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Optimization of Renal Organoid and Organotypic Culture for Vascularization, Extended Development and Improved Microscopy Imaging.

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TITLE:

Optimization of Renal Organoid and Organotypic Culture for Vascularization, Extended Development, and Improved Microscopy Imaging

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KEYWORDS:

kidney, organ culture, time-lapse, organoid, imaging, chick CAM, chicken embryo, chick ex ovo culture, vascularization, xenotransplantation

SUMMARY:

This work describes two methods for studying organ development, an improved xenotransplantation setup on chorioallantoic membrane (CAM) from avian embryos that allows for vascularization of cultured embryonic organs and organoids and a novel fixed z-direction organ culture method with modified experimental conditions that allows for high-resolution time-lapse confocal imaging.

ABSTRACT:

Embryonic kidney organotypic cultures, and especially pluripotent stem cell-derived kidney organoids, are excellent tools for following developmental processes and modelling kidney disease. However, the models are limited by a lack of vascularization and functionality. To address this, an improved protocol for the method of xenografting cells and tissues to the chorioallantoic membrane (CAM) of an avian embryo to gain vascularization and restoration of blood flow was developed. The grafts are overlaid with custom-made minireservoirs that fix the samples to the CAM and supply them with culture medium that protects the grafts from drying.

The improved culture method allows xenografts to grow for up to 9 days. The manuscript also describes how to provide optimal conditions for long-term confocal imaging of renal organoids and organotypic cultures using the previously published Fixed Z-Direction (FiZD) method. This method gently compresses an embryonic organ or organoid between a glass coverslip and membrane in a large amount of medium and provides excellent conditions for imaging for up to 12 days. Together, these methods allow vascularization and blood flow to renal organoids and organotypic kidney cultures with improved confocal imaging. The methods described here are highly beneficial for studying fundamental and applied functions of kidneys ex vivo. Both methods are applicable to various types of tissues and organoids.

INTRODUCTION:

Organotypic culture of embryonic kidneys became an important model to study nephrogenesis decades ago[1-3]. Renal organoids represent an advanced model system for studying development of healthy and diseased kidneys[4]. The main drawback for both methods, however, is that neither method recapitulates the main function of the kidney: blood filtration. Nephrons and renal vasculature develop in renal organoids and organotypic cultures similarly to early stage in vivo development; however, the glomeruli formed in vitro remain avascular[5]. Vascularization of ex vivo embryonic kidneys and renal organoids was previously demonstrated in transplantation experiments only under in vivo conditions. For example, transplantation of human pluripotent stem cell-derived renal organoids under a mouse kidney capsule allows development of the nephrons in the organoid to a functional stage[6].

An intermediate approach between purely in vitro cultures and in vivo transplantation methods is xenotransplantation to the CAM of avian embryos. Vascularization of intact mouse kidney primordia has been demonstrated previously using this system[7-8]. However, it was also shown that the renal vasculature in the xenotransplanted murine kidney was derived from the host endothelium, not the graft[9]. This observation significantly reduced the potential of chimeric (avian-mammalian) models of embryonic kidney to study development of the renal vasculature, because the experimental conditions were nonpermissive for the survival of donor-derived endothelial cells.

Presented in the first part of this protocol is an improved method for cultivation of mouse embryonic kidneys on CAM of avian eggs, combining microenvironmental conditions of organotypic culture and xenotransplantation. The main improvement to previous methods is that instead of placing the mouse embryonic kidneys and renal organoids directly on the CAM, the implantation area is overlaid with permeable minireservoirs filled with culture medium that supply the transplanted tissue with nutrients and protect it from drying. The success rate of the experiments significantly increases and the conditions for development of donor-derived vasculature improve. Application of this method to xenotransplant cultures results in the development of glomerular vasculature comprised of endogenous endothelial cells from donor kidneys.

Detailed analysis of cellular morphogenesis is another important application of kidney culture models. Previously reported methods of time-lapse image acquisition of kidney cultures are

sufficient only for analysis of overall morphology and patterning of embryonic kidney, but not for tracking individual cells[10]. Recently, a novel Fixed Z-Direction (FiZD) method aimed for high-resolution confocal 3D time-lapse imaging of renal organoids and organotypic cultures was described[11]. In this method, the organoids and embryonic organs are gently compressed between a glass coverslip and a permeable membrane of a transwell insert in a custom-designed plate until the thickness of the sample reaches 70 µm, providing optimal optical conditions for imaging. In the second part of the methods, a detailed protocol for the fabrication of a custom-designed plate and the setup of FiZD experiments for long-term organoid imaging is described.

PROTOCOL:

Animal care and procedures were in accordance with Finnish national legislation for the use of laboratory animals, the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS 123), and the EU Directive 86/609/EEC.

1. Fabrication of minireservoirs for cultivation of mouse embryonic kidneys and renal organoids on chicken CAM and setting up xenotransplantation experiments

1.1. Use transwell cell culture inserts designed for 6 well or 12 well plates.

NOTE: Depending on the particular experiment, large or small minireservoirs can be used. It is best to use small minireservoirs for xenografting up to eight embryonic kidneys or when several minireservoirs will be placed on same chicken embryo (e.g., experiment and control samples on same chicken CAM).

