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Corresponding Author:	Ye Yuan Colorado Center for Reproductive Medicine Lone Tree, CO UNITED STATES
Corresponding Author's Institution:	Colorado Center for Reproductive Medicine
Corresponding Author E-Mail:	yyuan@flcolo.com
Order of Authors:	Deirdre M. Logsdon
	Rebecca A. Kile
	William B. Schoolcraft
	Rebecca L. Krisher
	Ye Yuan
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TITLE:

Single Cell Collection of Trophoblast Cells in Peri-implantation Stage Human Embryos

AUTHORS AND AFFILIATIONS:

Deirdre M. Logsdon[1], Rebecca A. Kile[1], William B. Schoolcraft[1], Rebecca L. Krisher[1], Ye Yuan[1]

[1]Colorado Center for Reproductive Medicine, Lone Tree, CO, USA

Email addresses of co-authors:

Deirdre M. Logsdon (dlogsdon@flcolo.com)
Rebecca A. Kile (bkile@flcolo.com)
William B. Schoolcraft (bschoolcraft@colocrm.com)
Rebecca L. Krisher (rkrisher@flcolo.com)

Corresponding author:

Ye Yuan (yyuan@flcolo.com)

KEYWORDS:

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SUMMARY:

Here, we describe a method for warming vitrified human blastocysts, culturing them through the implantation period in vitro, digesting them into single cells and collecting early trophoblast cells for further investigation.

ABSTRACT:

Human implantation, the apposition and adhesion to the uterine surface epithelia and subsequent invasion of the blastocyst into the maternal decidua, is a critical yet enigmatic biological event that has been historically difficult to study due to technical and ethical limitations. Implantation is initiated by the development of the trophectoderm to early trophoblast and subsequent differentiation into distinct trophoblast sublineages. Aberrant early trophoblast differentiation may lead to implantation failure, placental pathologies, fetal abnormalities, and miscarriage. Recently, methods have been developed to allow human embryos to grow until day 13 post-fertilization in vitro in the absence of maternal tissues, a time-period that encompasses the implantation period in humans. This has given researchers the opportunity to investigate human implantation and recapitulate the dynamics of trophoblast differentiation during this critical period without confounding maternal influences and avoiding inherent obstacles to study early embryo differentiation events in vivo. To characterize different trophoblast sublineages during implantation, we have adopted existing two-dimensional (2D) extended culture methods and developed a procedure to enzymatically digest and isolate different types of trophoblast cells for downstream assays. Embryos cultured in 2D conditions have a relatively flattened morphology and may be suboptimal in modeling in vivo three-dimensional (3D) embryonic architectures. However, trophoblast differentiation seems to be less affected as demonstrated by anticipated morphology and gene expression changes over the

course of extended culture. Different trophoblast sublineages, including cytotrophoblast, syncytiotrophoblast and migratory trophoblast can be separated by size, location, and temporal emergence, and used for further characterization or experimentation. Investigation of these early trophoblast cells may be instrumental in understanding human implantation, treating common placental pathologies, and mitigating the incidence of pregnancy loss.

INTRODUCTION:

Human implantation and the emergence of the early placenta are historically difficult to investigate and remain largely unknown because human tissues are inaccessible at this stage when pregnancy is clinically undetectable. Animal models are inadequate, as human placentation has its own unique features compared to other eutherian mammals. For example, human placenta invades deeply into the decidua with some trophoblast cells reaching at least the inner third of the uterine myometrium while other cells remodel the uterine spiral arteries. Even our closest evolutionary ancestors, the non-human primates, show differences in placental morphology and trophoblast interactions with the maternal decidual tissues[1-3]. Obtaining pre-implantation human embryos in vitro was not possible until in the 1980's when clinical human in vitro fertilization (IVF) started as a routine practice for treating infertility[4]. Now, human blastocysts can be grown in vitro to allow for the selection of more viable embryos for transfer, as well as enable safe genetic testing. Improvement in embryo culture techniques, as well as the increasing use of IVF has yielded many surplus blastocysts which remains after patient's treatment cycles have been completed. With patient consent, IRB approval, and with certain restrictions, these blastocysts may be utilized for research studies. They have become an invaluable resource that were used for the derivation of human embryonic stem cells[5], understanding the transition of inner cell mass to embryonic stem cells[6,7], and more recently, have been successfully cultured until day (D) 13 to remodel human implantation[8,9]. By utilizing recently developed single cell omics approaches, the access to these implantation stage human embryo tissues has offered unique opportunities to describe the molecular mechanisms that regulate this highly dynamic cell differentiation process, which were previously impossible to explore[10-13].

Here, we describe the methods used in our recent publication characterizing the dynamics of trophoblast differentiation during human implantation[12]. This protocol includes the warming of vitrified blastocysts, extended embryo culture up to D12 post IVF, enzymatic digestion of the embryo into single cells, and cell collection for downstream assays (**Figure 1**). This extended culture system supports peri-implantation stage human embryo development without maternal input and recapitulates trophoblast differentiation that appears consistent with the observations made from histological specimens many years ago[14-17]. During implantation, the trophoblast population is comprised of at least two cell types: the mononucleated progenitor-like cytotrophoblast (CTB) and the terminally differentiated, multinucleated syncytiotrophoblast (STB). Upon trypsin digestion, the CTB are small, round cells that are morphologically indistinguishable from other cell lineages (**Figure 2A**, left panel). Separation of CTB from other cell lineages, such as epiblast and primitive endoderm, can be achieved by their distinct transcriptomic profiles revealed by single cell RNA sequencing. Syncytiotrophoblast cells can be easily identified as irregular shaped structures that are significantly larger than the other cell

types and mainly located at the periphery of the embryo (**Figure 2A**, middle panel; **Figure 2B**, left panel). Migratory trophoblast cells (MTB) are another trophoblast sublineage found during embryo extended culture and can be recognized as seemingly moving away from the main body of the embryo (**Figure 2A**, right panel, **Figure 2B**, right panel). Migratory trophoblast, although expressing many of the same markers as extra villous trophoblast (EVT), should not be referred to as EVT, since the villous structures in the placenta have not emerged at this very early stage of development.

Experimentally, we were able to collect small CTB and large STB that are easily distinguishable after embryos are digested into single cells at D8, D10, and D12 (**Figure 2A,B**). Migratory trophoblasts arise at the later stages of extended embryo culture and can be collected at D12 before enzymatic digestion of the whole embryo (**Figure 2A,B**). By phenotypically separating these three trophoblast sublineages before single cell analysis, we can identify specific transcriptomic markers and define the biological role of each cell type. Cytotrophoblast are highly proliferative and act as progenitor cells in supplying the STB and MTB differentiated lineages[10-13]. Syncytiotrophoblast are involved in producing placental hormones to maintain pregnancy and may be also responsible for the embryos burrowing into the endometrium[10-13]. Migratory trophoblast has even stronger features of an invasive, migratory phenotype and are likely responsible for deeper and more extensive colonization of the uterine endometrium[10-13]. After defining the transcriptomic signature of each cell type, clustering analyses have also revealed two additional subsets of cells that were morphologically indistinguishable from CTB and had transcriptomes with features of STB and MTB, respectively[12]. These intermediate stage cells are likely in the process of differentiating from CTB to either the MTB or STB sublineages and would have been overlooked if embryos were blindly digested and cells were separated by transcriptome alone.

The protocol described here utilizes a two-dimensional (2D) culture system and may not be optimal for supporting three-dimensional (3D) structural development, as suggested by a recent publication describing a newly developed 3D culture system[13]. Nevertheless, in this 2D system the differentiation of early trophoblast seems to be consistent with observations made from in vivo specimens[14-17]. This protocol may also be easily adapted for the use in the recently described 3D culture system[13] with minimal changes. All steps are carried out with a handheld micromanipulation pipette with commercially available disposable tips or a mouth pipette from a finely pulled glass pipette attached to rubber tubing, a filter, and a mouthpiece.

