Laser Scanning Confocal Imaging of Abnormal or Arrested Human Preimplantation Embryos

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Purpose: The improved resolution and optical sectioning of a confocal microscope make it an ideal instrument for extracting three-dimensional information, especially from extended biological specimens such as human embryos. The staining of actin together with chromatin allowed us to specify the architecture of the embryo and the appearance of the nucleus.

Methods: F-Actin and chromatin distributions were visualized using laser scanning confocal microscopy in "fresh" and "cryopreserved" human preimplantation embryos obtained by in vitro fertilization.

Results: The current study revealed a high rate of multinucleation in arrested or poor-quality embryos (89%, in grade IV embryos).

Conclusions: Confocal microscopy revealed high levels of multinucleated blastomeres, suggesting that the probable cause of arrested development in these embryos was due to multinucleation of blastomeres.

KEY WORDS: laser scanning confocal imaging; human preimplantation embryos; F-actin; chromatin.

INTRODUCTION

Laser scanning confocal microscopy (LSCM) has become essential for much of developmental biology research (1). Paddock clearly demonstrated that confocal microscopy is a powerful tool in specialized fields, providing light microscope imaging of fluorescent-labeled embryos (2). White *et al.* (3) have compared confocal versus conventional imaging of biological

structures and emphasized the advantages of using LSCM for the observation of biological specimens, as illustrated with optical sections which are thin enough to study the whole sample.

Briefly, there are two major advantages of using LSCM in comparison with conventional epifluorescence light microscopy for research in developmental biology. Fluorescent flare from out-of-focus structures in the specimen is much reduced, and image resolution is effectively increased both laterally and axially. Confocal microscopy is a valuable tool for examining difficult specimens, such as fluorescently labeled multicellular embryos.

The major application of the LSCM is related to improved resolution of the distribution of specific fluorescent probes within oocytes and embryos. Most embryos are impossible to observe after the two-cell stage using conventional epifluorescence microscopy because, although cell numbers rise, the overall volume of the embryo remains roughly the same. In consequence, increased fluorescence from the more closely packed cells outside the focal plane of interest interferes with image resolution.

The aim of this study was to observe fluorescent-labeled human preimplantation embryos using LSCM. As stated in national ethical guidelines, we cannot use supernumary normally developing "fresh" embryos. Therefore, we analyzed "fresh" arrested preimplantation embryos. In vitro studies suggest that a considerable rate of embryonic loss occurs during preimplantation stages (4). Early arrest of fertilized eggs represents the main cause of failure after embryo implantation since it has been estimated that less than 50% of fertilized eggs reach the blastocyst stage, while the others remain predominantly arrested at the eight-cell or earlier stages (5). We also analyzed freeze-thawed embryos displaying no intact blastomere, which were consequently inappropriate for transfer,

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and embryos that had been cryopreserved 10 years ago and then given later to research by couples who no longer had any parental plans.

We used nitrobenzoxadiazole (NBD)/fluorescein-labeled phalloidin (6,7), which binds specifically to polymerized actin for imaging cell shape, and the monomeric nucleic acid dye propidium iodide to stain the nucleus in order to observe both cortical architecture and nuclear abnormalities in "fresh" and freeze-thawed human embryos. This technique allowed us to quantify rapidly, simply, and accurately the number of nuclei per blastomere and also revealed different morphological shape patterns of fragmented or arrested human embryos.

MATERIALS AND METHODS

Spare human preimplantation embryos were obtained from patients undergoing in vitro fertilization (IVF) treatment for infertility at our Center for Reproductive Medicine and Infertility (Lyon, France). They consisted of either totally fragmented embryos, or embryos arrested in their development. Patients gave their informed, written consent to donate their embryos, which could not be transferred nor frozen, for research.

