Noncontact, Laser-Mediated Extraction of Polar Bodies for Prefertilization Genetic Diagnosis

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Purpose: We tested an entirely noncontact polar body-extraction method using an ultraviolet laser beam for laser zona drilling and a near infrared laser beam for polar body (PB) trapping and extraction.

Methods: A hole was drilled into the zona pellucida of an oocyte. Then, the PB was trapped with optical tweezers and dragged through the drilled hole.

Results: Bovine first PBs could be extracted in 49 out of 63 oocytes (78%) using this method. In human oocytes, PB extraction was successfully demonstrated, which however was more time consuming. A number of extracted PBs were dried on a special membrane, circumcised with the laser microbeam, and successfully catapulted into the lid of a microfuge tube (laser pressure catapulting).

Conclusions: This solely laser-mediated extraction method allows convenient procurement of PBs without the danger of contamination and is a promising approach that might replace standard micromanipulation methods in the future.

KEY WORDS: Laser microdissection; laser pressure catapulting; laser trap; optical tweezers; polar body.

INTRODUCTION

Genetic analysis of polar bodies (PBs) (prefertilization diagnosis) offers the opportunity to detect certain genetic or chromosomal disorders (aberrations, mutations) in oocytes before fertilization is completed. This prefertilization diagnosis is a unique possibility to exclude genetically altered oocytes before fusion of the male and female pronucleus and initiation of embryo development. This is especially important in countries, in which preimplantation genetic diagnosis

by embryo biopsy is not allowed by law, or for patients, who cannot accept, from their ethical point of view, that embryos would be discarded in case of a genetic alteration.

Extraction of PBs is most commonly performed by mechanical manipulation using glass pipettes with or without prior chemical treatment of the zona pellucida. All manipulation methods have to ensure unimpaired oocytes to allow ongoing development and intact PBs for unambiguous genetic analysis.

The first method published for extraction of PBs in prefertilization diagnosis was using a thin (15–18 μm), beveled (45°) sharp pipette, which was pushed through the zona pellucida and moved towards the PB using a mechanical micromanipulation setup (1). The PB was then aspirated into the pipette. To decrease the risk of oocyte or PB damage, acidic Tyrode's solution (pH 2.35) was used for partial zona dissection prior to PB extraction by a sharp pipette (2).

These mechanical and chemical procedures require extensive practice as well as time-consuming pipette

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preparation. For each PB a separate glass pipette is required to avoid contamination with DNA of other origin, which may stick to the glass surface. Furthermore, the tip of the manipulation pipette may easily contaminate or damage the PB or oocyte, despite using sucrose in some cases as a precaution to shrink the oocyte and to enlarge the perivitelline space.

Cutting Lasers

Different laser systems have been developed to facilitate the opening of the zona pellucida of oocytes or embryos. Laser zona drilling with a 193 nm excimer laser was described (3), while others (4,5) used an 2900 nm infrared Erb:Yag (erbium–yttrium–aluminum–garnet) laser. The disadvantage of all those is that none of the applied laser wavelengths can be guided through glass and thus cannot be coupled into conventional microscopes. For that reason, special capillaries for light transportation are required. Furthermore, holding pipettes have to be used to keep the oocytes in position during laser application. The excimer laser wavelength is located in the UV-c range and the Erb:YAG laser within the far infrared. Both laser wavelengths are highly absorbed in biological specimen and DNA fractionating or overheating might occur, which could negatively influence further development. A survey of different laser types used for zona drilling is given in Table I, indicating their features and side effects.

Noncontact laser zona drilling was first demonstrated using a tunable laser (6). This laser wavelength could be guided through a routine research microscope and focussed through the objective. Laser cutting occurred within the narrow laser focus spot only, but the expanded laser was transmitted through biological specimen without causing damage. Thus, this laser method allowed tangential application of the laser beam with respect to the manipulated specimen. Therefore no pressure force pushed towards the oocyte. No holding pipettes were required to keep the oocyte in position and, since the laser diameter was controlled by the quality of the laser and the focussing objective, cutting was precisely possible yielding cuts as small as 1 μm . However, this laser wavelength is still located within the UV-b range, and problems in subsequent embryo development have been reported (7). A further laser system was introduced in 1996 (8). A continuous wave 1.48 μm diode laser was applied for zona drilling in human and mouse oocytes and zygotes. Using this wavelength, water absorption still takes place and subsequent overheating is

critical, which in some cases caused swelling and plebbling of the cells. This laser system cannot be focussed to less than 3–4 μm and therefore seems to be restricted to zona drilling purposes. The use of this continuous wave 1.48 μm diode for laser zona drilling was combined with the use of a blunt-ended extraction pipette for facilitation of PB extraction in mouse oocytes (9) and blastomere extraction in human embryos (10).