PROTOCOL:

All human embryos have been donated with consent for use in research. Protocols for extended human embryo culture have been approved by the Western Institutional Review Board (study no. 1179872) and follow international guidelines. Any use of human embryos must be reviewed by the appropriate ethics and governing bodies associated with the research institution using this protocol.

1. Preparation

1.1. Prepare media and recovery plates one day prior to embryo warming in a sterile laminar flow hood.

1.1.1. Prepare 2 mL of blastocyst culture media (BM) with 10% v/v serum protein substitute (SPS).

NOTE: Blastocyst culture media may or may not contain added protein. Here, we used a medium that must be supplemented with 10% of the indicated albumin protein. Check the manufacturer guidance for variation in this supplementation.

1.1.2. Fill two center-well organ culture wash dishes with 500 µL of BM with 10% v/v SPS covered with 500 µL of embryo culture grade oil.

1.1.3. In a 60 mm tissue culture dish, layer 8 mL of embryo culture oil and then anchor 20 µL drops of BM with 10% v/v SPS to the bottom of the culture plate. Make 1 drop for each embryo warmed.

NOTE: More media, drops, and wash dishes may be made as needed. Drops may also be added to the dish before the oil is layered. Anchoring drops under the oil will help reduce evaporation and any subsequent changes in osmolality.

1.1.4. Equilibrate BM with 10% v/v SPS wash dishes and recovery plate in an incubator at 37 °C, 6% CO_2, 5% O_2 for at least 4 h.

1.1.5. Thaw one vial of the first step of extended culture media (IVC1) in 4 °C or on the bench top.

1.1.6. Prepare one wash dish of 500 µL of IVC1 with no oil overlay. Aliquot approximately 4 mL of IVC1 into a 5 mL snap cap tube.

1.1.7. Equilibrate IVC1 wash dish and small snap cap tube of IVC1 in 37 °C, 6% CO_2, and atmospheric O_2 for at least 4 h.

1.2. Prepare extended culture plates one day prior to embryo warming in a sterile laminar flow hood.

1.2.1. Dilute fibronectin from human serum in phosphate buffered saline (PBS) to 30 µg/mL. 250 µL of 30 µg/mL fibronectin per embryo will be needed to coat the chambers.

1.2.2. Open the 8 well chambered coverslip package under the hood while taking care not to touch the wells. Gently pipette 250 µL of 30 µg/mL fibronectin into each well.

1.2.3. Return the lid to the chambered coverslip and place in 4 °C for 20-24 h.

1.3. Prepare extended culture plate in the morning of embryo warming.

1.3.1. Retrieve the chambered coverslip with fibronectin and place in laminar flow hood. Remove the fibronectin mixture with a 1 mL pipette and discard into the waste container.

1.3.2. Retrieve warmed, equilibrated IVC1 media from small 5 mL snap cap tube.

1.3.3. Pipette 300 µL of equilibrated IVC1 into each well. Be careful not to let any fluid touch the lid as this will increase the risk of contamination.

1.3.4. Return the chambered coverslip with IVC1 to incubate in 37 °C, 6% CO_2, and atmospheric O_2 until removal of the zona pellucida in step 4.

2. Warming vitrified D5 human embryos

NOTE: Other manufacturers may have slightly different protocols according to their own vitrification technology. See manufacturer's instructions for use when applicable.

2.1. Warm 3.0 mL of thawing solution (TS) in 35 mm dish to 37 °C. Bring 300 µL of dilution solution (DS) and two wells of 300 µL of washing solution (WS) in a 6 well plate to room temperature.

2.2. Using forceps, carefully remove the cryo device sleeve while ensuring that the vitrified embryo always remains under liquid nitrogen.

2.3. Quickly move the cryo device from liquid nitrogen and plunge the tip of the cryo device in TS at 37 °C. Set a timer for 1 min and keep the cryo device submerged until the embryo detaches into the TS.

NOTE: Warm embryos one at a time to keep track of embryo identities as they may relate to patient demographic information in downstream analysis.

2.4. During the 1 min incubation in TS, carefully remove the cryo device and gently pick up the embryo and move to the opposite side of the TS dish.

NOTE: If culturing multiple embryos, be diligent to keep embryos separate to ensure proper identities are maintained.

2.5. After 1 min, gently pick up the embryo with a small amount of TS, approximately 2 µL. Move the embryo to the bottom of the 300 µL DS well while covering the embryo in a thin layer of TS from the pipette. Set a timer for 3 min and place the 6-well plate on the bench top at room temperature.

2.6. After 3 min, pick up the embryo with a small amount of DS, approximately 2 µL, and move the embryo to the bottom of the next well containing 300 µL of WS. Again, gently layer a small amount of DS from the pipette over the embryo. Set a timer for 5 min and return to the bench

top.

2.7. After 5 min, pick up the embryo with minimal volume of WS and move to the top of the final well of 300 µL WS. The embryo will slowly fall and wash through the WS to the bottom. Expel any retained WS from the pipette into an empty well.

2.8. Pick up the embryo with minimal volume and return to the top of the same 300 µL well of WS. The embryo will once again fall to the bottom of the well. Set a timer for 1 min and return the 6-well plate to the bench top.

3. Recovery of warmed embryos

3.1. One at a time, move warmed embryos into a center-well organ tissue dish containing 500 µL of equilibrated BM with 10% v/v SPS under 500 µL of equilibrated embryo culture grade oil.

3.2. Wash embryos by picking up each embryo and moving them around to several areas in the dish while blowing out old media between moves. Pick up the embryo and move it to an individual 20 µL culture drop of equilibrated BM with 10% v/v SPS under oil.

3.3. Let the warmed embryos recover for 2 h in an incubator at 37 °C, 6% CO_2, 5% O_2.

4. Zona removal

4.1. After a 2 h recovery, assess embryos for re-expansion and take pictures of each embryo.

4.2. Move embryos individually in 500 µL of a 3-(N-morpholino)-propanesulfonic acid (MOPS)-buffered handling medium with 5% (v/v) fetal calf serum (FCS) prior to the treatment with acidic Tyrode's solution.

4.3. Move one embryo quickly through 300 µL of warmed acidic Tyrode's solution while actively watching through the microscope. The zona pellucida will visually start to dissolve. This will take approximately 5 s.

4.4. Immediately move the embryo with dissolving zona into 300 µL of warmed MOPS buffered medium to quench the acid Tyrode's solution.

4.5. Move the embryo with minimal volume of MOPS buffered medium into a center-well organ tissue dish containing 500 µL of equilibrated BM with 10% v/v SPS under 500 µL of equilibrated embryo culture grade oil to wash.

4.6. Return the embryo to the 20 µL recovery drop from step 3.2.

NOTE: Upon visual examination following zona removal, any embryos with retained zona pellucidae may be further treated with acidic Tyrode's solution if necessary, by repeating steps

4.2-4.6. Minimizing exposure to the Tyrode's solution is desired.

5. Blastocyst extended culture

5.1. Move the embryos individually to the wash dish with equilibrated IVC1 media from step 1.1.6. Carefully move one embryo to one well of the chambered coverslip with equilibrated IVC1 and maintain embryo identification.

NOTE: This step needs to be finished as quickly as possible to minimize medium evaporation.

5.2. Return chambered coverslip to an incubator set to 37 °C, 6% CO_2, atmospheric O_2 for 2 days.

NOTE: Be sure to thaw and equilibrate media in 37 °C, 6% CO_2, atmospheric O_2 for the media exchange 4 h in advance.