Ovarian Stimulation, Oocyte Retrieval, and Embryo Culture

Ovarian stimulation was carried out using a gonadotropin-releasing hormone [luteinizing hormone releasing hormone (LHRH); triptorelin; Ipsen Biotech, France] agonist in a long protocol and human gonadotropins [purified follicle-stimulating hormone (FSH); Serono, France]. Both IVF and embryo transfer were performed using standard techniques as described previously (8). Ovum retrieval was carried out after 36 hr, following the injection of 10,000 IU human chorionic gonadotrophin (hCG; Profasi, Serono). After retrieval, oocytes from 28- to 38-year-old women were cultured in BM1 medium (Ellios Bio Media, France) at 37°C in an atmosphere of 5% CO₂ and 95% air. Insemination was carried out 3 hr after oocyte recovery with 100,000 motile spermatozoa per ml.

Classification and Grading of Embryos

Between 18 and 22 hr after insemination, oocytes were examined for the presence of two pronuclei. Only monospermic embryos developing from dipro-

nucleated zygotes were used for this study. All the embryos were submitted to embryo grading, and no multinucleation was detected by standard phase contrast microscopy.

On the morning of embryo transfer, embryos were examined and the number of cells was determined. Each embryo was graded according to Dawson *et al.* (9): grade I—perfectly symmetrical with no fragmentation; grade II—uneven blastomeres and/or slight fragmentation; grade III—uneven with one blastomere fragmented; grade IV—at least one blastomere still intact, but the remainder of the preembryo showing extensive fragmentation and no other discernible cellular structure; and grade V—totally degenerate.

The majority of embryo transfers were performed on day 2, corresponding to two- to four-cell-stage embryos. Some embryos (mostly from women with previous repeated implantation failures) were allowed to grow in a coculture system with Vero cells (10), and only those which reached the blastocyst stage by days 5–7 were transferred. The embryos used for subsequent analysis were either spare two- to four-cell-stage grade IV embryos, or cocultured embryos arrested in their development from the two-cell to the uncompact morula stage. Assessment of embryo quality and developmental stage was recorded daily until embryos showed cleavage arrest with no further progress compared with the previous 24 hr.

Controls. Supernumerary embryos reaching the four-cell stage on day 2 were frozen following a standard protocol using 1,2-propanediol (PROH) (1.5M) (Sigma, St. Louis, MO) combined with sucrose (0.1 M) (Sigma) (11). On the day of transfer, embryo thawing was performed and a maximum of four embryos with at least one intact blastomere was transferred. Fifteen supernumerary frozen-thawed embryos that were not transferred were placed in BM1 medium for 2 hr and were analyzed, with the informed written consent of couples. In 20 cases, we were able to use embryos that had been frozen 10 years earlier and donated for research by the couples. Among these 20 cryopreserved embryos, 10 were thawed and allowed to develop in culture for 1 day and 8 were normally cleaving at the six- to eight-cell stages.

Actin and DNA Immunofluorescent Staining

Arrested normally fertilized embryos were first processed for immunocytochemical detection of actin and DNA. To conserve the embryo's 3D morphology, the samples were attached to poly-L-lysine-coated coverslips (Bio-Rad, München, Germany). The zona pel-
lu-

cida was removed from embryos after a brief exposure to acid Tyrode's solution (pH 2.0) (Sigma) at 37°C.

Fixation. After a 30-min recovery at 37°C, zona-free embryos were fixed for 10 min in phosphate-buffered saline (PBS) containing 0.1% glutaraldehyde (Sigma) at 37°C and were then permeabilized with 1% Triton X-100 (Sigma) in PBS for 10 min (1). At the time of staining, samples were washed once in PBS containing 0.1% Triton X-100 and treated with 0.1% freshly dissolved sodium borohydride (Sigma) in PBS, pH 8.0, for 30 min to reduce cellular autofluorescence.

