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A novel embryo identification system by direct tagging of mouse embryos using silicon-based barcodes

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BACKGROUND: Measures to prevent assisted reproductive technologies (ART) mix-ups, such as labeling of all labware and double-witnessing protocols, are currently in place in fertility clinics worldwide. Technological solutions for electronic witnessing are also being developed. However, none of these solutions eliminate the risk of identification errors, because gametes and embryos must be transferred between containers several times during an ART cycle. Thus, the objective of this study was to provide a proof of concept for a direct embryo labeling system using silicon-based barcodes.

METHODS: Three different types of silicon-based barcodes (A, B and C) were designed and manufactured, and microinjected into the perivitelline space of mouse pronuclear embryos (one to four barcodes per embryo). Embryos were cultured *in vitro* until the blastocyst stage, and rates of embryo development, retention of the barcodes in the perivitelline space and embryo identification were assessed every 24 h. Release of the barcodes after embryo hatching was also determined. Finally, embryos microinjected with barcodes were frozen and thawed at the 2-cell stage to test the validity of the system after cryopreservation.

RESULTS: Barcodes present in the perivitelline space, independently of their type and number, did not affect embryo development rates. The majority of embryos (>90%) retained at least one of the microinjected barcodes in their perivitelline space up to the blastocyst stage. Increasing the number of barcodes per embryo resulted in a significant increase in embryo identification rates, but a significant decrease in the barcode release rates after embryo hatching. The highest rates of successful embryo identification (97%) were achieved with the microinjection of four type C barcodes, and were not affected by cryopreservation.

CONCLUSIONS: Our results demonstrate the feasibility of a direct embryo labeling system and constitute the starting point in the development of such systems.

Key words: assisted reproductive technologies / embryo labeling / IVF mix-ups / traceability / silicon microtechnologies

Introduction

The increasingly high number of patients undergoing assisted reproductive technologies (ART) treatments worldwide (Wright *et al.*, 2008; Nyboe Andersen *et al.*, 2009) prevents the performance of totally individualized clinical and laboratory procedures. The simultaneity of independent ART cycles is unavoidable and, because of their caseload, fertility clinics cannot allocate separate work, incubation or storage areas for each patient sample. As a result, sample identification and mismatching errors may occur. In fact, since the first known case of an ART mix-up in 1987 in Manhattan, USA (Liebler, 2002), the accidental use of incorrect gametes or embryos during ART procedures has been reported in several centers around the world (Spriggs, 2003; Bender, 2006). Many of these mix-ups were detected because couples gave birth to babies of different skin color from their own or because fertility clinics later informed patients of the mistake, but it is possible that other cases could be going unnoticed.

Even though the occurrence of ART mix-ups is rare, their consequences are devastating for both the couples and fertility centers involved, leading to complex paternity suits and legal actions against the clinics which may end up in economic compensations. Therefore,

mechanisms to prevent these unintended accidents are currently being sought. Critical points during the clinical and laboratory procedures, when mismatching of gametes and embryos is most likely to occur, have been indentified: collection of oocytes and sperm, fertilization of oocytes by mixing them with sperm (IVF) or by injecting them with sperm (ICSI), transferring gametes or embryos between tubes or dishes, freezing and thawing of gametes or embryos, and embryo transfer into a patient (Magli *et al.*, 2008). Medical-scientific societies such as the European Society for Human Reproduction & Embryology (ESHRE) or the Federación Latinoamericana de Sociedades de Esterilidad y Fertilidad (FLASEF), and regulatory bodies such as the Human Fertilisation and Embryology Authority (HFEA) in the UK, propose/mandate in their guidelines and codes of practice the permanent labeling of all labware to identify the source of the biological material inside the tube or dish, and the application of witnessing protocols to double check the identification of samples and the patients or donors to whom they relate, at all aforementioned critical points of the clinical and laboratory procedures. These measures, when rigorously followed, certainly minimize the risk of sample mismatching due to human error, but they do not eliminate it completely and they increase the already high workload of embryologists and clinicians and the costs of ART procedures. In fact, the efficacy of double-witnessing as a safeguard in the context of ART laboratories has been questioned, as errors can still occur due to involuntary automaticity. In addition, because embryologists must be continuously interrupted from their tasks by the need to double witness for other embryologists, this system may even have a side effect of increasing risk by creating distractions and interruptions to an embryologist's own work (Brison *et al.*, 2004; Mortimer and Mortimer, 2005).

Recently, technological solutions for electronic witnessing that allow automation of the process of recognition and verification of sample identity and matching have been developed as an alternative to manual double witnessing. They include barcodes (Matcher™, Fertility QMS Ltd, UK) and radio frequency identification (RFID) labels (IVF Witness™, Research Instruments, UK) that can be attached to all labware containing gametes or embryos from a particular patient and automatically detected by a scanner or RFID reader connected to a computer, reducing the need for human intervention. The use of these electronic systems, especially RFID technology, is rapidly extending to fertility clinics worldwide (Schnauffer *et al.*, 2005; Glew *et al.*, 2006) and, in the UK, it is supported by the HFEA to substitute some manual witnessing steps. However, because gametes and embryos must be transferred from one container to another several times during the course of an ART cycle, the possibility of misidentification errors still exists.