5.3. At outgrowth D2 (D7 post fertilization), carefully examine the attachment of embryos under the microscope and perform media exchange.

5.3.1. Note which embryo is attached to the dish before exchanging media. Gently tap the plate and examine whether an embryo has securely attached to the plate under the microscope.

5.3.2. Remove the lid and carefully remove 150 µL of IVC1 and discard while not disturbing the attached embryo. If an embryo has not yet attached to the plate, do not exchange the media, as the serum in IVC1 will aid in embryo attachment.

5.3.3. Pipette 150 µL of equilibrated extended culture media, IVC2, slowly into each well and return the lid to the chambered coverslip.

NOTE: Ensure that removing the lid from the chambered coverslip is minimized to avoid medium evaporation.

5.4. Carefully return the chambered coverslip to the incubator without splashing any media on the lid. Repeat media exchange and attachment check every day of extended culture until embryos are ready for fixation or single cell digestion.

6. Optional collection of spent media

6.1. Optionally, collect spent media during the exchange of culture media after D7 or any day thereafter. During step 5.3.2, rather than discarding the medium snap-freeze the 150 µL of removed IVC1 into a sterile, low-bind 0.5 mL tube for future analysis.

7. Optional fixation for immunofluorescence

7.1. Use a 200 µL pipette to wash embryos with ½ media exchanges of PBS for 3x before fixation

to remove any extracellular debris. Washing away excess debris and protein will help to optimize clarity and reduce background in images obtained with immunofluorescence.

7.2. Remove all media and slowly add 200 µL of 4% paraformaldehyde (PFA) in PBS to the well. The embryo will want to stick to the surface of the fluid. Multiple 150 µL washes with 4% PFA before removing all fluid will help to minimize any damage to the embryo.

7.3. Incubate the embryos in 4% PFA for 20 min for fixation.

7.4. Wash embryos 3x for 10 min per wash with 200 µL of 0.1% v/v polysorbate 20 in fresh PBS.

7.5. Store fixed embryos in 0.1% v/v polysorbate 20 in PBS at 4 °C before proceeding with the immunofluorescence protocol.

8. Single cell digestion with Trypsin

NOTE: Fresh (not fixed) embryos are used for single cell digestion.

8.1. Wash the embryo once with 200 µL of PBS and add 200 µL of trypsin solution to each well. Return the chambered coverslip to the incubator for 5 min.

8.2. Remove the chambered coverslip from the incubator and examine the embryos under a stereoscope. Cells on the periphery of the embryo will start to retract and MTB should still be attached to the plate where they are remotely located from the embryo.

8.3. Use a small pipette or finely pulled mouth pipette to pick up individual MTB before breaking apart the whole embryo. Skip ahead to step 9.1 to save MTB and return to step 8.4 after step 9.3.

8.4. Use a handheld micromanipulation pipette or mouth pipette to gently dissociate the embryo by aspirating up and down.

8.5. Cells will begin to dissociate from the whole embryo. Continue to aspirate the embryo gently and repeatedly using a smaller diameter pipette tip or mouth pipette until the embryo has been incubated for a total of 10 min in trypsin.

9. Single cell selection and sample collection

9.1. With minimal trypsin, move the dissociated cells through three wash drops of 20 µL PBS + 0.1% polyvinylpyrrolidone (PVP) under embryo culture oil with care not to lose any cells.

9.2. After washing the cells, use a finely pulled glass pipette to select one cell. Carefully pipette the single cell into a sterile 0.2 mL low-bind tube with minimal volume of PBS+0.1% PVP.

9.3. Snap freeze single cells in liquid nitrogen (LN_2), and store in -80 °C for future use.

REPRESENTATIVE RESULTS:

Healthy embryos exhibited continued proliferation over the course of extended culture (**Figure 2B**). Abnormal embryos began to retract from their outer edges and disintegrate (**Figure 2C**). From our experience, approximately 75% of embryos were attached to the bottom of the fibronectin coated dish at 48 h and the attachment increased to approximately 90% by 72 h in culture. The success of embryo attachment may be largely impacted by the initial quality of the blastocysts. Embryos not attached by 72 h likely will not survive.

At D8 post fertilization (D3 of extended culture), most cells in the embryos were CTB that were positive for trophoblast marker GATA3 (**Figure 3A**). Cytotrophoblasts had already began to differentiate into multinucleated STB on the periphery of the embryo (**Figure 2B**, left panel, dotted line). These STB had a sheet-like appearance and were stained positive for the human chorionic gonadotropin subunit beta (hCGB; **Figure 3A**, center panel). The embryos quickly grew, between D8 and D10, suggesting a rapid cell proliferation of CTB during this period. At D10, the formation of CGB positive MTB was at a maximum, which could be confirmed by the upsurge of hCG production at this time (**Figure 3B**). Migratory trophoblasts also began to emerge and migrated away from the embryo body and were stained positive for the EVT marker, HLA-G (**Figure 3A**, right panel). By D12, STB differentiation was in decline and MTB production became more prominent, suggesting a shift of emphasis from hormone production on D10 to cell migration on D12. These changes were observed by time-lapse video obtained during this peri-implantation period (**Movie 1**). Our single cell RNA sequencing data also reflected such dynamic changes in cell function. Together, these data suggest early trophoblast differentiation and the emergence of the early placenta is a dynamic process, during which the embryo can prioritize highly specialized cell functions at very specific time points to achieve successful implantation.

FIGURE LEGENDS:

Figure 1: **Procedural schematic of extended culture and single cell sample collection.** Workflow of warming vitrified embryos, zona pellucida removal, extended embryo culture, enzymatic digestion, and isolation of cytotrophoblast (CTB), syncytiotrophoblast (STB), and migratory trophoblast (MTB). This figure has been modified from West et al.[12].

Figure 2: Morphologies of trophoblast cells and embryos during extended culture (**A**) Representative images of different trophoblast cell types after enzymatic digestion with trypsin. Small CTB on the left, large STB in the middle, and MTB on the right. (**B**) Representative images of healthy embryos at D8 (left), D10 (middle) and D12 (right). Dashed lines in left panel outline presumably proliferative CTB population whereas dotted line outlines the flattened STB. Pink circles in the right panel outline MTB that were remotely located from the embryo. (**C**) Representative images of abnormal embryos beginning to retract and disintegrate at D8 (left), D10 (middle), and D12 (right). Scale bars = 200 µm. Some images have been adapted from West et al.[12].

Figure 3: Representative images of trophoblast marker expression and hCG production during extended culture. (**A**) Representative immunofluorescence images of embryos showing

expression of a CTB marker GATA3 at D10 (left), STB marker CGB at D10 (middle), and MTB marker HLA-G at D12 (right). Scale bars= 200 µm. (**B**) Representative hCG concentration (mIU/mL) over the course of extended culture from D8 to D12 (mean± SEM, n = 3). Statistical analysis was performed with One-Way ANOVA followed by Tukey's test ($P < 0.05$). These images have been adapted from West et al.[12].

Movie 1: Time-lapse video of a human embryo in extended culture from D8 to D12. The video demonstrates the collapse of the blastocoel, the formation of the STB (indicated by the green circles), and then eventual differentiation and migration of MTB (indicated by the orange circles). This has been adapted from West et al.[12].

DISCUSSION:

The development of the protocol to culture human embryos through implantation has allowed scientists to explore a previously uncharted time in development[8,9]. Here, we use an extended culture system to culture human embryos and study early trophoblast differentiation before the formation of villous placenta. The methods described here allow us to collect different TB sublineages for use in downstream single-cell analysis. This work allows the scientific community a chance to understand this critical and enigmatic period in human development, and may open new opportunities for therapeutic treatments for miscarriage or other placental pathologies such as pre-eclampsia, as well as provide new insights about early human development.