Staining. Ten microliters (33 ng) of NBD-phalloidin (Sigma) was evaporated and redissolved in 200 μ l of PBS. F-Actin microfilaments were stained with 5 IU/ml fluorescein isothiocyanate (FITC)/NBD-labeled phalloidin in PBS for 1 hr at room temperature and then washed in PBS. DNA was stained with a propidium iodide (5 μ g/ml) (PI; Molecular Probes, Eugene, OR) solution containing RNase (100 μ g/ml) (Sigma) for 20 min at room temperature and washed in PBS. The slides were mounted in an antifade medium containing 1,4-diazabicyclo-(2, 2, 2)-octane (DABCO) to retard photobleaching. Fifty human arrested embryos and 10 frozen-thawed embryos were examined.

Controls. Embryos that were not treated with Triton X-100 were stained by the same method, and it was confirmed that there were no antibodies attached to the surface of the embryos (data not shown).

After fixation, controls for autofluorescence were performed and these demonstrated a background fluorescence which was trivial in comparison to the fluorescence obtained with the staining (data not shown).

For each embryo, we quantified the number of blastomeres, the number of nuclei per blastomere, the nuclei's morphology—with or without chromatin condensation or fragmentation—and the presence of anucleated blastomeres.

Confocal Microscopy

Whole-mounted samples were examined on an LSM 10 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). The excitation source was a 488-nm wavelength argon laser, and fluorescence emission was recorded by two detectors situated behind a 590-nm long pass, a filter, and a 515- to 545-nm band pass. Both excitation and emission light were focused through a 40 $\times$ Plan-Neofluar oil immersion objective with a numerical aperture of 1.3 and an additional 30 $\times$ electronic zoom produced the definitive magnification. Total embryo height was evaluated, and the stage was moved at 1- μ m intervals. Optical sections were

acquired, each one with an image resolution of 512 $\times$ 512 picture elements (pixels), providing single planes or projections of multiple images. The images are single planes, but projections of multiple images ("splitscreen") can be obtained to visualize the whole sample in one image. Photographs were taken from digitalized images with a 100 ASA Ektachrome slide film (Kodak).

RESULTS

Application of Laser Scanning Imaging in the Observation of Human Embryos

Double FITC-NBD-phalloidin and propidium iodide staining allowed the coobservation and localization of F-actin microfilaments and chromosomes, respectively. The analysis of consecutive optical sections through embryos at 1- μ m intervals permitted an easy and rapid count of the number of nuclei in each blastomere. Indeed, the analysis of 25 or 16 sequential images allowed effective discrimination of multinucleated blastomeres (Figs. 1A–C). Interestingly, in some cases, binucleated blastomeres as verified by LSCM had been classified as "mononucleated" when using conventional microscopy. LSCM also provides useful stereoscopic representation of cells, which allows further analysis of the number of nuclei present in each blastomere of a whole embryo (Figs. 2A and C).

F-Actin Microfilament Labeling in Arrested or Highly Fragmented Embryos

In the majority of arrested embryos, a regular actin cortex of uniform thickness in each blastomere was observed. Similarly, cytoplasmic F-actin microfilaments were organized in a regular pattern (Fig. 3A). In grade IV, two- to four-cell-stage embryos, numerous cellular fragments were visible, of variable size (Fig. 3B). All of them were surrounded by a regular intact actin cortex and, occasionally, contained pieces of chromatin (Fig. 3C). In grade V totally degenerate embryos, probably undergoing "lysis," a complete disorganization of the cellular actin cortex was observed, with irregularities or complete disruption of the cellular membrane (Fig. 3D).

Multinucleated and Anucleated Blastomeres in Human Embryos

Table I reports the morphological characteristics of 50 arrested embryos. In grade III–IV embryos, the

Fig. 1. (A) Demonstration of the optical sectioning property of the confocal microscope. Section distance, 1000 nm. Staining by nitrobenzoxadiazole (NBD)-phalloidin and propidium iodide. Sixteen optical sections of an arrested three-cell embryo with two multinucleated blastomeres (arrowed). (B) The same embryo clearly shows one multinucleated blastomere. (C) The same embryo shows a single nucleus per blastomere. Original magnification, $\times 1200$.

rates of multiple abnormalities were very high and increased with the number of cells; abnormalities consisted mainly of multinucleated (MNBs) and anucleated blastomeres and nuclear fragmentation.