In 1994, we described the successful application of a noncontact 337 nm nitrogen laser for laser zona microdissection, sperm tail cutting, and blastomere fusion (11). This laser is of high beam quality and can be focused to less than 1 μm in diameter. As its wavelength is located within the UV-a range, no impact to biological specimen has been found in previous numerous cell biological experiments (12,13). UV-a laser zona drilling was successfully demonstrated in mice and reported to be safe for assisted embryo hatching (14). Already several babies have been born following nitrogen laser-assisted embryo hatching (15). We demonstrated UV-a laser zona drilling and subsequent PB extraction by blunt-ended pipettes in bovine zygotes without negative effects on further cleavage rates and embryo development and with a decrease in oocyte degeneration rates, if compared to PB extraction by a sharp-ended pipette only (16,17).

Laser Pressure Catapulting (LPC)

An important invention within the laser micro-manipulation procedures was the noncontact sample capture using the pressure force of the cutting laser. So far, no method existed which allowed capture of material from the object plane without using any microtool or mechanical attachment. The pressure force, which develops underneath the sample on the slide, is used to eject a selected single cell chromosome or a small cell cluster and to catapult it directly into a routine microfuge tube (18,19). This noncontact LPC has been successfully demonstrated in numerous different publications within molecular medicine as well as in cell and developmental biology.

Trapping Lasers

Optical tweezers have been used for cell sorting, segregation of bacteria, or force measurements *in vitro* as well as *in vivo* (20,21). The force within the laser focus is strong enough to even hold vigorously

Table I. Comparison of Different Laser Types for Cutting the Zona Pellucida of Oocytes

Laser type	Wavelength (nm)	Spectral range ^a	Features	Side effects
Excimer	193	UV-c	Cutting size 3–5 μm Absorbed by glass: laser transmission through glass capillaries necessary Energy transfer vertical to oocyte surface; pushing force holding pipette required to fix oocyte in position (contact mode) Cutting size 1 μm	Critical: impact on oocyte development because of high absorption in biological material Laser application difficult to control: eventually detrimental to oocytes
Excimer	308	UV-b	Laser transmission through glass (microscope lenses) is possible: laser focusing through objectives (noncontact mode) Energy transfer tangential to oocyte surface: no pushing force No holding pipette required (noncontact mode)	Critical: still some DNA and protein absorption Impact on embryo development was reported (7)
Erb:Yag	2900	Far infrared	Cutting size 20 μm Absorbed by glass: laser transmission through glass capillaries necessary Energy transfer vertical to oocyte surface; pushing force Holding pipette required to fix oocyte in position (contact mode)	Critical: heating effect because of water absorption may impact oocyte development Laser application difficult to control: eventually detrimental to oocytes
Diode	1480	Near infrared	Cutting size 3–4 μm Laser transmission through glass (microscope lenses) is possible: laser focusing through objectives (noncontact mode) Energy transfer tangential to oocyte surface: no pushing force No holding pipette required (noncontact mode)	Critical: heating effect because of water absorption may impact oocyte development (plebbling of diode laser treated oocytes has been observed—personal communication) No absorption in biological material (sufficient far away from DNA or protein absorption)
Nitrogen	337	UV-a	Cutting size: less than 1 μm Laser transmission through glass (microscope lenses) is possible: laser focusing through objectives (noncontact mode) Energy transfer tangential to oocyte surface: no pushing force No holding pipette required (noncontact mode)	No heating effect (“cold ablation”) No absorption in biological material (sufficiently far away from DNA or protein absorption) Cutting size and laser application is easy to control: no detrimental effects on oocyte

^a UV-c: 200–290 nm: DNA mutations, protein, and DNA absorption; UV-b: 290–320 nm: impact on embryo development; and UV-a: 320–400 nm: no negative side effect.

moving sperm and thus allows force measurement of sperm motion (22,23). Furthermore, it allows introduction of sperm through a previously laser-drilled hole into the perivitelline space for entirely laser-mediated fertilization (24).