This protocol requires skills in quickly manipulating embryos during embryo warming, zona removal and plating to minimize embryo stress prior to extended culture. Minimizing media evaporation and thus an increase in medium osmolality by limiting the time of exposure during media exchange is essential. Taking care to use proper aseptic technique when exchanging medium and moving dishes will help minimize contamination. Stabilizing the dish when in use to minimize any mechanical disturbance once embryos have attached is also important. Embryos that become stressed will start to recede their syncytial projections and begin to apoptose. It is also critical to avoid allowing the meniscus of the culture media to touch the surface of the embryo, and to minimize any oil in the wells. Either of these scenarios may destroy the embryo.

Culturing human embryos beyond the blastocyst stage is a rapidly evolving field. A recent study[13] demonstrated that a novel culture system which contains 10% of an extracellular matrix protein mixture and a medium with additional supplementation of sodium pyruvate, lactate, and rho-associate protein kinase (ROCK) inhibitor is advantageous in modeling in vivo 3D embryonic architectures and yielded significantly more viable embryos at later stage of extended culture compared to the extended culture system described here. Validation of this new system to demonstrate its reproducibility will be required. We believe the procedures described here can be adapted to this new 3D system with minor changes and may, therefore, benefit the advancement in methodology in this field.

This protocol may also be adapted to allow investigation into IVF culture media optimization. We have recently shown that in mouse, embryo development during extended culture may predict the success of fetal development after embryo transfer[18]. Abnormally fertilized and discarded

human zygotes with 3 pronuclei (PN) can be cultured to blastocysts on D5 in different conditions that may then influence their development during extended culture. Donated D5 human blastocysts may be cultured for 48 h in novel conditions before being placed into the extended culture system for performance evaluation. Routine measurements of epiblast cell number, total cell number, outgrowth area, and hCG have allowed our laboratory to better understand how different culture media conditions may better support human pre-implantation embryo development and influence their post-implantation success.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

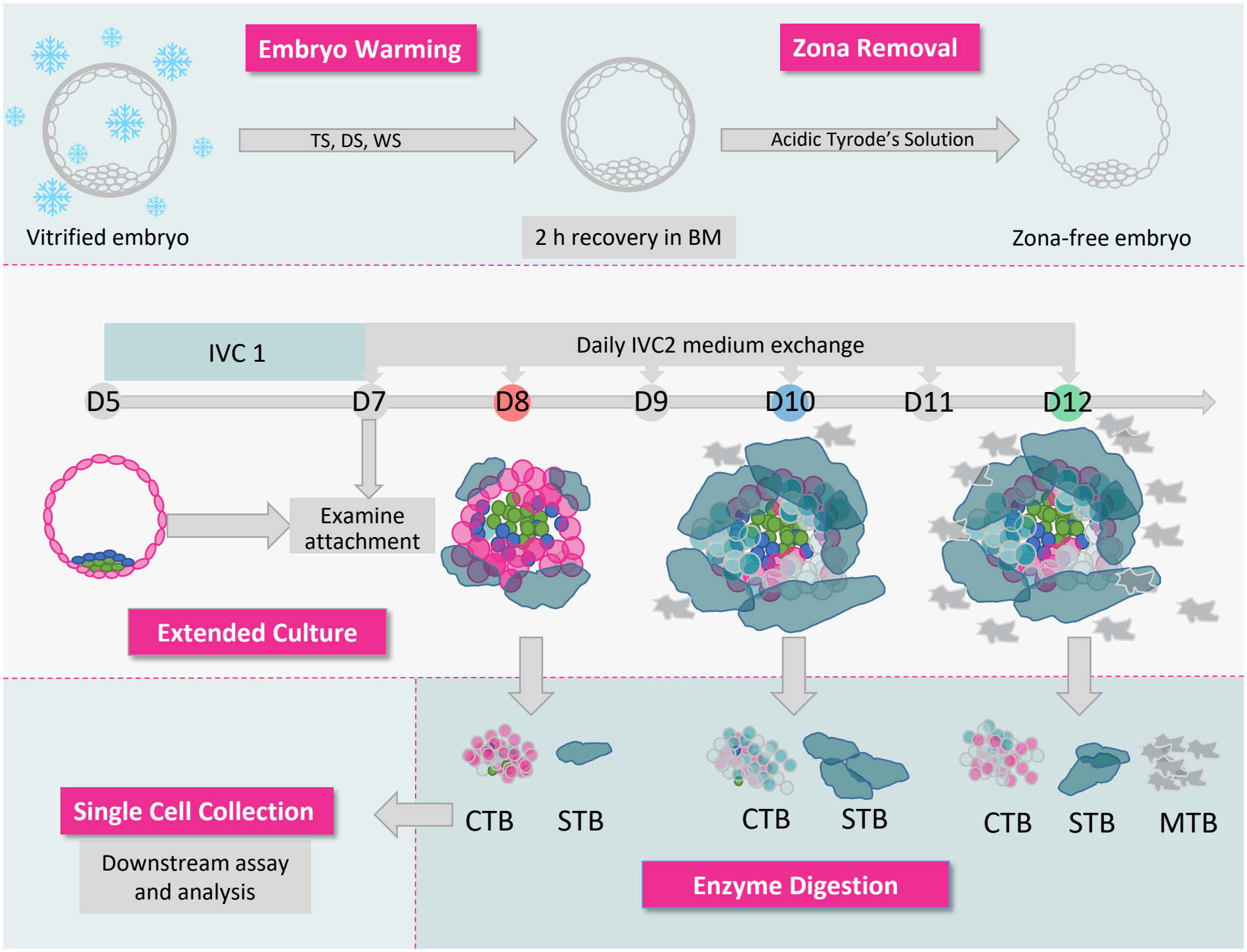
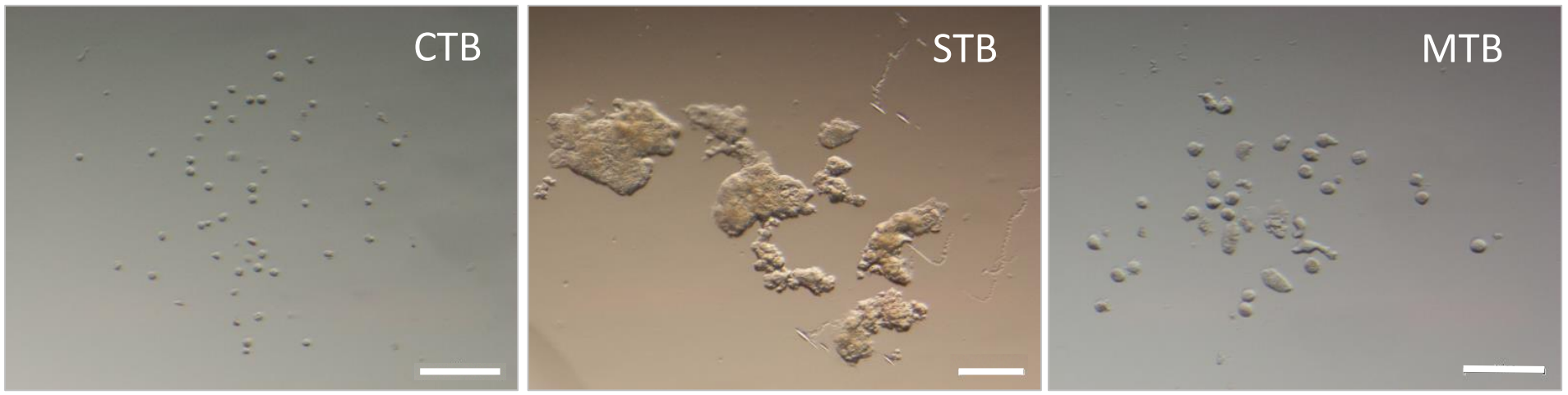