Embryos arrested early in their development (three or four cells at 72 hr or later) revealed few actin abnormalities. However, approximately 60% showed chromatin abnormalities such as a solitary binucleated blastomere. The nuclei in binucleated blastomeres were the same size as those observed in mononucleated cells from the same embryo. Five grade III-IV arrested embryos (10%) showed one blastomere with chromosomes spread out in prometaphase as shown in Fig. 3E.

Approximately 90% of arrested embryos blocked at a later stage (> 4 cells) contained at least one blastomere with two or more nuclei of equal size (Fig. 2A). Half of them exhibited anucleated blastomeres.

Most of the arrested embryos displayed nuclear abnormalities such as chromatin condensation and fragmentation.

Particular Aspects of Cryopreserved Embryos

Fifteen cryopreserved embryos that exhibited no intact blastomere after thawing were not transferred. They were placed in suitable culture medium and were then fixed and submitted to analysis. The actin cortex was disrupted and F-actin microfilaments had widespread ooplasmic dispersion. The blastomeres appeared to be separated and lacked connections. A total of 15 totally "lysed" embryos was analyzed by LSCM, and all exhibited mononucleated blastomeres; the nuclei did not exhibit any chromatin condensation or fragmentation (Fig. 4A).

Conversely, a total of 20 embryos that had been frozen 10 years ago and then donated for research by couples were thawed, removed from the cryoprotectant, placed in BM1 medium, and allowed to develop in culture for 1 day. Eight cleaved normally at the six- to eight-cell stages (Fig. 4B). The majority of these freeze-thawed embryos, which continued to divide

Fig. 2. (A) A grade II eight-cell arrested embryo with two multinucleated blastomeres. (B) Stereoscopic representation of the four nuclei in the same blastomere of this eight-cell arrested embryo. The distribution of chromatin in this cell has been visualized by means of the propidium iodide. This spatial distribution helps to quantify accurately the number of nuclei per cell. (C) LSCM provides useful stereoscopic presentation of the cells, allowing further analysis of the number of nuclei present in each blastomere.

after thawing, exhibited a thin regular actin cortex identical to "fresh," i.e., non cryopreserved, grade I or II arrested embryos, without cytoplasmic fragments, but with a distribution of ooplasmic actin similar as in "totally lysed" thawed embryos. Each blastomere contained a single nucleus and neither chromatin condensation nor fragmentation could be observed (Fig. 4C).

DISCUSSION

The current study presents some of the applications of LSCM in human embryo research. Our observations

showed a high rate of multinucleation in arrested or poor-quality embryos, and this was especially high in grade IV embryos (89%). Since 1982, multinucleation has been well described by numerous authors, with an incidence that varies from 17 to 69% (4,12-24) (Table II). Transmission electron microscopy (TEM), inverted phase-contrast microscopy, and epifluorescence microscopy were commonly used for the visualization of multinucleated blastomeres. The preparation of fixation and serialized sections, which is necessary before TEM analysis, is delicate and time-consuming. Disaggregated blastomeres were labeled with fluorochromes and then observed with an epifluorescence microscope

Fig. 3. (A) Four optical sections of a grade II four-cell arrested embryo with regular actin cortex and one nucleus per blastomere. Phase-contrast (B) and confocal (C) image of a grade IV fragmented embryo. (D) A single section of a four-cell embryo undergoing lysis or already lysed. (E) A fragmented grade III three-cell arrested embryo with one nuclear fragmentation and one multinucleated blastomere. The chromosomes appear to be in prometaphase.