The effect of laser traps on specimens is dependent on their wavelength, showing maximum photodamage in the range between 870 and 930 nm (25). A laser trap with 1064 nm did not show any negative impact on sperm motion in our own experiments or in bacterial cloning procedures (21).

Combining the cutting ability of the laser microbeam, the dragging force of optical tweezers and the pressure force of the microbeam for catapulting, entirely laser-mediated PB extraction might be possible in future. This could help to minimize the danger of contamination with nonselected material, to facilitate the tedious method, and to speed up the time-consuming procedure of PB extraction.

MATERIALS AND METHODS

Bovine Oocytes

Bovine oocytes were collected from fresh ovaries (slaughterhouse material). Follicles with a diameter between 2 and 8 mm were punctured by a needle connected to a suction pump (Labotect, Göttingen, Germany), with a maximum negative pressure of 80 mm Hg. Cumulus/oocyte-complexes were selected, washed, and collected in Ham's F 10 medium + 10% fetal calf serum + Gentamycin (ICN Biomedicals, Aurora, OH). In vitro maturation of the selected oocytes was performed in 400 μ L drops (30 oocytes per drop) of modified Parker's medium (MPM) + 10% fetal calf serum + 0.01 U/mL FSH + 0.01 U/mL LH (SIOUX, Iowa, USA) within the next 20–22 h. For selection of matured oocytes, cumulus masses were removed by exposure to Trypsin/EDTA (1%/0.8% in phosphate buffered saline) and gentle pipetting. Oocytes showing an extruded PB were classified as matured and selected for the laser experiments.

To obtain oocytes with two extruded PBs, in vitro fertilization was induced in matured oocytes by adding 1 Mio/mL of frozen-thawed and capacitated sperm, which had undergone a swim up procedure for 1 h (30 cumulus/oocyte-complexes in 500 μ L drops). Oocytes and sperms were subsequently cultured in modified Parker's medium + 10% fetal calf serum for the next 20 h. After removal of the granulosa cells

in Trypsin/EDTA, oocytes showing two extruded PBs were selected for laser experiments.

Human Oocytes

For the experiments, discarded human oocytes from the routine IVF-program were used, which did not show signs of fertilization within 24 h after IVF or intracytoplasmic sperm injection (no pronuclei, no second PB). Furthermore, one oocyte was used which had extruded a second PB, but did not show pronuclei at any time.

Patients were informed and gave their written consent as a prerequisite for the use of their oocytes. The study was proofed and accepted by the Ethic Committee of the Ludwig-Maximilians-University (No. 182/98).

Laser Equipment

Experiments were performed with a P.A.L.M.[®] Robot-CombiSystem (P.A.L.M. Microlaser Technologies AG, Bernried, Germany), consisting of a pulsed nitrogen laser (337 nm, UV-a) and a continuous-wave, diode-pumped Nd:YAG (Neodymium: yttrium-aluminum-garnet) laser (1064 nm, infrared, max. 2 W). The UV-a laser was used for cutting the zona pellucida (laser zona microdissection) and for LPC. Catching and moving of PB was performed with the infrared laser (laser tweezers) (Fig. 1).

Both lasers were coupled into an inverted microscope and their focus spot (less than 1 μ m) was adjusted to the optical focus of the microscope. For all experiments, an oil objective of high numerical aperture (100 $\times$) was used, resulting in a 1000-fold magnification. Laser manipulation was performed keeping the lasers in a fixed position, but moving the specimen into the laser beams via a motorized stage. Control of the laser manipulation was performed with a computer mouse and could be observed on a monitor.

For laser manipulation, one oocyte was set into a 50 μ L drop of Hepes-buffered medium (Ham's F 10 + 10% fetal calf serum (bovine) or 10% human serum albumin (human)) onto a glass slide (thickness 0.17 mm). After manipulation, the oocyte was immediately transferred back into culture medium. Manipulation time was recorded from onset of laser zona drilling until the extraction of first or second PB was completed.