Figure 2

A



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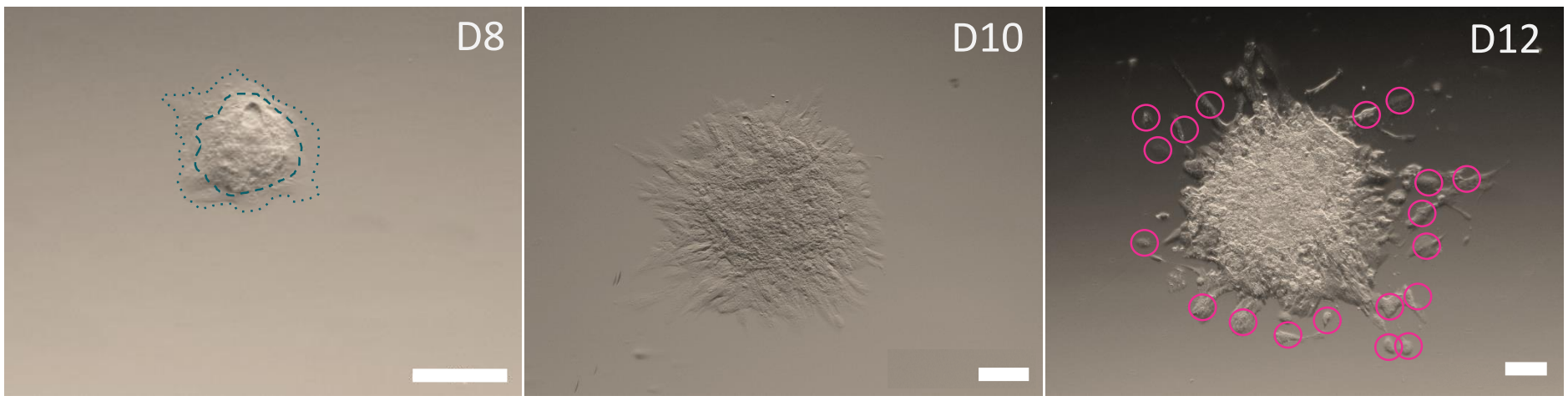

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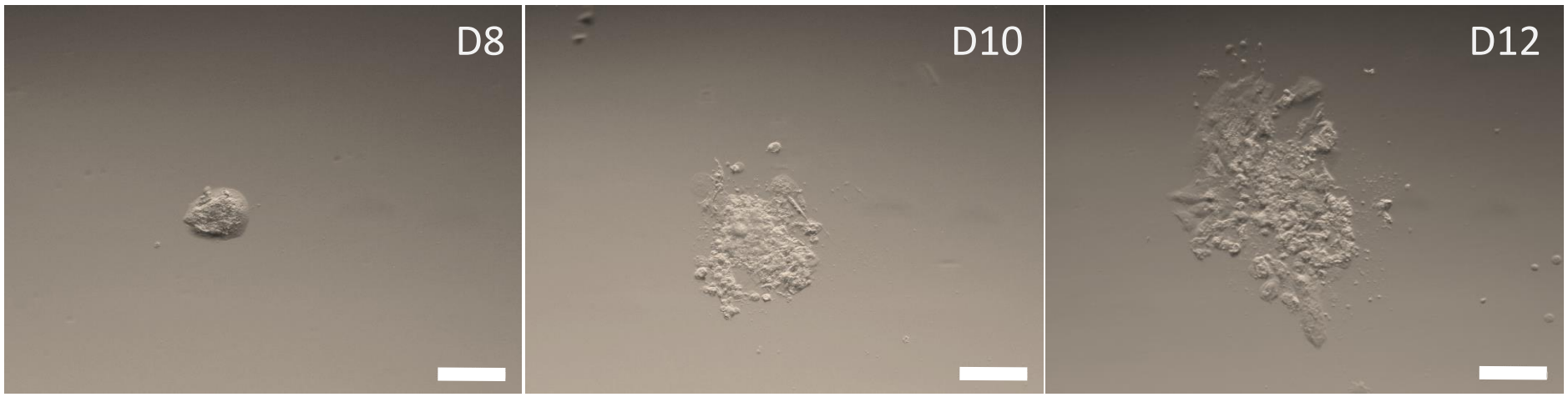
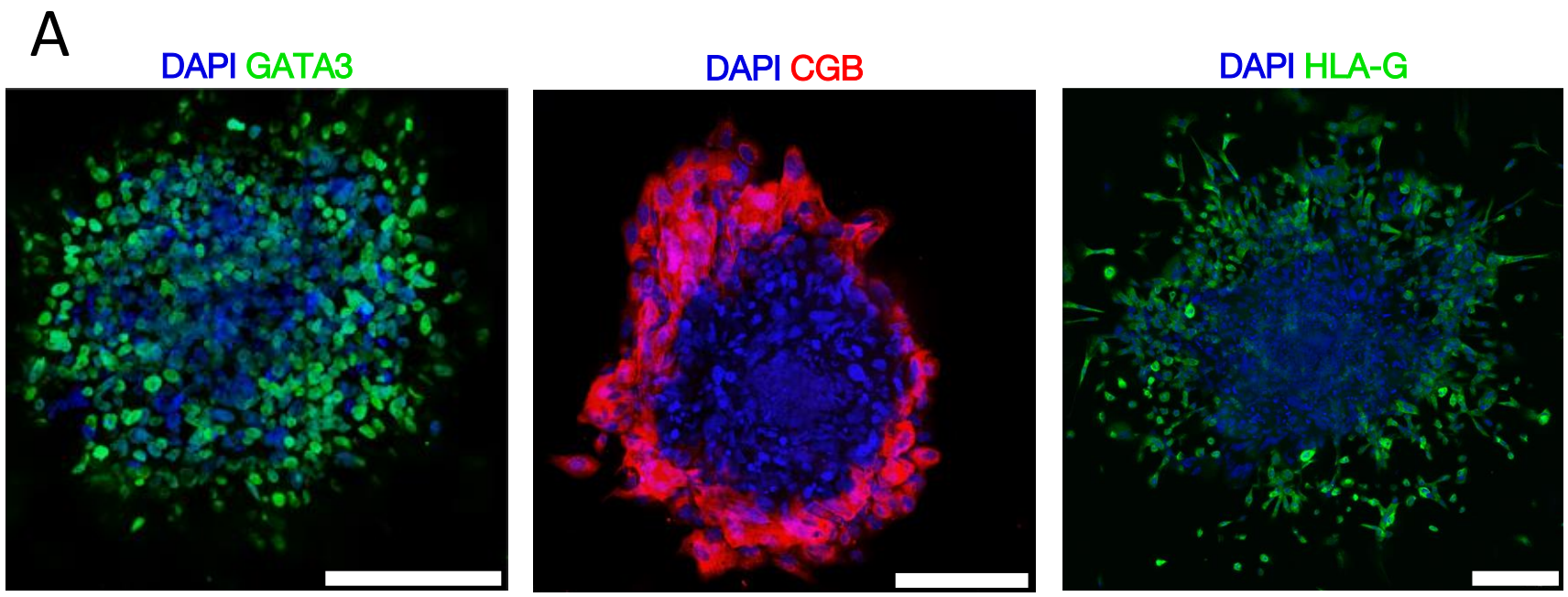
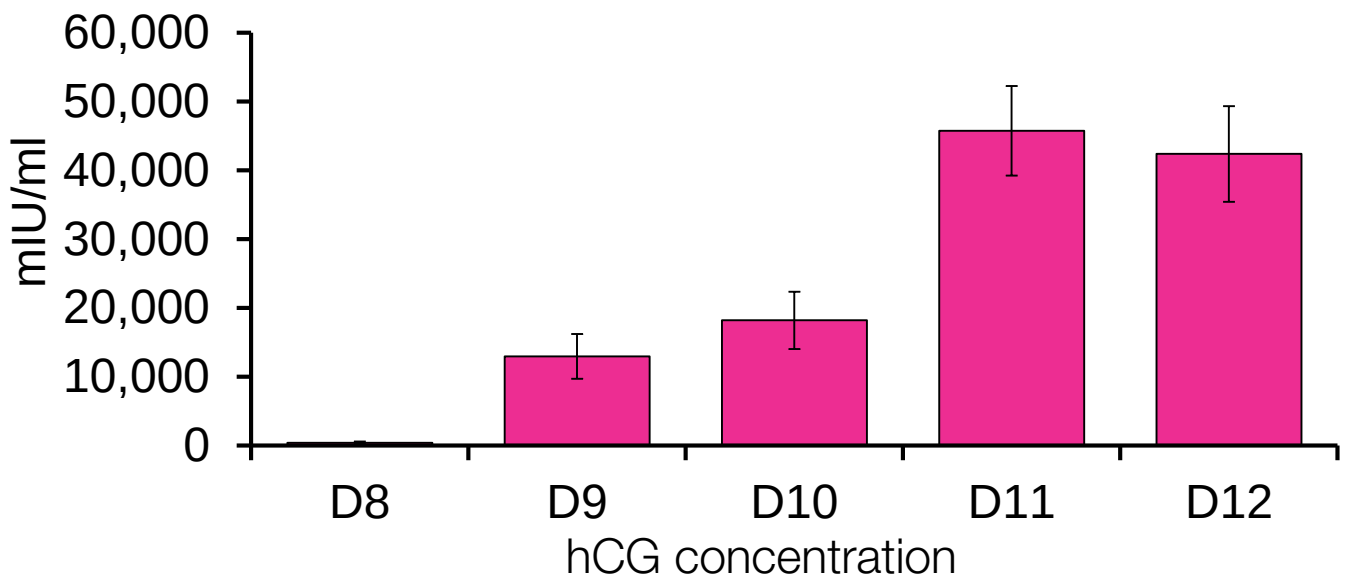


