduction, there is no room for mistakes’. The Human Fertilisation and Embryology Authority considers an embryo mix-up as a grade A incident (the most serious incident). Even if the percentage of mix-ups reported up to now is low (0.04%, [Shaikh, 2009]), the estimation of the nonreported could be much higher. Therefore, all efforts should be made to minimize the occurrence of these traceability errors. The semen sample labelling approach presented here, together with the previous reported approach for the labelling of oocytes and embryos (Novo et al., 2014), allows tagging female and male reproductive samples, using the same codification
number for a couple. Gametes and embryos could therefore be easily indentified at any step of the ART laboratory procedures, helping to decrease the risk of mix-ups.

In conclusion, a new barcode-based approach for the labelling of sperm samples has been developed and has proved to be harmless and efficient. With this approach, human semen samples could be tracked along the laboratory procedures typically conducted during an ART cycle, without any effect on sperm viability, quality, cryopreservation or motile fraction selection. In addition, using an animal model, tagged spermatozoa was shown to be able to produce pregnancies at a normal rate and live offspring.

Together with barcode-tagging of oocytes and embryos, this system could provide enhanced security in the traceability of both gametes and embryos during the application of human ART.

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