A protocol for embryonic stem cell derivation by somatic cell nuclear transfer into human oocytes

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Method Article

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Abstract

Here we describe detailed methods that allowed us to derive embryonic stem cell lines by nuclear transfer of fibroblasts from a newborn and from a type 1 diabetic adult. The protocol is based on the insight that 1) agents for cell fusion can act as potent mediators of oocyte activation by compromising maintaining plasma membrane integrity; minimizing the concentration at which they are used, and at least transiently remove calcium from the medium can improve developmental potential. 2) and on the insight that an efficient stimulus for oocyte activation is required; 3) and that histone deacetylase inhibitors facilitate reprogramming after nuclear transfer. A realistic expectation is that this protocol will result in about 10% of the oocytes developing to the blastocyst stage, most of which will be suitable for stem cell derivation. While most aspects of the protocol can be well controlled, variation in developmental efficiency when using oocytes of different donors should be expected. Note: this protocol is a guideline that allows ES cell derivation after somatic cell nuclear transfer. The author strongly discourages the use of this protocol or aspects of it in attempts for human reproductive cloning.

Introduction

The derivation of embryonic stem cell lines by somatic cell nuclear transfer yields stem cells that are genetically identical to the donor of the somatic cell nucleus. In the late ’90s, it was proposed that such cells might be useful to generate replacement cells and tissues, a concept referred to as ‘therapeutic cloning’ \[1, 2]. Although nuclear transfer in many animal species had been successful for several years \[3], establishing a nuclear transfer protocol in human eggs proved to be more challenging. One aspect impeding progress was the ability to procure high-quality human oocytes. Only few groups had access to oocytes for stem cell research. The publication of guidelines on oocyte donation for research by the International Society for Stem Cell Research \(ISSCR)\[4, 5], by the American Society for Reproductive Medicine \(ASRM) \[6], and by the Human Fertilization and Embryology Authority in the UK \[7], as well as the demonstration that recruitment of oocyte donors without any form of compensation was not practical \[8], paved the way for compensated oocyte donation programs in both the United States \[9, 10] and in Britain \[11]. Besides the logistical limitations to obtain good quality oocytes, several lines of evidence suggested that protocols resulting in efficient development to the blastocyst stage in various mammalian species were either not efficient or failed entirely when applied to human oocytes. Researchers working with Advanced Cell Technology reported developmental arrest at the cleavage stage after somatic cell nuclear transfer \[12-14], and though parthenogenetic blastocysts could be obtained, they were of poor quality and did not result in embryonic stem cell lines \[15]. Another group at Seoul National University in South Korea also had access to a significant number of high-quality human oocytes, and was able to derive a parthenogenetic embryonic stem cell line. Originally reported as a nuclear transfer ES cell line, others discovered that the cell line was of parthenogenetic origin \[16]. Apparently, oocyte enucleation followed by somatic cell nuclear transfer did not result in development that would allow ES cell derivation. Other groups similarly reported difficulties in obtaining development beyond the cleavage stage following somatic cell nuclear transfer, though not all had access to high quality human oocytes \

[9, 17-21], and few used stringent standards to genotype the embryos obtained. The protocols used for nuclear transfer were all reminiscent of protocols that had been established using sheep or cow oocytes \[22]: the use of somatic cell fusion by an electrical pulse, followed by oocyte activation with a calcium ionophore and the kinase inhibitor 6-dimethylaminopurine \(6-DMAP) had been successful in sheep and in bovine \[22, 23], but was apparently very inefficient with human oocytes. And even modifications that had resulted in rhesus macaque nuclear transfer ES cell lines \[24], including the use of spindle birefringence instead of Hoechst and UV for oocyte enucleation \[25], did not significantly improve development after human somatic cell nuclear transfer \[9]. Initially shown to increase developmental potential after nuclear transfer in mouse oocytes \[26, 27], histone deacetylase inhibitors also appeared to improve development after somatic cell nuclear transfer in human oocytes \[28]. Further improvement in developmental potential was observed when calcium ionophore used for oocyte activation was substituted with an electrical pulse, resulting in the derivation of diploid ES cell lines after nuclear transfer of fetal \(embryonic) fibroblasts \[29]. Here we describe methods that allowed the derivation of nuclear transfer ES cell lines from fibroblasts of a newborn and of an adult type 1 diabetic.