Figure 3





Click here to access/download

Video or Animated Figure

Movie-JoVE.mp4

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
3130 or 3110 Forma Series II water-jacketed CO2 incubator	Thermo Fisher Scientific	13-998-078	
35 mm Corning Primaria tissue culture dish	VWR	62406-038	Case of 500
5 mL snap cap tube	VWR	60819-295	Pack of 25
60 mm Corning Primaria tissue culture dish	VWR	25382-687	Case of 200
6-well dish	Agtech Inc.	D18	Pack of 1, 10, or 50
Acidic Tyrode's solution	Millipore Sigma	T1788	100 mL
Biotix 1250 µL pipette tips	VWR	76322-156	Pack of 960
Blast, blastocyst culture media	Origio	83060010	10 mL
Dilution Solution	Kitazato	VT802	1 x 4 mL
Disposable Borosilicate Glass Pasteur Pipets	Thermo Fisher Scientific	1367820D	5.75 in. (146mm); 720/Cs
Dulbecco's Phosphate Buffered Saline	Millipore Sigma	D8537	
Embryo culture paraffin oil OvOil	Vitrolife	10029	100 mL
Eppendorf PCR tubes 0.2 mL	VWR	47730-598	Pack of 1,000
Eppendorf PCR tubes 0.5 mL	VWR	89130-980	Case of 500
Fibronectin from human plasma. Liquid .1% solution	Millipore Sigma	F0895	1 mg
Gilson 1 mL Pipetteman	Thermo Fisher Scientific	F123602G	1 Pipetteman 200-1000 µL
Gilson 20 µL Pipetteman	Thermo Fisher Scientific	F123602G	1 Pipetteman 2-20 µL
Gilson 200 µL Pipetteman	Thermo Fisher Scientific	F123602G	1 Pipetteman 50-200 µL
G-MOPS handling media	Vitrolife	10129	125 mL
Handling media	Origio	83100060	60 mL
Ibidi 8 well chambered coverslip	Ibidi	80826	15 slides per box
IVC1/IVC2	Cell Guidance Systems	M11-25/ M12-25	5-5mL aliquots
K System T47 Warming Plate	Cooper Surgical	23054	
MilliporeSigma Millex Sterile Syringe Filters with Durapore PVFD Membrane	Fisher Scientific	SLGVR33RS	Pack of 50
Mouth pieces	IVF Store	MP-001-Y	100 pieces
Oosafe center well dish	Oosafe	OOPW-CW05-1	Case of 500

Quinn's Advantage SPS	Origio	ART-3010	12x 12 mL
Rubber latex tubing for mouth pieces	IVF Store	IVFS-NRL-B-5	5 ft.
Stereomicroscope	Nikon	SMZ1270	
Stripper tips	Cooper Surgical	MXL3-275	20/pk 275 µm
Thawing Solution	Kitazato	VT802	2 x 4 mL
The Stripper Micropipetter	Cooper Surgical	MXL3-STR	
TrypLE Express Enzyme (1X), no phenol red	Thermo Fisher Scientific	12604013	1 x 100 mL
Tween20	Millipore Sigma	P1379-25ML	25 mL bottle
VWR 1-20 µL pipette tips	VWR	76322-134	Pack of 960
VWR 1-200 µL pipette tips	VWR	89174-526	Pack of 960
Washing Solution	Kitazato	VT802	1 x 4 mL

Responses to reviewers' comments

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Response: The manuscript was thoroughly proofread and all spelling or grammar issues were corrected.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Response: The manuscript was formatted as requested.

3. Please make the title concise.

Response: The title was changed to "Single cell collection of trophoblast cells in preimplantation stage human embryos".

4. Please include at least 6 keywords or phrases.

Response: "Trophoblast Cells" was added as a keyword.

5. Please expand all abbreviations during the first time use.

Response: all abbreviations were explained when they first appeared.

6. JoVE cannot publish manuscripts with commercial terms. Please use generic terms instead. Please move all commercial terms to the table of materials. E.g., Ibidi, Cryotop, TrypLE, etc.

Response: All commercial terms were removed from the main text.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: All text in the protocol section was examined and only imperative tense was used.

8. The Protocol should contain only action items that direct the reader to do something.

Response: This has been done.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Response: This has been done.

10. Please include composition of all the buffers if made in house as a separate table in .xlsx format. Please upload this table separately to your editorial manage account and reference wherever applicable.

Response: No in-house media is listed.

11. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: This has been done.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response: We have decreased highlighted text to meet the 2.75 page limitation.

13. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

Response: This has been done.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: We have copyright permission to reuse any figures from our previous publication (https://www.pnas.org/page/about/rights-permissions). This was stated clearly in the letter to the editor and was uploaded as a separate document.

15. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Response: This has been done.

Reviewers' comments:
Reviewer #1:
It was interesting to read the manuscript of Logsdon et al and I have only minor concerns and suggestions.

29 "Human implantation, the apposition, adhesion, and invasion of the blastocyst into the maternal" - the apposition and adhesion are towards the surface epithelium, not the decidua / stroma.