To further minimize this risk, a method of labeling the gametes or embryos directly could be devised, so that the label would travel with the biological material throughout the entire ART process, from collection to transfer back to the patient. The labels should be made of a biocompatible material and should be small enough not to compromise gamete fertilization and embryo developmental potential, but large enough to hold a sufficient amount of information for sample identification purposes that could be read under a standard inverted microscope. In this sense, silicon-based barcodes on the low micrometer size range could be useful as embryo identification tags, as they fulfill all the aforementioned requirements. Moreover, they have already been successfully used as intracellular tags for human macrophages in culture, demonstrating their utility for individual cell tracking without affecting cell viability (Fernández-Rosas *et al.*, 2009).

The aim of the present work was to provide a proof of concept for such a direct oocyte/embryo labeling system, by tagging pronuclear mouse embryos with silicon-based barcodes and monitoring them during *in vitro* culture. Several types of barcodes were designed and tested, and embryo labeling was accomplished by means of their microinjection into the perivitelline space. Rates of development, embryo identification, retention of barcodes in the perivitelline space during culture and release of barcodes after blastocyst hatching were determined to demonstrate the validity of this labeling approach. Moreover, the effectiveness of the labeling system after embryo cryopreservation was also investigated.
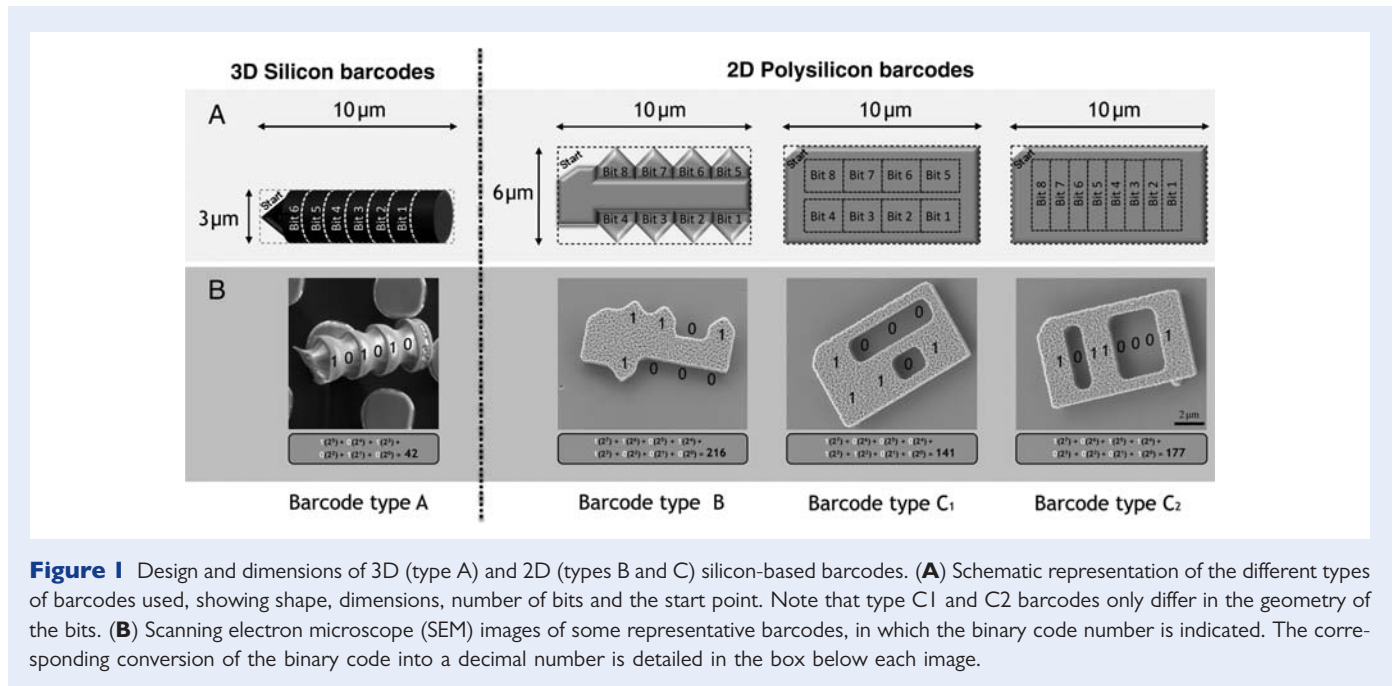
Materials and Methods

Animal care and procedures used in this study were conducted according to protocols 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 de la Generalitat de Catalunya.

Collection of mouse embryos

Eight- to 12-week-old female mice of the hybrid strain B6CBAF1 (C57BL/6J × CBA/J) were used as embryo donors. Females were induced to superovulate by intraperitoneal injection of 5 IU of pregnant mare serum gonadotrophin (Intervet, Spain) followed 48 h later by a second injection of 5 IU of human chorionic gonadotrophin (hCG; Farma-Lepori, Spain), and mated with males of the same strain. One-cell embryos were collected from the oviducts 25 h after hCG administration, and incubated for 5−10 min at 37°C in Hepes-buffered potassium simplex optimized medium (H-KSOM; Biggers *et al.*, 2000) supplemented with 156 U/ml of hyaluronidase (Sigma, Spain) for dispersion of cumulus cells. Denuded embryos were then washed twice in fresh H-KSOM and embryos with two pronuclei and a good morphology were selected. Selected embryos were incubated in KSOM culture medium containing both essential and nonessential amino acids and 1 mg/ml of bovine serum albumin (Embryo-Max, Millipore, Spain) at 37°C in a 5% CO_2 atmosphere until their use.