For LPC, one extracted PB was transferred in a small amount (0.5 μ L) of highly purified water onto a polyethylene naphthalene (PEN-) membrane, which

Fig. 1. Scheme of the noncontact, laser-mediated extraction of a polar body; (a)–(c): Laser zona drilling; (d)–(f): Polar body extraction. (a) Oocyte before manipulation, polar body in the 3 o'clock position. (b) the UV-a laser beam drills a small channel into the zona pellucida. Therefore, the oocyte is moved backwards and forwards into the laser beam. (c) Completed laser zona drilling. The hole is slightly bigger than the polar body. (d) The laser tweezers (infrared light) are switched on. The polar body is trapped by the laser light. (e) The laser tweezers are moved, and the polar body is dragged through the previously drilled hole. (f) After complete extraction of the polar body, the laser tweezers are switched off, and the extracted polar body sinks onto the slide.

had been mounted on a glass slide (0.17 mm thickness). After drying, the fixed PB was prepared for LPC: The UV-a laser beam was used to cut the membrane around the fixed PB. For targeted catapulting, a small membrane connection was left between the PB-containing membrane island and its surroundings. For PB collection, the lid's inner surface of a 0.5 mL polymerase chain reaction-tube (PCR-tube, flat lid with clear surface, rim 2 mm) was spread with 1 μ L of mineral oil. The lid was placed upside down over the microdissected area in the PEN-membrane with a microscope-mounted cap holder. It was lowered until it nearly touched the membrane. With one single laser shot at the site of the membrane connection, the entire PB-containing membrane island was catapulted into the lid of the tube.

RESULTS

Bovine and human PBs could be extracted contact-free solely using laser-mediated manipulation. An opening was drilled into the zona pellucida adjacent to the PB by a series of UV-laser pulses (frequency 30 pulses per second). The opening was made slightly bigger than the PB (Fig. 2) by moving the oocyte backwards and forwards into the laser beam and thus drilling small, 1 μ m thin paths through the zona pellucida.

Then, the focus of the laser tweezers was oriented to the center of the PB, and the optical trap was switched on. This induced attraction visualized by a fine movement of the PB, which indicated that the PB was not sticking to the plasma membrane of the oocyte. By

Fig. 2. Laser zona drilling in a bovine oocyte with an UV-a laser beam. (a) Oocyte before manipulation. (b) A small channel is visible, drilled by moving the oocyte backwards and forwards into the laser beam. (c) After laser zona drilling; the size of the hole is slightly bigger than the polar body to avoid sticking while subsequent polar body extraction. (d) Intact oocyte after laser zona drilling.

movement of the microscope stage (computer mouse-controlled), the laser trap could drag the trapped PB through the laser-drilled hole out of the perivitelline space into the surrounding medium (Fig. 3).

After its complete extraction, the laser trap was switched off, and the extracted PB sank onto the slide. If the PB was sticking to the membrane of the oocyte or to the zona pellucida, in most cases it could be released by a few, carefully located laser shots at the contact area. In case of a fertilized oocyte, the second PB was subsequently trapped and extracted through the same laser-drilled hole.

Bovine Polar Bodies and Oocytes

In total, 49 first PBs could be extracted out of 63 bovine oocytes (78%) using laser light-mediated manipulation (Table II). In the group of the

49 oocytes, in which the PB could be extracted, 41 oocytes (84%) had remained in a morphological intact shape. In 14 oocytes (22%) the PB could not be extracted because of tight sticking to the plasma membrane or to degeneration of the oocyte or PB. Five minutes after manipulation, 58 of the 63 oocytes (92%) remained morphologically intact, and 5 (8%) showed signs of degeneration (loss of the plasma membrane structure, disappearance of the perivitelline space). When observing the oocytes 30 min after manipulation, 43 of the 63 manipulated oocytes (68%) appeared morphologically intact, and 20 oocytes (32%) showed signs of degeneration. Thirty-eight of the extracted 49 PBs (78%) remained in their shape after manipulation, whereas 11 PBs (22%) showed signs of shrinkage. This shrinkage occurred when UV-a laser pulses were triggered to microdissect sticking areas between the plasma