Reagents

Rock inhibitor Y27632 \(Stemgent, 04-0012-02). Thiazovivin \(Stemgent, 04-0017). Origio Humagen micropipets \(Piezo-18-25 and MPH-SM-25). Glass Bottom Dish \(MatTek Corporation, P50G-1.5-30-F). Polyvinylpyrrolidone \(PVP) Solution with HSA - 7% \(Irvine Scientific, 90121) Light Mineral Oil for Embryo Culture \(Irvine Scientific, 9305) LifeGlobal medium \(IVF online, LGGG-050) Global total \(IVF online, LGGT-030) G-MOPS plus medium \(Vitrolife, 10130) or Global total with HEPES \(LifeGlobal, LGTH-50). ES cell tested FBS \(tested by investigator). Should be compatible with clonal human ES cell growth after enzymatic splitting and plating on mouse embryonic fibroblasts. Somatic cells. Somatic cells may be marked with a cytoplasmic or nuclear GFP to monitor cell fusion. DMEM/F12 \(11320-033), Knockout-SR \(10828028), bFGF \(13256029), KnockOut DMEM \(10829-018), Penicillin Streptomycin \(15070063), DMEM \(11885-092), Glutamax \(35050-061), Standard FBS \(10437-036), TrypLE \(12605-010), 1000x 2-mercaptoethanol \(21985023), all LifeTechnologies. Irradiated mouse embryonic fibroblasts \(GlobalStem CF-1, 2M IRR). Microcapillary tube 100ml \(Sigma-Aldrich, P1174-1PAK). Pipet filler \(Sigma-Aldrich, P4925) or mouth pipettes \(Sigma-Aldrich, A5177-5EA). Engraving pen \(Sigma-Aldrich, Z225568-1EA). Click-iT® EdU Alexa Fluor® 488 Imaging Kit \(Life Technologies, C10337). CryoTube vials \(ThermoScientific, 377267). Nunc 4-Well Dishes for IVF \(Thermo Scientific, 12-566-300). Nunc Cell-culture treated multidishes 4-well \(Thermo Scientific, 176740). CytochalasinB \(Sigma-Aldrich, C2743), and further diluted to 5mg/ml in DMSO \(Sigma-Aldrich, D2650) to make a 1000x stock solution. Stored at -20 deg. Histone deacetylation inhibitors NCH-51 \(Tocris Bioscience, 3747) and Scriptaid \(Sigma-Aldrich, S7817) or TrichostatinA \(Sigma-Aldrich, T1952). Inactivated Sendai virus \(GenomeOne, Cosmo Bio) reconstituted in 260ml suspension medium. Store at -80 deg in aliquots of 1-5ul and then further diluted in suspension medium at 1:10 to 1:20 ratio when thawed. Caffeine \(Sigma-Aldrich, C0750). Human Serum Albumin \(HSA)-Solution, \(Vitrolife, 10064). 0.1% Gelatin \(EMD Millipore, ES-006-B).

Equipment

Nikon TE-2000U equipped with Hoffmann modulation contrast optics. Equipped with 4x, 20x and 40x long working distance objectives. \(Or use equivalent brand). NT-88-V3 Narishige Micromanipulation System for IVF. Tokai hit Thermoplate TP-108R05 \(order number depends on microscope system) CRI-O-PLI-400 Oosight Meta Imaging System, Camera and Software Minicentrifuge \(e.g. Biorad #166-0603EDU) Lykos Laser of Hamilton Thorne, 40x objective equipped with RED-i target locator. Thermo Scientific Heracell 150i incubator. Desktop computer to run Laser and Oosight programs. Nikon SMZ1500 with Tokai hit heating plate TP-SMZSL. Portable incubator \(CryoLogic, BioTherm INC-RB1). Traceable Double Thermometer \(Control Company Cat No 4137). Standard Tissue Culture equipment, including Biological Safety Cabinet, fridges and freezers, Bunsen-Burner, Gilson pipettes or equivalent. Nepagene Super Electro Cell Fusion Generator ECFG21 \(or LF201) with CUY5000P1 fusion chamber.