Design and fabrication of silicon-based barcodes

Three different types of silicon-based barcodes (A, B and C) with a binary code numerical representation were designed, fabricated and tested in this study (Fig. 1). Type A are three-dimensional (3D) silicon barcodes with a cylindrical shape and divided by engraved zones, allowing a total of six alphanumeric digits (bits) and, therefore, 64 different combinations (numbers 0−63). They are 10 μm in length and 3 μm in diameter. Type B and type C are two-dimensional (2D) polysilicon barcodes based on a horizontal matrix representation defined by either pentagonal (type B) or rectangular (type C) bits. Both types of 2D barcodes are 10 μm in length and 6 μm in width and have a thickness of 1 μm. They allow a total of 8 bits and, therefore, of 256 different combinations (numbers 0−255). However, because type C barcodes can be designed with either square (subtype C1) or rectangular (subtype C2) bits, the different combinations offered by this type of barcode is doubled (512 different barcodes). To allow the reading of the data in its correct orientation, all the barcodes are asymmetric and contain a start marker. The binary data contained in the barcode design can be easily converted to a decimal number (Fig. 1).

Figure 1 Design and dimensions of 3D (type A) and 2D (types B and C) silicon-based barcodes. (**A**) Schematic representation of the different types of barcodes used, showing shape, dimensions, number of bits and the start point. Note that type C1 and C2 barcodes only differ in the geometry of the bits. (**B**) Scanning electron microscope (SEM) images of some representative barcodes, in which the binary code number is indicated. The corresponding conversion of the binary code into a decimal number is detailed in the box below each image.

The three types of barcodes were fabricated on 4″ p-type (100) silicon wafers using silicon microtechnologies used for microelectromechanical systems (MEMS). The fabrication process for type B and C barcodes has been previously described (Fernández-Rosas *et al.*, 2009). Briefly, a plasma-enhanced chemical vapor deposition silicon oxide layer was deposited on the front side of the wafer to be used as a sacrificial layer for later release of the barcodes. Next, a 1 μm thick low-pressure chemical vapor deposition polysilicon layer (device layer) was deposited and the barcodes were patterned by a photolithographic step and a vertical polysilicon dry etching. The photoresist was removed by plasma etching, and the barcodes were released by the etching of the silicon oxide sacrificial layer in vapors of hydrofluoric acid.

Type A barcodes were fabricated using a similar process, but in this case a simple photolithographic step with 3 μm spot pattern on a previously grown silicon oxide layer, followed by sequential dry etching, was used to produce the cylindrical shape of the barcodes. Controlling vertical and non-vertical etch conditions allowed the definition of the binary code along the axis. The final fabrication step was a large non-vertical etching to release the barcodes (Gómez-Martínez *et al.*, 2009).

Microinjection of the barcodes into the perivitelline space

An Eppendorf TransferMan NK2 micromanipulator, a Burleigh Piezodrill and an Olympus IX71 inverted microscope were used to microinject the barcodes into the perivitelline space of the pronuclear stage embryos. Embryos were placed into a drop of H-KSOM medium in the micromanipulation dish and barcodes were transferred into a separate drop of 3% (w/v) polyvinilpirrolidone (Sigma, Spain) in H-KSOM, to avoid their precipitation and facilitate their aspiration with the injection micropipette. Several barcodes were first introduced into a blunt-ended microinjection pipette with an outer diameter of 10 μm. The pipette was then moved to the drop containing the embryos and used to drill a hole in the zona pellucida of an embryo with the help of a few piezo pulses. Next, the barcodes (1–4) were expelled into the perivitelline space of the embryo, as far away from the hole as possible to prevent

their escape, and the pipette was gently withdrawn. Microinjection of the barcodes in 20 embryos took ∼10 min.

Injected embryos were returned to the KSOM culture drops in the incubator and cultured until they hatched. Non-injected embryos were cultured in parallel as a control of development.

Embryo freezing and thawing

Embryos microinjected with barcodes and control non-injected embryos were frozen after 24 h of *in vitro* culture using a slow-freezing protocol (Costa-Borges *et al.*, 2009). Briefly, 2-cell embryos were first incubated for 7 min in H-KSOM containing 1.5 M propylene glycol (PROH; Fluka, Spain) at room temperature (RT). Embryos were then transferred to a drop of H-KSOM containing 1.5 M PROH and 0.1 M sucrose (Merck, Spain) and immediately loaded into 0.25 ml French-type straws (Minitube, Germany). Twelve to fifteen embryos were loaded per straw. The straws were thermo-sealed and placed in a controlled-rate freezer (Kryo 360, Planer, UK). Embryos were initially cooled at a rate of $-2°C$/min from RT to $-7°C$, the temperature at which manual seeding was performed. Next, they were cooled from -7 to $-30°C$ at rate of $-0.3°C$/min, and then fast cooled to $-150°C$ at a rate of $-35°C$/min (Lassalle *et al.*, 1985). Finally, the straws were directly plunged into liquid nitrogen at $-196°C$ for storage.

At 1–7 days after cryopreservation, the straws were thawed by keeping them for 40 s at RT followed by 40 s at 30°C in a water bath. The embryos were then released from the straws and incubated for 15 min at RT in H-KSOM containing 0.3 M of sucrose. Finally, the embryos were incubated for 15 min in H-KSOM at 37°C and then transferred to KSOM culture medium and cultured at 37°C and in a 5% CO_2 atmosphere until they hatched.

Statistical analysis

All experiments were repeated at least three times on separate days and the results obtained in the replicated experiments were pooled. Data collected were analyzed by χ^2 test or Fisher's exact test. A probability value of $P < 0.05$ was considered to be statistically significant.