Fig. 3. Laser-mediated extraction of the first polar body in a bovine oocyte. (a) After switching the laser trap on, the polar body is dragged through the laser-drilled hole out of the zona pellucida. (b) The polar body is almost dragged out of the zona pellucida.

membrane of the oocyte and the PB. If one pulse hit the PB membrane, the PB slightly shrunk, but no loss of material could be observed. This was different to the oocytes, in which hitting of the membrane induced leakage and subsequent degeneration. The use of the laser trap alone never induced shrinkage of any PB, even if it had been trapped for several minutes. The average duration for extraction of the first PB was 5 min 35 s per oocyte (173 min 15 s for 31 time-registered oocytes). In 10 fertilized, bovine oocytes we tried to extract both, the first and second PB by use of laser light (Fig. 4).

All 10 first PBs could be extracted. Extraction of the second PB was successful in eight oocytes, whereas in two oocytes the second PB degenerated during laser manipulation (Table II) and thus could not be trapped by optical tweezers.

All oocytes and extracted first PBs remained intact during and after manipulation. On average, manipulation procedure for both PBs was completed within 5 min 36 s per oocyte (time was registered for 5 oocytes: total time 28 min). If the PBs were sticking tightly, duration was prolonged up to 10 min, if they were loose, manipulation could be completed within 3 min.

Human Polar Bodies and Oocytes

In human oocytes, experiments were performed in 15 oocytes, 14 of them showing one PB and 1 showing two PBs. Laser-mediated extraction of the first PB was successful in five oocytes (Table II).

In 10 oocytes, the PB could not be extracted because of its intensive sticking to the plasma membrane. Three of the oocytes degenerated within 5 min after laser manipulation. Thirty minutes after manipulation 10 oocytes were classified as morphologically intact and five as degenerated. Of the five successfully PB-extracted oocytes, four were morphologically intact when checked 30 min after manipulation, and one oocyte was degenerated. Three of the five extracted PBs remained in their shape, whereas two shrunk as described in bovine PBs. In the oocyte with two PBs, both PBs could be successfully extracted and remained morphologically intact like the oocyte. In average, manipulation time was 6 min 38 s (93 min for 14 time-registered oocytes) for extraction of the first PBs. LPC was performed in six extracted first PB. With one single shot, the circumcised PB on the PEN-membrane fragment was successfully catapulted into the lid of the tube (Fig. 5).

The presence of the catapulted, PB-containing membrane island sticking on the surface of the lid could be confirmed in all cases by reidentification under the microscope.

DISCUSSION AND CONCLUSIONS

The experiments were performed to examine an innovative laser-based PB extraction method as an alternative to conventional PB extraction methods using glass micropipettes. Micropipette-based PB extraction is time consuming and expensive, as glass pipettes have to be self-produced or bought, and bear the risk of oocyte or PB damage or contamination by direct contact of the pipettes with the oocyte and PB. If subsequent analysis of the extracted PB include polymerase chain reaction (PCR) for amplification

Table II. Results of Laser-Mediated Extraction (LEX) of Polar Bodies

LEX	No. of oocytes	LEX		Status oocyte 5 min after manipulation		Status oocyte 30 min after manipulation		Status PB		Average manipulation time per oocyte
		Successful	Failed	Intact	Degen.	Intact	Degen.	Intact	Degen.	
Bovine										
I. PB	63	49	14	58	5	43	20	38	25	5 min 35 s ^a
Total (%)		78	22	92	8	68	32	60	40	
LEX ^b (%)						84	16	78	22	
I. and II. PB	10	8	2	10	0	10	0	10/8 ^c	0/2 ^c	5 min 36 s ^d
Total (%)		80	20	10	0	100	0	100/80 ^c	0/20 ^c	
LEX ^b (%)						100	0	100/100 ^c	0/0 ^c	
Human										
I. PB	15 ^e	5	10	12	3	10	5	6	9	6 min 38 s ^f
Total (%)		33	67	80	20	67	33	40	60	
LEX ^b (%)						80	20	60	40	

Note. Degen. – degenerated, PB – polar body.

^a Total 173 min 15 s for 31 time-registered oocytes (range: 4–15 min per oocyte).