Procedure

Preparation of donor cells Preparation of somatic cells: Plate somatic cells in DMEM containing 8-10% Standard FBS \(antibiotics may also be used) approximately 10 days before the planned experiment. Allow somatic cells to reach confluence. Change medium every 2-3 days. The cells will be confluent about 5 days before the experiment and less than 0.5% should be in S-phase at the time of the nuclear transfer experiment. The number of S-phase cells can be determined by EdU staining. Media Preparation Media Preparation is as described in Reference \[30] and provided here for convenience. Preparation of master salts: Begin with 980 mL ultrapure H2O in sterile 1 L bottle. Add dry components: NaCl 4760 mg \(81mM) Sigma S-5886, KCl 360 mg \(5mM) Sigma P-5405, MgSO4• 7H2O 290 mg \(1.18mM) Sigma M-2773, KH2PO4 160 mg \(1.17mM) Sigma P-5655, EDTA 2NA 40 mg \(0.1mM) Sigma E-6635, Glucose \(D) 1000 mg \(5.5mM) Sigma G-6152. Add liquid Components: Na-lactate \(lactic acid) 5.3 mL Sigma 44263, Pen’Strep 10 mL Gibco 15140-122. For preparation of calcium-free HCZB: Start with 500 mL master salts. Add 50 mg polyvinyl alcohol \(PVA) \(cold-soluble; Sigma P-8136). Stir for 30-60 min and sterile filter. Use 99ml of this mixture to add Hepes-Na 520 mg Sigma H-3784 and NaHCO3 42 mg Sigma S-5761. Swirl until dissolved. Adjust pH to 7.5 with 1N HCl. Add 1ml of HSA solution. Sterile filter. For preparation of calcium-free mCZB: To 99ml master salts, add NaHCO3 211 mg Sigma S-5761, Na-pyruvate \(pyruvic acid) 3 mg Sigma P-4562, L-Glutamine 15mg Sigma G-8540, add 1ml HSA solution, Swirl until all components are dissolved. Sterile filter. Check pH after equilibrating in CO2 atmosphere. Oocyte manipulations 1. Retrieval: Oocytes are retrieved 35 hours post induction of ovulation. Oocyte handling and preparation, including removal of cumulus cells, is best done according to established practice at your IVF center. 2. Transport: If manipulation occurs in a different facility than oocyte retrieval, oocytes can be transported \(e.g. by car or train) in a portable incubator heated to 37°C. The incubator is turned on 1h before retrieval, with the tubes loaded in the incubator, so it has at least 1 hour to equilibrate temperature. Tubes inside the incubator may be ThermoScientific CryoTube vials loaded with 1ml-1.5ml of G-MOPS PLUS. Caffeine at 1mM may be added to this medium. Quality controls of the temperature of the incubator should be confirmed prior to the experiment with a temperature probe. A quality control run