Experimental design

To test the validity of the proposed embryo labeling system and to select the most appropriate barcode design, a first set of experiments was performed in which a single barcode (type A, B or C) was microinjected into the perivitelline space of pronuclear stage embryos. Microinjected embryos were maintained in culture, together with a control group of non-injected embryos, until the blastocyst stage (96 h) and were monitored every 24 h under an inverted microscope to assess their developmental progression (development rate) and the presence of the microinjected barcode in the perivitelline space (retention rate). In addition, the number of developed embryos in which the barcode could be clearly read under the inverted microscope ($200\times$ magnification) was recorded (identification rate). It is important to point out that barcode reading was performed only by adjusting the focus on the microscope, without embryo manipulation. Therefore, only embryos with barcodes in the correct orientation could be successfully identified.

Once the most appropriate type of barcode was selected, a second set of experiments was performed in order to increase the identification rate. With this aim, two, three or four barcodes of the selected type were microinjected into the perivitelline space, and the microinjected embryos, together with a control group of non-injected embryos, were maintained in culture until the blastocyst stage (96 h). The same rates as in the previous experiments were determined every 24 h of culture (development, retention and identification rates) and, in this case, retention and identification rates were calculated considering only embryos that retained all the microinjected barcodes. In addition, in this set of experiments, blastocysts were kept in culture until they hatched and the number of hatched blastocysts that were totally free of the barcodes was determined (barcode release rate). Because some embryos were not able to complete hatching on their own, a short incubation with pronase (35 U/ml) was performed in these cases to help the zona pellucida digestion.

The last set of experiments was designed to test the validity of our embryo labeling system after an embryo freezing–thawing process. Pronuclear stage embryos were microinjected with the type and number of barcodes selected in the previous experiments and, after 24 h in culture, cleaved embryos that retained all the microinjected barcodes were cryopreserved. Two-cell embryos were thawed 1–7 days after freezing and maintained in culture until hatching. As in the previous set of experiments, the embryos were assessed every 24 h and the development, retention and identification rates, as well as the barcode release rate after hatching, were determined and compared with those obtained with the equivalent group of barcode-tagged embryos that were not cryopreserved.

Results

Selection of the optimal barcode design for embryo labeling

A total of 240 pronuclear-stage mouse embryos were microinjected, each with a single barcode (80 embryos per barcode type), and cultured in parallel to a group of 76 non-injected control embryos for 96 h. Development rates of barcode-tagged and control embryos were similar at all time points examined (Table I and Fig. 2), indicating that neither the microinjection process nor the presence of the polysilicon barcode in the perivitelline space affect embryo developmental potential.

Barcode retention rates during culture were higher than 90% in all three groups of tagged embryos, and no significant differences were detected among them (Table I). Retention rates did not differ significantly along the time points examined for each particular type of

Table I Rates of *in vitro* development and barcode retention in embryos microinjected with different types of barcodes.

Group	n	24 h Development (%)	24 h Retention* (%)	48 h Development (%)	48 h Retention* (%)	72 h Development (%)	72 h Retention* (%)	96 h Development (%)	96 h Retention* (%)
Control	76	65 (85.5)	–	65 (85.5)	–	65 (85.5)	–	64 (84.2)	–
Barcode A	80	70 (87.7)	70 (100)$^{\alpha}$	70 (87.7)	69 (98.6)$^{\alpha,\beta}$	70 (87.7)	66 (94.3)$^{\alpha,\beta}$	67 (83.8)	62 (92.5)$^{\beta}$
Barcode B	80	72 (90.0)	70 (97.2)	68 (85.0)	64 (94.1)	68 (85.0)	64 (94.1)	63 (78.8)	59 (93.7)
Barcode C	80	71 (88.8)	71 (100)	71 (88.8)	71 (100)	71 (88.8)	71 (100)	66 (82.5)	66 (100)

*Number of developed embryos that retain the microinjected barcode.
$^{\alpha,\beta}$Values with different superscripts within the same row differ significantly between time points ($P < 0.05$).
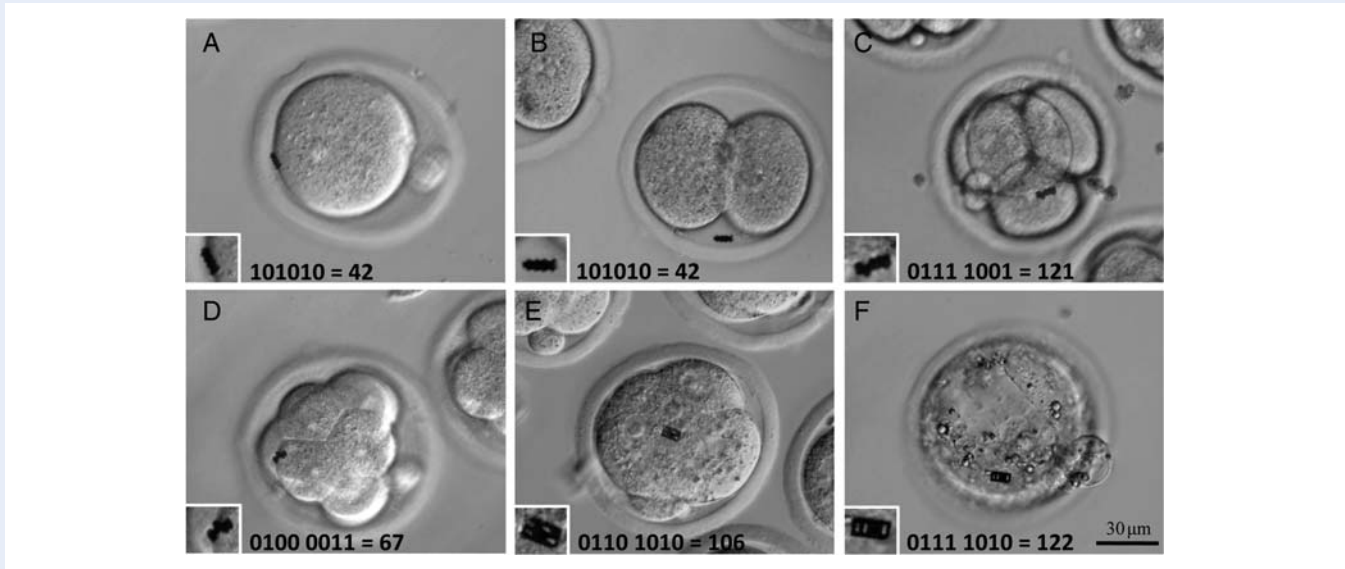
Figure 2 *In vitro* development of embryos microinjected with different types of polysilicon barcodes into their perivitelline space. (**A** and **B**) One- and 2-cell embryos labeled with type A barcodes. (**C** and **D**) Four-cell and compacting 8-cell embryos containing a type B barcode. (**E** and **F**) Morula and hatching blastocyst labeled with a type C barcode. Magnified images of the barcodes (insets) and their corresponding binary and decimal numbers are shown for each embryo.