Table I. The Number and Percentage of Embryos Exhibiting Blastomeres with Multiple Nuclei (MNBs), Fragmented Nuclei, or No Nucleus in Normally Fertilized but Arrested Human Preimplantation Embryos After Confocal Laser Scanning Microscopy Observation^a

Morphology	Stage (cell)	Number of embryos (n)	MNBs ^b	Anucleated fragments	Fragmented nuclei	"Normal embryos" ^c
Grades I-II	2-4	6	3	0	2	2
	>4	2	1	0	0	1
Grades III-IV	2-4	20	12 (60%)	2 (10%)	7 (35%)	2 (10%)
	>4	22	18 (89%)	12 (54%)	9 (41%)	3 (17%)
Total		50	34 (68%)	14 (28%)	18 (36%)	8 (16%)

^a A single embryo can exhibit multiple abnormalities.^b Multinucleated blastomeres.^c Embryos without MNBs, anucleated cytoplasmic fragments, or fragmented nuclei.

by Hardy *et al.* (4) and Pickering *et al.* (20). The embryo, being one of the largest and most voluminous mammalian cells, needs special treatment for fixation, staining, and microscopy to provide maximum optical clarity. The present study used confocal microscopy for the observation of nuclei within the whole intact embryo. This observation requires a simple preparation step consisting of fixation with glutaraldehyde. Confocal microscopy allowed us to observe high rates of MNBs, which could possibly be missed using conventional epifluorescence. Rather than physically cutting sections of multicellular embryos, the LSCM measures the exact thickness of samples and produces fast automated "optical sections" that are thin enough to resolve structures of interest. The analysis of such automated optical sections through embryos at 1- μ m intervals combined with stereoscopic representation improved the observation of the samples and made the detection of any abnormal cells easy (25). On the other hand, a trained embryologist can detect multiple nuclei in some embryonic blastomeres with classical fluorescence microscopy or high-resolution light microscopy, but this requires a very careful and time-consuming examination by varying the focus and can lead to an under-evaluation of some abnormally nucleated cells.

Multinucleation in human embryos is considered to be a pathologic event, probably associated with chromosomal abnormalities. A recent analysis of chromosomes X, Y, 18, and 13/21 indicated that MNBs are generally abnormal, as well as some of their mononucleated siblings (26). Defective embryos may certainly be abnormal as far as other chromosomes are concerned.

Embryos containing MNBs are generally considered to be inappropriate for producing viable fetuses, leading to developmental arrest in the case of transfer. In

contrast to this general assumption, Balakier and Cadesky (23) clearly demonstrated that although defective embryos contribute to pregnancy losses, in rare cases they may retain full developmental capability and lead to healthy babies. The interest of high-power inverted microscopy in identifying MNBs in living embryos and thus discarding them for replacement is emphasized by these authors, who observed a relative high rate of blastomere multinucleation within two- and four-cell-stage good-quality embryos (15%) developing after monospermic fertilization. But they admitted that nuclei could remain undetected in the case of mitosis (nuclei not seen) or when there were difficulties in their visualization (granular cytoplasm, cytoplasmic fragments, overlapping blastomeres). Confocal microscopy can easily resolve most of the latter difficulties and allow the visualization of metaphases, although this method is an invasive method, more relevant to analysis of fluorescently labeled (and fixed) specimens, and is subsequently of great interest in research but of no value in embryo selection on the day of the transfer.

However, the experience of Mottla *et al.*, who injected a biocompatible fluorescent conjugated dextran (Texas Red-lysine-dextran) into individual blastomeres, goes some way in modifying this point of view (27). Their experiment demonstrated both the successful use of lineage tracers in human normally developing preembryos and the application of scanning confocal microscopy in early human embryologic investigations, showing that individual early blastomeres are not already committed to the formation of either trophectoderm or inner cell mass but can instead form both rudiments.