with mouse oocytes is also highly recommended. Our transport time was between 20 and 30 minutes. If oocytes are transported, two people are required to carry out the experiment. One person picks up the oocytes, while the other prepares the micromanipulator. 3. Preparations: set up 4-6 4-well Dishes containing 0.75ml Global medium each well the night before the experiment to equilibrate in the incubator at 37degree Celsius, 5-6% CO2. Dishes are used for washes, and temporary storage during the manipulation. In the early morning, the micromanipulator is prepared, with humagen needles attached and oosight system started and a background image taken. Prepare approximately 10 capillaries by pulling them over a Bunsen burner, then cut the capillaries with the engraving pen \(See Movie). These should be changed frequently during the manipulations to avoid medium carry over and stickiness. Manipulation dishes \(Matek glass dishes) are set up by adding 1) one drop of PVP, where the manipulation needles can be lubricated, 2) three drops of Globaltotal w Hepes medium containing 5µg/ml cytochalasinB, 3) one drop of Sendai virus diluted in suspension buffer 1:10 to 1:20, and 4) three drops of calcium-free HCZB \(Figure 1). The cytochalasinB \(CytoB) containing medium is used for oocyte enucleation, the CytoB free medium for nuclear transfer. Caffeine at 1mM may be added to both enucleation and transfer solutions. Warm up the up the dishes on the heated stage before placing oocytes in them. PVP is used to lubricate the pipettes. Aspirate a small amount of PVP and expel it again. There should be little PVP left in the pipette. When the pipette gets sticky during manipulation, repeat this process, or exchange the pipette. Confluent somatic cells are trypsinized using TrypLE resuspended in medium containing DMEM, 10% Standard FBS, spun down and placed on ice in about 50µl of medium. 4. Manipulation: Upon arrival, take oocytes out of the vials using a 1ml tips on a Gilson pipette, and placed in a 30mm dish on the heated stage of a Stereomicroscope. Oocytes are then placed in the equilibrated medium of the 4-well dishes and placed in the incubator at 37 degree Celsius and 5% CO2 \(or 6% CO2, 89% N2, 5% O2). 2-3 oocytes are placed on the stage heated to 37 degree Celsius, in cytochalasinB free medium and the zona is drilled using the Lykos laser near the spindle visualized by birefringence\[25]. Drilling is performed at low laser power, of 200µs, 90% power. The drilling in the absence of cytochalasinB serves to prevent oocyte leakage. Two small openings in the zona pellucida are made 90-180 degrees apart from each other. The first polar body may be ablated using 2-3 direct laser pulses or removed at this point to avoid later confusion with the somatic cell to be transferred. The oocyte is then transferred into cytochalasinB containing medium \(5µg/ml), turned until a spindle can be visualized using microtubule birefringence \(Oosight), the zona is again drilled with the laser to gain access to the spindle. Be careful to have the spindle, the focus of the laser on the zona and the enucleation pipette all in the same plane of focus. If this is not the case, the spindle cannot be removed or seems to be moving away from the pipette when trying to aspirate it. After enucleation, oocytes are allowed to recover for 5-10 minutes from the cytochalasinB prior to fusion. CytochalasinB makes the cytoplasm look uneven, but is reversible within a few minutes. They may be washed in Global medium and returned to the incubator, or left on the heated stage the drops without cytochalasinB. While recovering from cytochalasinB, a new batch of 2-3 oocytes is enucleated. Nuclear transfer is performed as follows: a small drop of somatic cells is placed in one of the cytochalasinB free drops. Enucleated oocytes are placed in calcium-free medium. One somatic cell is aspirated into the pipette, and exposed to the virus for 10-20s \(Figure 2a). There is no need to expel the somatic cell entirely. They become very sticky and more difficult to handle if