barcode, except for a specific difference between 24 and 96 h for type A barcodes ($P = 0.026$). Therefore, the majority of microinjected barcodes, independently of their size and shape, remain in the perivitelline space from the pronuclear to the blastocyst stage.

Finally, with regard to embryo identification rates, values ranging from 30.5 to 58.6% were achieved and no significant differences were observed at any time point according to the type of barcode used (Table II). However, when the total number of identification processes performed during culture for each group of tagged embryos was considered, the rate of successful embryo identification was significantly higher when using type A (53.2%) than type B (41.2%) barcodes ($P = 0.008$), and the use of type C barcodes produced an intermediate result (48.0%). Comparison of identification rates along the time points examined only revealed significant differences for type B barcodes between 24 and 96 h ($P = 0.017$).

The results obtained in this first set of experiments indicated that none of the three types of barcodes tested was clearly superior to the others in terms of the parameters analyzed and, therefore, that all of them would be suitable for embryo tagging. In this context, we selected type C barcodes to proceed with the development of the embryo labeling system because this design allows for the highest number of combinations and it is the easiest to read under the inverted microscope.

Optimization of embryo identification rates

In a second set of experiments, aimed at increasing embryo identification rates, pronuclear stage embryos were microinjected with two, three or four type C barcodes into their perivitelline space (80 embryos per group) and cultured in parallel to a group of 49 non-injected control embryos for up to 120 h. Ideally, each embryo should have been injected with various copies of the same barcode, to simulate an eventual real situation in a clinical setting in which all

Table II Identification rates of embryos microinjected with different types of barcodes.

Group	Identification (%)*				
	24 h	**48 h**	**72 h**	**96 h**	**Total**
Barcode A	41/70 (58.6)	35/69 (50.7)	38/66 (57.6)	28/62 (45.2)	142/267 (53.2)[a]
Barcode B	37/70 (52.9)[α]	26/64 (37.5)[α,β]	27/64 (42.2)[α,β]	18/59 (30.5)[β]	106/257 (41.2)[b]
Barcode C	36/71 (50.7)	33/71 (46.5)	33/71 (46.5)	32/66 (48.5)	134/279 (48.0)[a,b]

*Number of embryos that were successfully identified from those that developed and retained the microinjected barcode.
[α,β]Values with different superscripts within the same row differ significantly between time points ($P < 0.05$).
[a,b]Values with different superscripts within the same column differ significantly between groups ($P < 0.05$).

embryos from the same patient or couple would be tagged with a unique barcode number. However, because type C barcodes were fabricated in all possible combinations in a single silicon wafer (including both subtypes C1 and C2) and they were mixed upon release, this was not possible at this stage of the research and the various barcodes injected into each embryo corresponded to different codes.

Rates of embryonic development up to the blastocyst stage (96 h) were similar among embryos microinjected with two, three or four barcodes and control non-injected embryos (Table III). When compared with embryos injected with a single type C barcode in the previous experiments (82.5% blastocyst rate, Table I), significant differences ($P = 0.022$) were only observed at 96 h for the group injected with four barcodes, which surprisingly showed a higher

Table III Rates of *in vitro* development and barcode retention in embryos microinjected with different numbers of type C barcodes.

Group	n	24 h Development (%)	24 h Retention* (%)	48 h Development (%)	48 h Retention* (%)	72 h Development (%)	72 h Retention* (%)	96 h Development (%)	96 h Retention* (%)
Control	49	45 (91.8)	–	45 (91.8)	–	45 (91.8)	–	44 (89.8)	–
Barcode Cx2	80	73 (91.3)	66 (90.4)	73 (91.3)	66 (90.4)	73 (91.3)	63 (86.3)	70 (87.5)	58 (87.5)
Barcode Cx3	80	70 (87.5)	63 (90.0)	70 (87.5)	63 (90.0)	70 (87.5)	58 (82.9)	68 (85.0)	57 (83.8)
Barcode Cx4	80	76 (95.0)	69 (90.8)	76 (95.0)	69 (90.8)	76 (95.0)	64 (84.2)	76 (95.0)	64 (84.2)

*Number of developed embryos that retain all of the microinjected barcodes.

percentage of embryos that reached the blastocyst stage (95.0%, Table III).