Response: Text was changed to "Human implantation, the apposition and adhesion to the uterine surface epithelia and subsequent invasion of the blastocyst into the maternal decidua, is a critical yet…"

53 "Animal models are inadequate, as human placentation has its own unique features compared to other eutherian mammals" - own unique features such as, please elaborate.

Response: The following text was added "For example, the human placenta invades deeply into the decidua with some trophoblast cells reaching at least the inner third of the uterine myometrium while other cells remodel the uterine spiral arteries. Even our closest evolutionary ancestors, the non-human primates, show differences in placental morphology and trophoblast interactions with the maternal decidual tissues. "

63 "extended culture of peri-implantation stage human embryos was reported in 2016" Extended culture of human blastocyst was reported earlier, here are the references:
* O'Leary et al (2012). Tracking the progression of the human inner cell mass during embryonic stem cell derivation. Nature Biotech. 30: 278-282.
* O'Leary et al (2013). Derivation of human embryonic stem cells using a post-inner cell mass intermediate. Nature Protocols 8: 254-264.

Response: These articles have been added, thank you for this valuable information. The text now reads "They have become an invaluable resource that were used for the derivation of human embryonic stem cells [5], understanding the transition of inner cell mass to embryonic stem cells [6, 7], and more recently, have been successfully cultured until day (D) 13 to remodel human implantation [8, 9]".

71 Figure 1 - graphically Figure 1 is, let's say, not very pretty…

Response: We have reconfigured Figure 1 to improve flow and clarity for the reader. Thank you.

92 What markers (as an example) for each lineage were used to define transcriptionally the STB, MTB and CTB?

Response: CGB, HLA-G, and GATA3 were used as markers for STB, MTB, and CTB sublineages, respectively. These markers were discussed in details in the “Representative Results” section on lines 368-375. There were also stained with immunofluorescence and represented in Figure 3A.

Please, use the same abbreviations throughout the manuscript - for example, SyncytioTB or STB, choose only one.

Response: Changes were made to include only CTB, MTB, and STB.

93-98 There are a lot of statements, more references are needed to support them.

Response: These statements were made based on our findings from single cell transcriptomic data of the early trophoblast cells from extended cultured embryos. We have made detailed discussion on these statements in our original publication (West et al., 2019). No other earlier publications described these trophoblast cells from peri-implantation stage human embryos.

132 "blastocyst culture media (BM)" - what are these media? Reference?;

Response: It is a commercial embryo culture media. This detailed product information is listed in the materials table. A more descriptive name was added to improve clarity in the materials table. The protocol text restricts the use of any commercial names and thus, a generic description was used.

133 "prepare approximately 2 ml of this media" Do you mean medium? How to prepare these media?

Response: Thank you. The text has been changed to read:
“Prepare 2 mL of blastocyst culture media (BM) with 10% v/v serum protein substitute (SPS).

> NOTE: Blastocyst culture media may or may not contain added protein. Here, we used a medium that must be supplemented with 10% of the indicated albumin source. Check the manufacturer guidance for variation in this supplementation.”

138 "In a 60 mm tissue culture dish, layer 8 mL of embryo culture oil and prepare 20 µL drops of BM with 10% v/v SPS to the bottom of the culture plate." So what comes first - place oil and then make 20ul droplets or first the droplets are prepared and then they are covered with oil?

Response: In our lab, we first layer oil before anchoring the drops beneath the oil, as indicated. Text within the note was added to clarify “Drops may also be added to the dish before the oil is layered. Anchoring drops under the oil will help to reduce evaporation and any subsequent changes in osmolarity.”

142 "Equilibrate BM" For how long?

Response: “for at least 4 h.” was added.

145 - IVC1 , How to prepare this medium? Reference?

Response: This is a commercial product. More detailed product information can be found in the table of materials.

150 Equilibrate for how long? What is the reason to use 6% CO2?

Response: “for at least 4 h.” was added.

The original papers (Shahbazi, M. N. et al. 2016, and Deglincerti et al., 2016) used 5% CO2. However, one study found that in vitro culture of human embryo under 6% CO2, resulted in significantly higher developmental grades compared to 5% CO2 (https://www.fertstert.org/article/S0015-0282(08)02195-X/fulltext). 6% CO2 is now regularly used for human embryo culture in IVF clinics. 6% CO2 maintains the media at appropriate pH (7.2-7.4). The most recently published 3D human extended culture protocol also uses 6% CO2 (Xiang et al., 2020). However, no direction comparison between 5% vs 6% CO2 has been made.

176 "dezonated" - I would say removal of Zona pellucida or something like this, dezonated is used in the literature but not very often.

Response: “dezonated” was removed throughout the text.

190 "to retain embryo identity" - What do you mean by "identity"?

Response: Text was changed to read “to keep track of embryo identities as they may relate to patient demographic information in downstream analysis”.

191 "if the technician is comfortable." I am not sure how to comment on this… it just doesn't sound right… it sounds like it is taken from a lab book protocol.

Response: This sentence was removed.

312 "Hold" , use "store".

Response: Done.

326 Where are steps 10.1 and 10.3 ?

Response: They were changed to 9.1 and 9.3.

384 "zonal removal" - zona removal ?

Response: Yes, changed.

Reviewer #2:
Manuscript Summary:
Thank you very much for giving me the opportunity to review manuscript JoVE61476. The aim of this manuscript was to describe the methods of West et al. who showed that characterizing the dynamics of trophoblast differentiation during human implantation. It was interesting to read your findings. The article will be of interest to people in the field.

Minor Concerns:
Introduction
1. Please consider to remove the manuscript of Edwards et al. (reference 1) from reference list (line 54). I think that the citation of this manuscript is not appropriated because this manuscript reported "fertilization and maturation in vitro".

Response: Thank you. This reference is meant to support a statement made later. This redundant reference is now reference 4.

2. You should confirm that reference 5 because reference 5 is identical to reference 1 (line 56).

Response: We deleted reference 1 and reorganized references to make sure they correspond to each statement.

Protocol
3. You should unify the writing of unit (min/h or minute/hour).

Response: All were changed to min/h.

Preparation
4. Please consider to insert the word of "incubator" into following sentence to better understand your protocol.
1.1.4 Equilibrate BM with 10% v/v SPS wash dishes and recovery plate in 37 °C, 6% CO_2, 5% O_2 (line 142).

Response: “an incubator at” was added.

5. Please consider to insert the word of "media" following IVC1 in following sentence like step 1.3.2 because "IVC1" was first used in this manuscript.
1.1.5 Thaw IVC1 in 4 °C or on the bench top. (line 145).

Response: “the first step of extended culture media” was added to 1.1.5 as well as parenthesis surrounding IVC1.

6. Please revise "1 ml" to "1 mL" in following sentence.
1.3.1 Retrieve chambered coverslip with fibronectin and place in laminar flow hood. Remove fibronectin mixture with a 1 ml pipetteman and discard into waste (line 168).

Response: Done.

Warming vitrified D5 human embryos
7. Please insert "space" between "30" and "µL" and between "Bring 300" and "µL" in following sentence.
2.1 Warm 3.0mL thawing solution (TS) in 35 mm dish to 37 °C. Bring 300µL dilution solution (DS) and two wells of 300 µL washing solution (WS) in a six well plate to room temperature (line 184).

Response: Done.

8. Please confirm DS in following sentences. I think WS.
2.7 After 5 minutes, pick up the embryo with minimal volume of WS and move to the top of the last well of 300 µL WS. The embryo will slowly fall and wash through the WS to the bottom. Expel any retained DS from the pipette into an empty well (line 213).

Response: Thank you. This is now corrected.

Recovery of warmed embryos
9. Please inserted space into between "2" and "h" in following sentence.
3.3 Let the warmed embryos recover for 2h in an incubator at 37 °C, 6% CO_2, 5% O_2 (line 230).