they are expelled entirely. Fusogenic agents like Sendai virus act by affecting the integrity of the plasma membrane, and therefore increase permeability to ions contained in the culture medium. The membrane of a cell becomes more fragile when exposed to Sendai virus and the cell is prone to undergo lysis. This adverse effect of the virus lasts longer than the immediate time of incubation with the virus. Therefore, the minimal amount of fusogenic agent required to achieve fusion is recommended, to preserve integrity of the plasma membrane of the oocyte as much as possible, while still achieving fusion. To minimize exposure of the egg to the virus, do not expose the holding pipette to the virus and on the way back to the drop containing the oocytes, pass through another drop to get rid of excess virus. Drill the zona and make a very small opening \(Figure 2b), insert the somatic cell with the side exposed to the virus facing the plasma membrane, and ensure that the zona pellucida presses on the somatic cell to ensure contact between the somatic plasma membrane and the oocyte plasma membrane. If the somatic cell is GFP positive, fusion can usually be confirmed within 5 minutes by briefly checking the fluorescence in the oocyte. The oocyte is returned to the incubator in calcium-free medium for up to 1h. Checkpoint: the investigator may want to fix a few oocytes to determine chromosome condensation. Before activation, the oocyte is returned to Global total for at least 10 minutes. 5. Activation: activate oocytes within approximately an hour or two post-transfer using an electrical pulse as described\[29]. Before delivering the pulse, wash the oocytes through two drops of fusion medium containing d-sorbitol \(0.25M), 0.1mM calcium acetate, 0.5mM magnesium acetate, 0.5mM HEPES, and 1mg/ml BSA \(Sigma A7030). They are ready for the electrical pulse when they sink to the bottom of the drop containing fusion medium. The presence of several zona openings avoids leaking out of the oocytes and possible loss. Oocytes are pulsed using an LF201 pulser \(Nepagene) set to deliver two times 2 pulses of 50μs width at 2.7kV/cm in a CUY5000P1 fusion chamber \(time between pulses, 1s). The Thereafter oocytes are washed in Global total and cultured in Global total containing 10μM puromycin, 2mM 6-DMAP, as well as the histone deacetylase \(HDAC) inhibitors scriptaid \(250nM, Sigma S7817) and nch-51 \(1μM, Tocris Bioscience 3747) \(trichostatin A at 10nM may likely be used instead of the former two), for 3.5-4 hours. Formation of small pronuclei should be apparent at 4 hours. Wash oocytes through 2 wells of medium to eliminate compounds used for oocyte activation. 6. Culture: culture activated constructs in HDAC inhibitor for an additional 10-14 hours, followed by culture in Global total medium until day1. On day1 in the early morning, pronuclei are monitored again. New 4-well dishes are set up for equilibration containing Global medium containing 10% FBS \(quality controlled by the investigator for compatibility with clonal human ES cell growth). Cleaved oocytes are transferred into this new medium. On day3, cleaved oocytes are again transferred to fresh medium. Developmental progression may be monitored every day, by briefly placing dishes on the heated stage of the microscope. Avoid evaporation of the medium while viewing. 7. Derivation: Derive in the evening of day 6 or in the morning of day 7 after nuclear transfer. About 70,000 mouse embryonic fibroblasts \(MEFs) per well of a 4-well dish are seeded 3-4 hours before derivation in warm fibroblast medium on a surface that had been coated for approximately 10 minutes with 0.1% gelatin. Time before derivation is just sufficient for the MEFs to attach to the bottom of a 4-well dish and spread out. The MEF layer should cover all plastic, but should not be dense. The timing of derivation from blastocysts should be on day two after blastulation is first observed. Trophectoderm cells are ablated with laser pulses at 400μs pulses, 90% power, as described\[31]. No more than one pulse per cell is