^b % referred to all successfully laser-extracted oocytes.

^c I. PB/II. PB.

^d Total 28 min for 5 time-registered oocytes (range: 3–10 min per oocyte).

^e One oocyte showed two PB, which were successfully extracted.

^f Total 93 min for 14 time-registered oocytes (range: 2–10 min per oocyte).

of DNA (single-cell PCR), one pipette per extracted PB is requested to decrease the risk of contamination and to ensure that the PB is safely deposited in the PCR tube. The tip of the pipette containing the extracted PB is usually broken into the PCR tube and remains there during PCR cycles. Taking into account that numerous PB extraction pipettes are required per patient, self-making of pipettes has to be considered to decrease the costs for PB analysis.

In our laboratory, the average time for the production of one sharp, beveled extraction pipette with additional peak of good quality is about 6–7 min. To identify genetically intact oocytes, a larger number of oocytes per patient are required for prefertilization diagnosis. Therefore, pipette production requires at least 1 h or more per patient if sharp-ended, beveled pipettes with a tip are used. Alternatively, a blunt-ended and fire-polished pipette, used after zona opening (by drilling or slitting), may be produced in a time range of about 2 min. Summarizing the time for the actual micromanipulation procedure, which takes about 6 min per PB for mechanical extraction versus 4 min for the combined laser/blunt-pipette PB extraction, gives all together 12 min per oocyte for the mechanical versus 6 min per oocyte for the combined laser technique (Table III).

The entirely laser light-mediated extraction method, if compared to recently published PB extraction methods, which use a blunt-ended pipette after zona slitting (26) or laser zona drilling (17),

shows no decrease in manipulation time or is even more time consuming (Table III). This seems to be due to the low-laser power and should be accelerated using a stronger laser trap. Manipulation time might be decreased if a stronger laser trap is used, as it will attract the PB more tightly and thus will enable its extraction in a shorter time period. Furthermore, this would reduce harmful exposure time of the oocyte outside the incubator. Further experiments will be performed, which will also test, whether higher laser power has any influence (i.e. overheating) on the oocytes or polar bodies.

It has to be pointed out that laser micromanipulation does not require intensive experience as compared to pipette-performed PB extraction. Once focussed correctly, the laser unit only needs to be switched on and off and delivers reproducible results. As the procedure is controlled by a computer mouse and observed on a monitor, intensive experience in micromanipulation is not required.

One interesting point to focus on in the future are the different sizes of the holes created in the zona pellucida, dependent on the method of PB extraction, and their influence on further embryo development and hatching. If extracted with a sharp-ended pipette, the hole is tiny and almost not to identify after withdrawal of the pipette. Combining zona drilling (either enzymatically or by laser) and extraction by a glass pipette, the size of the hole is drilled slightly smaller than a PB to enable easy pipette penetration.

Fig. 4. Laser-mediated extraction of the first and second polar body in a bovine oocyte. (a), (b) Before manipulation; note both polar bodies close to each other. (c) After laser zona drilling. (d) The first polar body is trapped and dragged through the hole in the zona pellucida; the second polar body is out of focus. (e) Oocyte after extraction of both polar bodies. (f) Both extracted polar bodies after manipulation on the bottom of the slide.

Compared to this, the laser-drilled hole for extraction by a laser trap has to be bigger than a PB, to avoid contact of the PB with the zona pellucida during the extraction procedure.

In the literature, only little information is given about the efficiency of PB extraction experiments referring to the degeneration rate of oocytes and PBs. The published data only refer to the percentage of

Fig. 5. Laser pressure catapulting of a human first polar body. (a) Polar body (arrows) swimming in a small amount of water. (b) After drying, the polar body can still be identified (arrows). (c), (d) Laser microdissection of the PEN-membrane around the polar body. (e) A small PEN-membrane bridge is left over to avoid accidental early catapulting. (f) After laser pressure catapulting: the edges of the leftover PEN-membrane are clearly visible.

fluorescence in situ hybridization (FISH) results in PBs in respect to the overall number of manipulated oocytes. In the last 5 years, this overall efficiency in FISH could be steadily increased from 68% (27) to 77% (28), and up to 82% (29). In PCR-related analysis the overall efficiency is lower (60% (30)). As no numbers of the manipulation process per se are given, a comparison with the efficiency of the laser-mediated extraction results therefore seems to be impossible. However, survival rates of oocytes and PBs are demanded to be able to compare different manipulation methods.