The problem of the "control," i.e., nonarrested, group is difficult to resolve. Because of strict national

Fig. 4. Cryopreserved embryos. (A) A freeze-thawed three-cell embryo with no intact blastomere. A single optical section obtained from the same lysed embryo, displaying no intact blastomere. The actin cortex has disappeared and actin microfilaments have widespread ooplasmic dispersion. Blastomeres appear to be separated, without connections. Therefore, each cell clearly exhibits one single nucleus, without chromatin condensation or fragmentation. (B) A three-cell embryo which had been frozen 10 years ago and donated for research. After thawing, the embryo was allowed to develop in culture for 1 day and normally cleaved at six-cell stage. (B) The six-cell embryo is surrounded with a thin regular actin cortex, without cytoplasmic fragments. (C) Optical sections of the embryo showed that each blastomere contained a single nucleus. Neither chromatin condensation nor fragmentation could be observed.

ethical guidelines, we cannot use normally developing "fresh" embryos, which, of course, would represent the ideal "controls." Therefore, we used both freeze-thawed embryos displaying no intact blastomere, and consequently inappropriate for transfer to the patient's uterus, and fresh embryos, which had been cryopreserved between 1987 and 1989 and given later to research by couples who no longer had any parental plans. The latter embryos were initially developing and of good quality, since poor-quality ones were sys-

tematically discarded from cryopreservation. Ten of these embryos were thawed and allowed to develop in vitro for 24 hr, and 80% of them cleaved at an expected rate. So we can assume that the development potential of these cryopreserved embryos was correct for the majority of them.

We showed that cryopreservation of embryos could in some cases adversely affect the cellular actin cortex and cytoplasmic network. The actin cortex was totally disrupted and F-actin microfilaments showed wide-

Table II. Summary of Some Studies Related to the Observation of Multinucleation in Human Embryos Obtained After In Vitro Fertilization*

	Materials	Methods	Observations
Sathananthan <i>et al.</i> (12)	8 human cleaving embryos	Fixation, serial sectioning, and TEM	Cytoplasmic anucleated fragments and MNBs in 2 embryos
Van Blerkom <i>et al.</i> (14)	2 preimplantation human embryos from polynuclear eggs	DNA labeling with DAPI (epifluorescent microscope) serial sectioning, and TEM	25% of blastomeres were MNBs
Troumson and Sathananthan (15)	8 normal and 10 abnormal (2- to 4-cell embryos)	Fixation, serial sectioning, and TEM and phase-contrast microscopy	Abnormal embryos Cytoplasmic and nuclear fragmentation MNBs (65%)
Tesarik <i>et al.</i> (17)	2 pronuclei preembryos from 2-cell to the morula stage	DNA labeling with ³ H-thymidine and ³ H-uridine (TEM), light and electron-microscope autoradiography	70% of preembryos with inappropriate numbers of nuclei
Winston <i>et al.</i> (18)	30 and 42 human cleaving preembryos at days 2 and 3 and 159 (5 days) cavitated preembryos (normally fertilized embryos)	Fluorescent DNA chromophore, DAPI, and fixation, section, and light microscopy	DAPI assessment was lower than serial sectioning Blastocyst (day 5): 6 to 45% of MNBs Day 3: 60% with polynucleate or anucleate cells Day 2: 60% with polynucleate or anucleate cells
Balakier and Casper (19)	26 polyspermic human embryos (abnormally fertilized embryos)	Inverted phase-contrast microscopy	Multinucleated blastomeres
Hardy <i>et al.</i> (4)	Disaggregated blastomeres from 200 preimplantation human embryos (abnormally and normally fertilized embryos)	Vital labeling with a DNA-specific fluorochrome (Hoechst 33342), fluorescence microscopy (epi-)	A single nucleus in the majority of blastomeres Binucleate blastomeres of equal size 2-4 cells (17%), >at least a single binucleate blastomere 9-16 cells (65%), >1 to 6 binucleate blastomeres Fragmented nuclei or anucleated blastomeres
Pickering <i>et al.</i> (20)	Disaggregated blastomeres	Fixation and DNA labeling with DAPI, quantitation of DNA by image analysis (Seescan)	Number of embryos with at least one MNB Day 2: 22-35% of embryos Day 3: 39-71% of embryos Nuclear morphology (binocular microscope) Day 2: 9-24% of MNBs and 8-28% with no nucleus visible Day 3: 10-23% MNBs and 6-24% with no nucleus visible
Wojcik <i>et al.</i> (21)	40 abnormal embryos (grade IV diploid or polyploid embryos)	Inverted phase-contrast microscopy, Tarkowski protocol	14 multipronucleate embryos Trippronucleate embryos: 43% MNB and 1 fragmentation From multipronuclei zygotes: 73% MNB and two fragmentations 26 abnormal diploid embryos: 60% MNBs of different sizes
Hardy <i>et al.</i> (22)	74 embryos (12 abnormally & 62 normally fertilized embryos)	Immunocytochemical techniques coupled with CLSM	Nuclear abnormalities is normally fertilized embryos of good morphology: binucleate cells and MNBs, anucleate cells