applied, and not all trophectoderm cells need to be ablated. If the blastocyst did not hatch from the zona pellucida on its own, laser pulses may be used to ablate the zona pellucida with gentle pipetting to remove remaining pieces. Plating is conducted in medium containing KnockOut DMEM/F12 supplemented with 10% Knockout-SR and 10% quality-tested FBS, 10μM Rock inhibitor Y-27632, 2μM thiazovivin, non-essential amino acids \(1%), 10ng/ml bFGF, Glutamax \(1%), PennStrep \(1%), and 1x 2-mercaptoethanol. Two days after plating, remaining trophectoderm cells are ablated using laser pulses at 400μs pulses, 90% power, which may be repeated until most trophectoderm cells are gone \(see Extended Data Figure 5 of Reference \[32]. Trophectoderm cells are rather flat and large and may inhibit growth of inner cell mass \(ICM) cells. If the investigator cannot see ICM cells, or cannot distinguish them from trophectoderm, wait another day and check again. Outgrowths can be apparent as soon as 3-4 days after plating, but may take up to two and a half weeks to be fully-grown and ready for picking. Medium changes are partial every 1-2 days. Once an outgrowth is apparent, partial medium changes should be conducted without Y-27632 or thiazovivin. Picking is done when colonies reach a size of about 1mm in diameter and colony fragments are plated into 2-3 wells of a 4-well dish coated with MEFs. MEFs must be fresh, meaning no older than 12 hours. If colonies emerge in several wells, enzymatic passaging may begin after just one manual passage. FBS-containing medium is gradually replaced by using standard ES cell medium \(substituting FBS with Knockout-SR and DMEM/F12 with Knockout-DMEM). Enzymatic passaging is done using 10μM Rock inhibitor Y-27632 for the first day after plating. 8. Verification: Quality control the cell lines derived, by analysis of pluripotency markers and differentiation potential. Protocols for performing such analysis are provided elsewhere. In addition, analyze the karyotype and the short tandem repeats of the nuclear transfer ES cell lines. There are commercially available services for doing that, such as at Cell Line Genetics in Madison, Wisconsin. The karyotype should be diploid. Short tandem repeats \(STR) are unique genetic features that provide an identifier of the cell lines derived. For instance, in the associated Nature paper, in Extended Data Table 2, we provide the STR genotypes of all nuclear transfer ES cell lines. These were found to be an exact match to the STR genotype made on the somatic cells prior to nuclear transfer. An example, the somatic cells prior to nuclear transfer \(ID 1018) is given here and as an attachment: Amelogenin X; vWA 16,17; D8S1179 10,15; TPOX 8; FGA 25,27; D3S1358 15,18; THO1 9.3; D21S11 28,30; D18S51 13,15; PentaE 12,14; D5S818 11,13; D13S317 9,11; D7S820 9,10; D16S539 11,13; CSF1PO 10,12; PentaD 9,13; The locus name is indicated before one or two numbers that indicate the identity of the allele. If there are two numbers for a given locus, it is because maternal and a paternal alleles differ from each other. Use ID numbers, and not patient names throughout all of your procedures.

Timing

Oocyte transport: 20-30 minutes. Oocyte manipulation: 2-3 hours, depending on the number of oocytes retrieved. Incubation: 1 hour. Oocyte activation: 4 hours. Oocyte incubation in histone deacetylase inhibitor: 12 hours. Culture: 6 days \(up to 7 if blastulation occurs only on day 6). Medium change on day1 and 3. ES cell derivation: one to two months. ES cell characterization: 1 month \(in vitro analysis only).

Troubleshooting

It is strongly recommended that the laboratory have an ongoing research program on animal oocytes. This will enable using essentially identical conditions for somatic cell nuclear transfer. Mouse oocytes are most readily accessible, and should be used to quality control all reagents and solutions. For mouse nuclear transfer, some species-specific changes to the nuclear transfer protocol are required. Mouse oocytes do not fuse well with somatic cells when using inactivated Sendai virus; the researcher may use direct injection for nuclear transfer. And the strength of the fusion pulse may need to be adapted. For how to obtain mouse oocytes, see reference \[34]. In parallel, the laboratory should have expertise at human ES cell derivation and iPS derivation. Somatic cells may be used to generate iPS cells to determine if the somatic cells are of good quality and can indeed be reprogrammed.

Anticipated Results

A realistic expectation is that this protocol will result in about 10-20% of the oocytes developing to the blastocyst stage, most of which will be suitable for stem cell derivation. Variation in development between oocyte donors should be expected. Establishing this protocol requires a significant institutional commitment and a dedicated research program with researchers skilled at manipulating oocytes and embryos.