In 1998, the combined use of a 1.48 μm diode laser for zona drilling and PB extraction by a blunt-ended pipette was described (9). The results, although no numbers are mentioned, showed that neither the oocytes nor the PBs were damaged using this procedure. If compared to PB extraction by a sharp-ended pipette, we observed a reduced degeneration risk in bovine oocytes, using an UV-a microbeam for laser zona drilling (17). In comparison, degeneration rates for oocytes and PBs are still too high if the laser-mediated extraction method is used. Optimized laser trap parameters and decrease of exposure time

Table III. Time Comparison (Min) for Different Procedures of Polar Body Extraction (Own Laboratory Experience)

Steps	Solely manual by sharp-ended pipette		Laser zona drilling and manual by blunt-ended pipette		Solely laser-mediated Not required
Pipettes	Commercial	Prepared	Commercial	Prepared	
Pipette preparation (min/pipette)	0	6	0	2	0
Manipulation procedure (min/oocyte)	6 ^a	6 ^a	4 ^{a,b}	4 ^{a,b}	6
Total time per oocyte (min)	6	12	4	6	6
Total time per patient (min) (estimated average of 15 oocytes)	90	180	60	90	90

^a Time including pipette change after each oocyte.

^b Time including laser zona drilling.

outside the incubator might overcome this problem in further experiments.

We have shown that PBs can be isolated solely by noncontact, laser-mediated extraction using a combined laser unit. First PBs could be extracted in 78% of the matured bovine oocytes and in all fertilized bovine oocytes. In human oocytes, PB extraction by means of laser light was also possible, but tight sticking of the PB to the plasma membrane and zona pellucida resulted in lower efficiency. One possible reason for this phenomenon was that in the bovine we could perform the experiments with fresh oocytes of good quality. This was a clear difference to the human oocytes, which were available only because of their failure in fertilization. This implies that the oocytes might be abnormal from the onset of the experiments and therefore might show different cellular characteristics if compared to fresh, normal oocytes. Additionally, the human oocytes were at least 1 or 2 days old when they were available for the laser experiments, and aging might have negatively influenced their resistance to laser micromanipulation. Therefore, we suppose that PB-extraction results in human oocytes may be significantly increased if fresh oocytes of good quality are available.

A problem in our experiments with human and some bovine PBs was their tight sticking to the plasma membrane of the oocyte. If the PB was loose, PB extraction by laser light was no problem at all. In some cases, single shots of the UV-a laser beam could cut off the sticking areas between the oocyte and the PB, and the PB could then be subsequently extracted. One possible reason for degeneration of the oocytes or PBs was that their membrane structure was hit and destroyed while trying to dissolve sticking areas. This problem might be overcome by using a stronger laser trap (3 or 4 W). The enhanced energy will lead to a stronger trapping ability, which can avoid surplus UV-a laser shots. Furthermore, several other parameters can be modified, such as pre-culture in Ca^{2+} -/ Mg^{2+} -free phosphate-buffered saline before manipulation, which loosens the cell contact. It has been shown that preculture in Ca^{2+} -/ Mg^{2+} -free medium does not lead to any detrimental effects on further development of mouse embryos (31). The time of PB removal referred to the onset of in vitro maturation (bovine) or oocyte retrieval (human) might additionally influence the characteristics of cell contact and response on laser treatment. In further experiments laser-mediated extraction of PBs will now be performed with the oocyte on the PEN-membrane, so

that the extracted PB may remain on the slide for subsequent laser pressure catapulting. This will combine noncontact PB extraction and noncontact PB transfer into PCR-tubes.

We conclude that these new laser-assisted or entirely laser-based techniques for PB extraction have good potential to substitute standard micromanipulation techniques in close future, once some parameters have been optimized. Even without intensive micromanipulation experience, the described methods allow a noncontact procurement of PBs with a reduced risk of contamination, especially needed for single-cell PCR in prefertilization genetic diagnosis.

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