A high percentage of developed embryos retained all the microinjected barcodes during culture (82.9–90.8%; Table III) and no significant differences in retention rates were detected at any time point among the three groups of embryos microinjected with a different number of barcodes. Nor did retention rates differ along time in culture for any particular group. Compared with the 100% retention rate achieved in the previous experiments in the group of embryos injected with a single type C barcode, retention rates were significantly decreased at all time points when two to four type C barcodes were injected ($P = 0.0138$ to $P < 0.0001$). However, because the vast majority of embryos which did not retain all the microinjected barcodes only lost one of them, when the percentage of embryos retaining at least one of the microinjected barcodes was considered (98.5–98.7%, 98.5–100% and 100% for embryos injected with two, three or four barcodes, respectively), no significant differences were found at any time point when compared with the group of embryos microinjected with a single type C barcode.

In order to compare embryo identification rates when a different number of barcodes is present in the perivitelline space, only embryos that retained all the microinjected barcodes were initially considered (Table IV). Positive embryo identification required the successful reading of at least one of the barcodes present in the perivitelline space (Fig. 3). No significant differences were detected for any group along the different time points examined, but identification rates significantly differed between groups at all time points, specially between the groups having two or four barcodes ($P = 0.010$ to $P < 0.001$; Table IV). When compared with the injection of one type C barcode (Table II), injection of two or more barcodes resulted in a significant increase in identification rates at all time points examined ($P = 0.006$ to $P < 0.001$). As expected from these results, the total identification rate (considering the total number of identification processes performed during culture for each group of embryos) significantly differed between all groups ($P = 0.013$ to $P < 0.0001$), increasing from 48% for embryos injected with a single type C barcode (Table II) to 97% for embryos injected with four type C barcodes (Table IV).

In this set of experiments, embryos that reached the blastocyst stage by 96 h were maintained in culture for another 24 h to

Table IV Identification rates of embryos microinjected with different numbers of type C barcodes.

Group	Identification (%)* 24 h	48 h	72 h	96 h	Total
Barcode Cx2	50/66 (75.7)[a]	49/66 (74.2)[a]	45/63 (71.4)[a]	46/58 (79.3)[a]	190/253 (75.1)[a]
Barcode Cx3	59/63 (93.6)[b]	50/59 (84.7)[a,b]	54/58 (93.1)[b]	49/57 (86.0)[a]	212/237 (89.4)[b]
Barcode Cx4	65/69 (94.2)[b]	67/69 (97.1)[b]	61/64 (95.3)[b]	64/64 (100)[b]	257/265 (97.0)[c]

*Number of embryos that were successfully identified from those that developed and retained all of the microinjected barcodes.
[a–c]Values with different superscripts within the same column differ significantly between groups ($P < 0.05$).
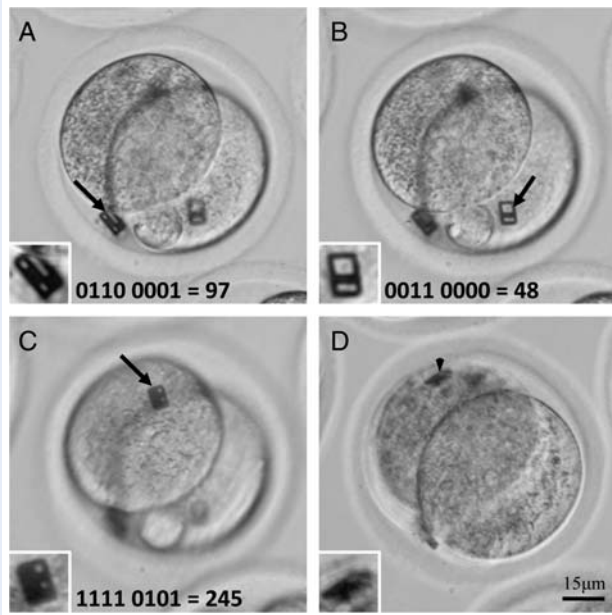
Figure 3 Identification process of a 2-cell embryo containing four type C barcodes in its perivitelline space. Identification was performed under an inverted microscope (200× magnification) by adjusting the focus without reorienting the embryo. (**A**–**C**) Images of various focal planes at which three of the barcodes could be clearly read (type C1 in A and C; type C2 in B). (**D**) The fourth barcode present in the perivitelline space could not be read, due to its incorrect orientation. Magnified images of the barcodes (insets) and, when readable, their corresponding binary code and decimal number are shown.

determine the fate of the barcodes after embryo hatching. In blastocysts derived from embryos injected with two barcodes, barcodes usually remained inside the empty zona pellucida or were released when embryos hatched, so that most of the blastocysts were totally free of the barcodes after hatching (44/58, 75.9% release rate). In the remaining blastocysts (14/58, 24.1%), at least one of the barcodes was firmly adhered to the embryo surface and could not be liberated even after rough pipetting of the hatched embryos (Fig. 4). Complete barcode release rates significantly decreased ($P = 0.026$ to $P < 0.001$) as the number of barcodes injected into the perivitelline space was increased: 54.4% (31/57) for embryos injected with three barcodes and 17.2% (11/64) for those injected with four barcodes.