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Figures

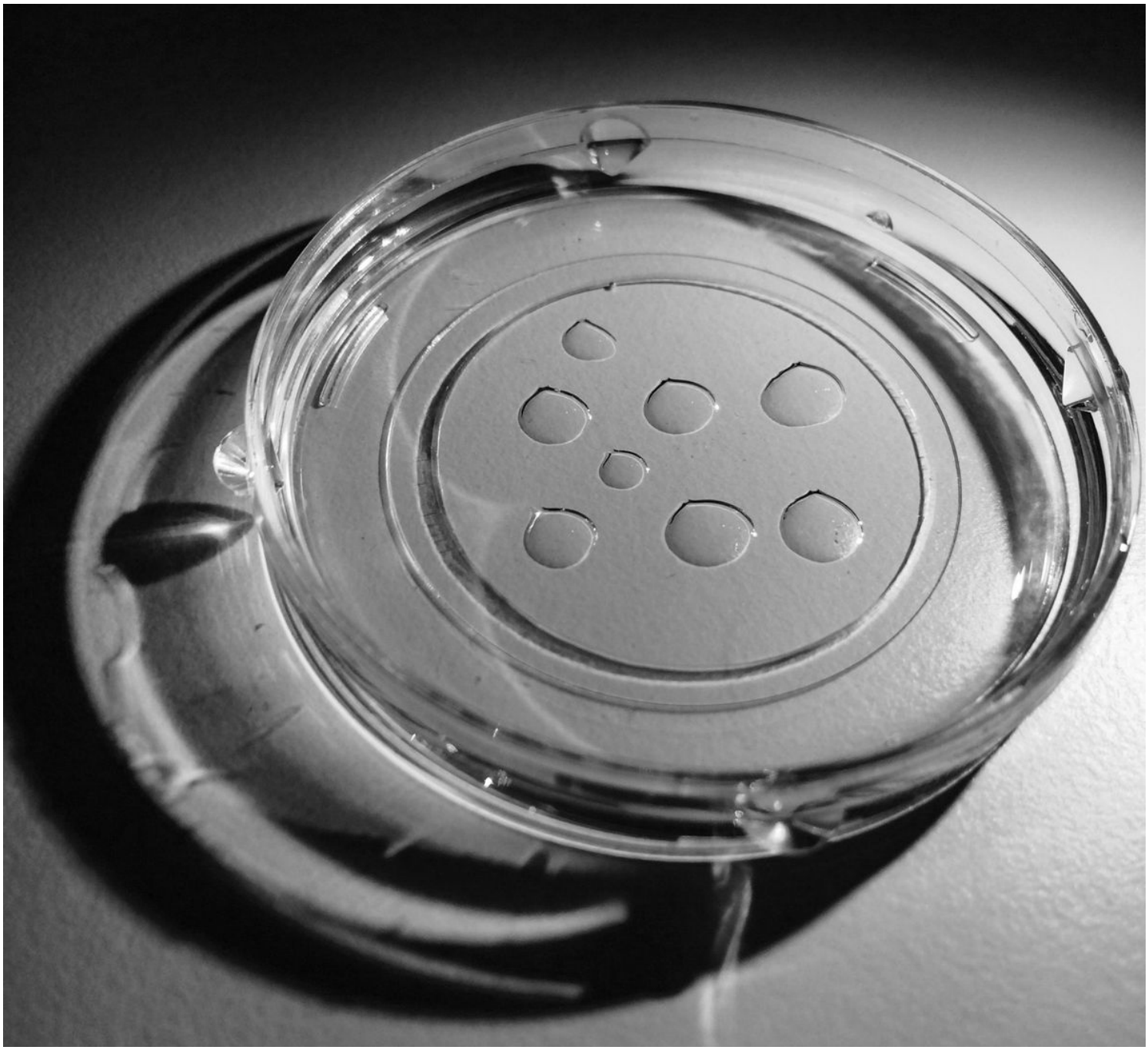

Figure 1

Example of possible setup of the manipulation dish Rows from top to bottom: 1 PVP drop, 3 drops with cytochalasinB, 1 drop with virus, 3 drops with calcium-free or calcium-chelated medium.