Response: Done.

Zona Removal
10. Please confirm "step 2.2" in following sentences. And please insert "space" between "20" and "µL".
4.6 Return the dezonated embryo to the 20µL recovery drop from step 2.2 (line 253).

Response: Thank you. We made corrections and changed to 3.2.

11. Please confirm "step 3.2-3.6" in following sentences. I think "step 4.2-4.6" or "step 4.3-4.6".
4.7 Upon visual examination following zona removal, any embryos with retained zona pellucidae may be further treated with acidic Tyrode's solution if necessary by repeating steps 3.2-3.6. Minimizing exposure to the Tyrode's solution is desired (line 255).

Response: Thank you. It has been changed to 4.2-4.6.

12. Please consider to insert the word of "incubator" into following sentence to better understand your protocol.
5.2 Return Ibidi chambered coverslip to 37 °C, 6% CO_2, atmospheric O_2 for two days (line 266).

Response: “an incubator set to” was added.

Optional Fixation for Immunofluorescenc
13. You should insert "(PFA)" between "paraformaldehyde" and "in" in following sentence.
7.2 Remove all media and slowly add 200 µL of 4% paraformaldehyde in PBS to the well. The embryo will want to stick to the surface of the fluid. Multiple 150 µL washes with 4% PFA before removing all fluid will help to minimize any damage to the embryo (line 303).

Response: Done.

Single cell digestion with Trypsin
14. Please confirm "step 10.1" and "step 10.3" in following sentences because there are not "step 10" in this manuscript.
8.3 Remove the chambered coverslip from the incubator and examine the embryos under a stereoscope. Cells on the periphery of the embryo will start to retract and MTB should still be attached to the plate where they are remotely located from the embryo. Use a small pipette or finely pulled mouth pipette to pick up individual MTB before breaking apart the whole embryo. Skip ahead to step 10.1 to save migratory trophoblast cells and return to step 8.4 after step 10.3 (line 322).

Response: Thank you. We made correction to Step 9.1 and 9.3.

Reference
15. Please confirm "et al." in reference 3, 4, 6, 8, 9, 10, 11 and location of A.D in reference 4. Moreover, punctuation such as period and comma are left out and mistyped in several location in reference section. Please confirm them follow "Instructions for Authors".

Response: Done.

"Instructions for Authors"
Citation formatting: Last name, first and middle initials (if available). List ALL authors. If there are six or more authors, list the first author and then "et al."
"Examples"
Journal article: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).
Book: Kioh, L. G. et al. Physical Treatment in Psychiatry. Blackwell Scientific Pubs. Boston, MA (1988).

Figure Legend
16. There are "Scale bars = 200 μm" 3 times in Figure 2 legend. Please consider to remove 1st and 2nd one.

Response: The first and second ones were removed.

17. What day is in GATA3 picture after insemination? Please consider to insert at D8, D10 or D12 into Figure 3A legend like CGB and HLA-G (line 499).

Response: Thank you. It is a D10 embryo. The information is added in the figure legend.

Reviewer #3:
Manuscript Summary:
Aberrant early TB differentiation may lead to implantation failure, placental pathologies, fetal abnormalities, and miscarriage. Studies of TB development in the peri-implantation stage is important for understanding these diseases using in vitro cultured human embryos. However, the manuscript is very primary. There are some issues that need to be addressed.

Major Concerns:
1) In the abstract, the authors clamed "TB differentiation seems to be less affected". The author should provide more evidences to support the condition can model TB development by evaluating temporal gene expression patterns of TB over development.

Response: The following text was added “as demonstrated by anticipated morphology and gene expression changes over the course of extended culture.”

2) The authors should use the OCT4, GATA6 and CK7 to evaluate whether cultured human embryos have normal development lineages, as described in Fig. 2B. Only morphology observation is not enough to determine the health state of cultured embryos. The communications between hypoblast, trophoblast and epiblast are very important for development and function of trophoblast. Any abnormal development may result in the abnormal trophoblast;

Response: Thank you for this comment. We agree that the interactions between different cell types orchestrated the normal development of a human embryo. The goal of this manuscript is to provide a detailed protocol that others could follow and collect single cells for downstream analysis. It is impossible to examine the markers for all cell lineages to ensure the normal development of an embryo and, at meantime, be able to collect unfixed cells for downstream analysis. It is unfortunate that morphology is probably the only criteria we could use to examine the health of an embryo when live staining of these markers is not available. In fact, morphology was used as the only criteria to determine the normal development of human embryo during extended culture until day 14 in the most recent publication (PMID: 31830756, Extended Data Fig. 1b and 1c).

3) STB is multinucleated cells. More clear pictures should be provided to evaluate the presence of STB.

Response: We have provided a new image for STB in the revision.

4) CGB and HLA-B represent STB and migrating TB cells, respectively. Double staining pictures should be provided to make sure whether they are different cell types.
Response: We did not perform double staining of CGB and HLA-G in our study. However, these markers were identified based on our single cell transcriptomic data published earlier (PMID: 31636193). This was also supported by findings made by other groups (PMID: 31830756; PMID: 31596842).

Minor Concerns:
Fig. 3B, statistical analysis should be provided. Experiment reproductivity should be validated.
Response: This information was included in the figure legends.

Reviewer #4:
Manuscript Summary:
The JoVE manuscript by Logsdon et al. describes 2D culture protocol of blastocysts into 14-day old peri-implantation embryoids. This is based on their recent paper published in the PNAS (2019) that studies trophoblast differentiation of human embryos in vitro.

Major Concerns:
As a JoVE paper, we wish to see more video clips that describe detailed procedures. Therefore, I suggest that the authors make more video clips available, so that other investigators can easily follow their experimental procedure. The authors may refer to videos on the JOVE website, especially in the area of stem cells and developmental biology (ISSUE 121 DOI: 10.3791/55268 Wang, J., Anguera, M. C. In Vitro Differentiation of Human Pluripotent Stem Cells into Trophoblastic Cells).

Response: JoVE will be sending out a video crew to record the protocol professionally. Highlighted text serves as the captions for the video that will be filmed upon the acceptance and completion of the peer and editorial review process.

Minor Concerns:
1 As the authors commented, recent 3D cultures of blastocyst embryos into 14 days may be more optimal. Would the authors discuss the pros and cons of 2D and 3D cultures in more details?

Response: The 3D culture protocol just came out early this year and our group has not had a chance to test the 3D protocol yet. Therefore, it will be difficult for us to make an objective comparison between these two protocol procedures in details. A number of advantages of 3D

culture were stated in their original publication (PMID: 31830756). We are looking forward to validating this 3D system and demonstrate its reproducibility. However, we have conducted a large amount of work using this 2D culture system including the work presentedin our PNAS publication, and as such we believe the 2D system has significant merit.

2. The authors listed their culture condition as 37 °C, 6% CO2. Is the 6% CO2 concentration essential when compared to a 5% CO2 condition? Did the authors have some data with 5% CO2, which is more frequently used in many other cell and tissue cultures.
Response: The original papers (Shahbazi, M. N. et al. 2016, and Deglincerti et al., 2016) used 5% CO2. However, one study found that in vitro culture of human embryo under 6% CO2, resulted in significantly higher developmental grades compared to 5% CO2 (https://www.fertstert.org/article/S0015-0282(08)02195-X/fulltext). 6% CO2 is now regularly used for human embryo culture in IVF clinics. 6% CO2 maintains the media at appropriate pH (7.2-7.4). The most recently published 3D human extended culture protocol also uses 6% CO2 (Xiang et al., 2020). However, no direction comparison between 5% vs 6% CO2 has been made.

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