Table II. Continued

	Materials	Methods	Observations
Balakier and Cadesky (23)	1885 2- to 4-cell human embryos	Inverted microscopy at $\times 320$ –400 magnification	44% of patients with embryos exhibiting MNBs, Good-quality embryos 2-cell embryos: 67% with MNBs 4-cell embryos: 25% with MNBs Defective embryos 2-cell embryos: 54% with 1 MNB, 46% with both MNBs 3-cell embryos: 48% with 1 MNB, 52% with 2 or 3 MNBs 4-cell embryos: 46% with 1 MNB, 46% with 2 MNBs, 8% with 3 or 4 MNBs
Current study	50 human embryos (grades III–IV, normally fertilized)	Immunocytochemical techniques coupled with CLSM	Number of embryos with at least one MNB: 60–89%. Number of embryos with no nucleus visible: 10–54%

^a JEM, transmission electron microscopy; CLSM, confocal laser scanning microscopy; MNBs, multinucleated blastomeres.

spread ooplasmic dispersion. The blastomeres appeared to be separated and lacked connections. This process apparently did not affect the nucleus structure, as the same aspects could be observed when the embryo exhibited intact or “lysed” blastomeres after thawing. The cytoskeleton, essentially made of F-actin microfilaments (and tubulin; not shown here), can be drastically disturbed by the freeze–thawing process, but with major variations seen within a cohort of embryos. This differential temperature sensitivity appeared to be limited to the cytoskeleton.

In 1990, Mandelbaum (28) showed that only 35 to 45% of cryopreserved human multicellular embryos were found to be fully intact after thawing. Recently, Kondo *et al.* clearly demonstrated that embryo quality was a major clinical factor for successful implantation of cryopreserved–thawed embryo transfer (29). This was confirmed by Van den Abbeel *et al.*, who found a birth rate per embryo transferred that was three times higher after transfer of fully intact embryos compared with transfer of partially damaged embryos (30).

The main problem consists of the loss of some, or even all, cryopreserved embryos after thawing, which results in an overall decline in the number of embryos transferred and in the embryo morphological grading after the freeze–thaw procedure. Further confocal microscopic observations of thawed embryos could help to specify the detrimental effects of cryopreservation, visualize the structures of the blastomere which are the targets of this process, and better define the “survival” of thawed embryos. LSCM could be a useful

tool to identify the main factors influencing the result of cryopreservation procedure or to determine the ideal developmental stage for embryo freezing.

In conclusion, the applications of laser scanning confocal microscopy for the observation of fluorescently labeled fixed human embryos are numerous. Multiple labeling is often necessary to map the distribution of more than one probe simultaneously in the same sample. This possibility provides accurate and valuable information for the elucidation of the developmental process such as in developmental failure or mechanisms involved in the freeze–thawing procedure. Furthermore, recent and ongoing advances in confocal technologies that allow the confocal imaging of living embryos will provide valuable help in embryo selection on the day of the transfer.

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