Cryopreservation of the barcode-tagged embryos

In the last set of experiments, embryos microinjected with four type C barcodes at the pronuclear stage were cultured for 24 h and cryopreserved at the 2-cell stage ($n = 148$), in parallel to control non-injected embryos ($n = 49$). After thawing, embryos were cultured for 72 h, and no significant differences were found between the two groups in development rates (Table V). Development of frozen-thawed embryos carrying four barcodes in their perivitelline space was also similar at all time points examined to that of their non-cryopreserved counterparts from the previous set of experiments (Table III).
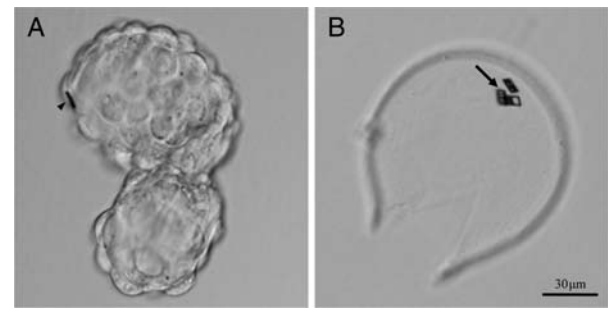
Figure 4 Adhesion of barcodes to the embryo surface. (**A**) Hatched blastocyst showing one type C barcode adhered to its surface (arrowhead). (**B**) In the corresponding empty zona pellucida, the three remaining barcodes are left.

The majority of cryopreserved embryos retained all four microinjected barcodes after thawing and culture (94.5–96.2%; Table V), and these retention rates were significantly higher than those obtained in the group of embryos microinjected with four type C barcodes that were not cryopreserved, at 48 h ($P = 0.014$) and 72 h ($P = 0.028$). However, because barcode loss mainly takes place during the 1-cell to 2-cell transition and because retention rates in cryopreserved embryos were calculated from the number of 2-cell embryos thawed, these differences can probably be attributed to the different starting point in the calculation of these rates for non-cryopreserved (1-cell) and cryopreserved (2-cell) embryos. In fact, in both groups of tagged embryos 100% of them retained at least one of the microinjected barcodes up to the blastocyst stage.

All the barcodes maintained their integrity after freezing and thawing. Identification rates of 90.5% (115/127), 93.4% (114/122) and 97.5% (118/121) were achieved after 24, 48 and 72 h of culture, respectively, of the frozen-thawed microinjected embryos. This means that out of the total number of identification processes performed during culture, in 93.8% (347/370) of the embryos at least one of the barcodes could be successfully read, a rate that was similar to that of the non-cryopreserved group (97%).

As in the previous set of experiments, the complete barcode release rate after embryo hatching (96 h of culture after thawing) was very low (16/121, 13.2%), demonstrating again that when four barcodes are present in the perivitelline space the probability that at least one of them remains adhered to the embryo surface after hatching is high.

Discussion

In this work, a first step towards the development of a safe and reliable direct oocyte/embryo identification system is presented. Once developed, such a system could minimize the risk of sample misidentification and mismatching errors during ART procedures and greatly reduce the steps in the clinical and laboratory processes at which manual double-witnessing should be performed.

This novel approach of direct sample labeling is based on the use of micrometer-sized silicon-based barcodes containing bits that are large enough ($\geq 1\ \mu m^2$) to be visible under a standard optical microscope.

Table V Rates of *in vitro* development and barcode retention after thawing of embryos microinjected with four type C barcodes and cryopreserved at the 2-cell stage.

Group	n	24 h		48 h		72 h	
		Development (%)	Retention* (%)	Development (%)	Retention* (%)	Development (%)	Retention* (%)
Control	49	43 (87.8)	–	41 (83.7)	–	41 (83.7)	–
Barcode Cx4	148	132 (89.2)	127 (96.2)	128 (86.5)	122 (95.3)	128 (86.5)	121 (94.5)

*Number of developed embryos that retain all of the microinjected barcodes.

Therefore, setting up such a system in an embryology laboratory would be straightforward as no special equipment would be needed, other than an inverted microscope equipped with a micromanipulator, a camera and a computer, which most embryology labs already have. Even though manual eye reading of the barcodes was performed in this initial work reported here, a computer software for the automatic reading of the barcodes is now being developed, which will facilitate a faster and more reliable reading.

As a proof of concept for this novel labeling system of oocytes and embryos, mouse embryos at the pronuclear stage were used as sample models and the barcodes were microinjected into the previtelline space. Because our laboratory routinely performs mouse nuclear transfer experiments, our microinjection setup is adapted to work with a piezodrill and this system was used for the experiments described in the present work. However, piezo-assisted microinjection is not a requirement for the microinjection of the barcodes, as long as beveled micropipettes are used. In fact, because the hole created in the zona pellucida with a blunt-ended piezo-driven pipette is much bigger than that created with a beveled pipette, the use of a conventional microinjection system with beveled pipettes for the microinjection of the barcodes may have resulted in a higher retention rate of the barcodes in the perivitelline space during embryo culture.

In the first part of our study, the validity of the approach was investigated and three different types of barcodes were tested to select the most appropriate for embryo labeling and identification. Barcodes microinjected into the pervitelline space of the embryos clearly had no effect on their *in vitro* developmental potential up to the blastocyst stage. These results were expected, as we had previously demonstrated that silicon and polysilicon microparticles internalized into human macrophages by phagocytosis do not affect cell viability and that no effect on *in vitro* development is seen when these microparticles are microinjected into the cytoplasm of mouse pronuclear embryos (Fernández-Rosas *et al.*, 2010). In addition, type B barcodes, the same as the ones used in the present work, had already been used successfully to label and track human macrophage cells in culture without any apparent effects on cell viability (Fernández-Rosas *et al.*, 2009).