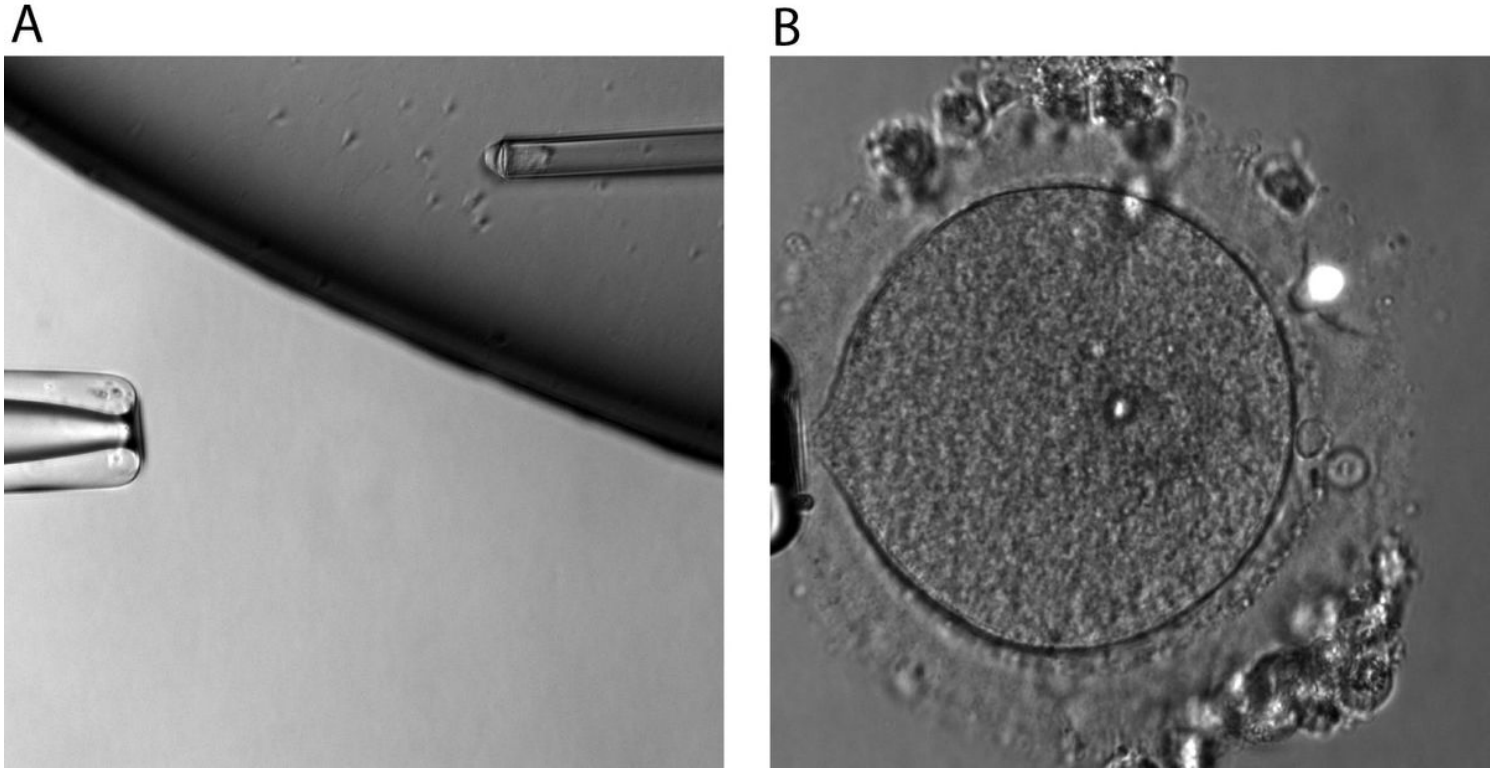

Figure 2

Virus incubation and zona penetration a) exposing only one side of the cell to the virus is sufficient for cell fusion. b) The laser is set at low power and only a very small opening is made for cell transfer so the zona can be used to confine the somatic cell between zona and the oocyte.

Supplementary Files

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