Most of the injected barcodes remained in the previtelline space during embryo culture and development, and 40–50% could be successfully read, allowing the correct identification of the corresponding embryos. It is important to note that embryo identification was performed without manipulating the embryos, because it was our intention to simulate the eventual situation in which an automatic reading system would be used. Therefore, successful reading of the barcodes in these circumstances is totally dependent on barcode orientation. It is probable that if embryos had been manipulated to change their orientation, all of the barcodes could have been finally read and embryo identification rates of 100% could have been obtained. In fact, anticipating the importance of barcode orientation for successful reading, 3D type A barcodes were designed as we reasoned that they would be easier to read than 2D type B and C barcodes in any orientation. This did not turn out to be the case, as identification rates for embryos containing type A barcodes were similar to those of embryos containing type C barcodes, and only slightly higher than those of embryos containing type B barcodes.

As all the parameters tested in the first set of experiments were similar for the three types of barcodes tested, our selection had to be based on other barcode properties. In their current design, 2D barcodes (with 8 bits) allow a higher number of combinations than 3D barcodes (with only 6 bits). Moreover, the bits of type C barcodes can be designed with two different geometries, allowing twice the number of combinations than type B barcodes. In addition, we found that type C barcodes were easier to read, at least manually, than type B or even type A barcodes. For all these reasons, we considered type C barcodes as the most appropriate for our embryo labeling approach, as a higher number of embryos could be potentially labeled.

Next, we focused on increasing embryo identification rates to a value as close as 100% as possible. Because correct orientation of the barcode is key for its successful reading, the number of barcodes microinjected per embryo was increased in order to maximize the probability that at least one of them remains properly oriented for its reading. Increasing the number of barcodes present in the perivitelline space up to four had no detrimental effects on embryo development, and the percentage of embryos that retained at least one of the microinjected barcodes during all preimplantation development (and, therefore, could be potentially identified) was similar, independently of the number of barcodes injected, and very close or equal to 100%. Nonetheless, as expected, identification rates significantly increased in parallel to the number of barcodes present, reaching a maximum of 97% for embryos receiving four type C barcodes.

Having determined that tagging embryos with four type C barcodes is the optimal condition in terms of identification rates, the next step was to determine whether barcodes would be able to withstand cryopreservation and whether the effectiveness of the embryo labeling system would be maintained. Cryopreservation of surplus embryos is a routine procedure at fertility centers, and because it involves multiple transfers of embryos from one container to another during both freezing and thawing processes, it is considered as one of the critical

points when mismatching of embryos may occur. Our results clearly show that embryo freezing and thawing have no detrimental effects on the developmental potential of tagged embryos, barcode integrity and retention, or embryo identification rates. Therefore, the barcode-based embryo labeling approach presented here is also reliable after cryopreservation.

One of the unexpected hurdles of the system was the low release rate of barcodes observed after embryo hatching, especially when four barcodes per embryo were used. Because adherence of barcodes to the embryo surface was never observed in pre-blastocyst stages, two non-excluding explanations for this phenomenon are possible. First, the reduction or even disappearance of the perivitelline space when blastocysts expand may facilitate a close contact between the barcode and the trophectoderm cells surface, thus inducing their adhesion. Second, changes in cell surface that accompany the formation of trophectoderm cells (Yamanaka *et al.*, 2006) may promote the adhesion of the barcodes. Barcodes should accompany embryos throughout the entire *in vitro* procedures, so that they can be indentified and tracked at every critical step, but they should be released from the embryos before implantation. Even though the effect of barcodes adhered to the blastocyst surface on embryo implantation has not been assessed, this situation should be avoided if an embryo labeling system like the one described here is to be eventually applied in a clinical setting. It is possible that coating of the barcodes surface with a hydrophobic biocompatible compound could prevent their adhesion to the embryos. Alternatively, a biodegradable material could be used for the fabrication of the barcodes.

In summary, the results presented here demonstrate the feasibility of a direct embryo labeling system and constitute the starting point in the development of such systems. Even though pronuclear embryos were used in the experiments reported here, the same barcode-based labeling approach could also be applied to embryos at later developmental stages and to oocytes. In fact, in a clinical setting, labeling of samples at an early point of the ART procedure would be the best approach to minimize the risk of sample mismatching, and microinjection of the barcodes could be performed in oocytes at the time of ICSI. In the case of embryos undergoing preimplantation genetic diagnosis, in which a relatively large opening is created in the zona pellucida during blastomere biopsy, a higher number of barcodes might be injected into the perivitelline space to reduce the possibility of losing all the barcodes.

Compared with current technological solutions for sample identification and tracking during ART procedures, a direct sample labeling approach would minimize even further the risks of human errors in sample identification and mismatching because the labels would travel together with the samples throughout all the steps in the process, even when samples are moved from one container to another. However, because the approach is not applicable to sperm cells, mismatching of sperm and oocytes at the fertilization step could not be avoided with this system. In a barcode-based system like the one described here, sample identification takes place under a standard inverted microscope, avoiding exposure of oocytes and embryos to potentially harmful radio waves or lasers and the need for expensive specialized equipment.

In spite of the promising results obtained so far, the approach reported here for direct embryo labeling has some limitations, such as barcode adhesion to the embryo surface after hatching or the need for micromanipulation to label each individual embryo. Current work in our laboratory is focused on overcoming these limitations, and alternative methods of barcode incorporation into oocytes/embryos are being pursued. In particular, modification of the barcode surface aimed at the selective attachment of barcodes to the outer surface of the zona pellucida by either physical or chemical means is being investigated.

Authors' roles

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