1.2. Cut down the sides of the inserts using a rotary multitool with a circular saw blade or a hand saw to create a 2 mm high plastic ring with a permeable membrane attached to it.

1.3. Polish the edges of these minireservoirs with a scalpel or a sharp knife.

1.4. Sterilize the minireservoirs in 70% ethanol for at least 1 h.

1.5. Wash the minireservoirs in autoclaved double-distilled water and let them dry in a laminar hood.

1.6. Rinse the minireservoirs in PBS and culture medium (high-glucose DMEM/10% FBS/1% penicillin-streptomycin).

1.7. Place the reservoir in a Petri dish, on top of a drop (600 µL/400 µL) of culture medium with the membrane facing up.

1.8. Arrange dissected ex vivo embryonic kidneys or renal organoids evenly on the membrane with the help of a pipette or a glass capillary tube. Avoid leaving a lot of liquid around the kidneys.

1.9. Let the samples attach to the membrane for 2–24 h in a cell culture incubator at 37 °C and 5% CO_2.

NOTE: Up to eight E11.5 embryonic mouse kidneys or kidney organoids can be arranged on the small insert. The large insert is recommended if either bigger pieces of embryonic tissue will be used for xenotransplant or a cell-hydrogel mixture on a larger area of the CAM.

1.10. Take 8-day-old ex ovo cultured embryonic chickens prepared according to previously published methods out of the cell culture incubator[12,13].

1.11. Transfer the minireservoirs to the CAM so that the grafts are facing the CAM, and the membrane overlays them. Place the minireservoirs in the periphery of the CAM, so that they are not covering the chicken embryo.

NOTE: When using the smaller minireservoirs fabricated from transwell inserts for 12 well plates, up to three minireservoirs can be placed on one CAM.

1.12. Add 500 µL (6 well insert) or 300 µL (12 well insert) of culture medium to the minireservoirs.

1.13. Cultivate the samples on chicken CAM for a maximum of 9 days. Replace the culture medium in the minireservoirs daily.

NOTE: Vascularization of the samples can be observed as soon as 24–48 h after transplantation.

2. Fabrication of custom-designed plates and setting up FiZD cultures

2.1. Drill 20 mm diameter holes in the bottom of a 6 well plate.

NOTE: For FiZD experiments, use 6 well plates with a 16 mm well depth (specified in **Table of Materials**).

2.2. Polish the rims of the holes (especially on the upper side) using an electric drill with a countersink drill bit, a scalpel, or a sharp knife.

2.3. Sterilize the plates in 70% ethanol for at least 1 h.

2.4. Wash the plates in autoclaved double-distilled water and let them dry in sterile conditions.

2.5. Thoroughly wash 22 mm x 22 mm glass coverslips with 70% ethanol or clean them according to the published protocol by Saarela et al.[11].

2.6. Glue the coverslips to the upper side of the holes in 6 well plates using a nontoxic tissue glue. Apply glue around the hole and gently place the coverslip to cover it. Let the glue dry.

2.7. Inspect the plates under a stereomicroscope and remove excess dried glue from the surface of the coverslips if needed.

NOTE: Check that there is no glue above the coverslip to ensure even compression of the samples.

2.8. Mix polystyrene beads (70 µm particle size) with an equal volume of hydrogel. A volume of 50–100 µL per well is sufficient.

NOTE: Keep the mixture of hydrogel and polystyrene beads on ice.

2.9. Place a transwell insert under a dissecting microscope with the membrane up.

2.10. Arrange the samples (e.g., ex vivo embryonic kidneys or renal organoids) evenly on the membrane.

2.11. Add the hydrogel/polystyrene bead mixture next to the samples carefully to avoid damage to fragile tissues.

2.12. Flip the transwell insert so that the membrane with the samples assembled on it is facing down.

2.13. Gently place the insert into a well of the modified 6 well plate and gently press it down so the samples are compressed to the level of the beads.

NOTE: Follow the progress of the compression using a microscope.

2.14. Keep the insert slightly pressed to the well with one hand and fix it to the plate by melting plastic with a soldering iron at three points at the periphery of the insert.

2.15. When the insert is fixed to the plate, add 2 mL of culture medium (DMEM/10% FBS/1% penicillin-streptomycin) to the well and continue assembling the remaining wells in the plate in the same way.

2.16. Transfer the finished plate to an on-stage incubator of an inverted microscope for time-lapse imaging.

2.17. Perform time-lapse imaging of the samples using appropriate experimental settings.

NOTE: Changing the culture medium in the wells of the plate during time-lapse experiments is not required but can be easily done through the holes on the sides of the insert.

REPRESENTATIVE RESULTS:

The CAM culture protocol presented here enabled highly efficient vascularization of renal organoids and embryonic kidneys as a result of xenotransplantation on chicken CAM (**Figure 1**, **Movie 1**). Minireservoirs containing culture medium supplied nutrients to donor tissue and protected it from drying during the time period preceding proper vascularization. This method provided permissive conditions for donor-derived endothelial cells to grow. Therefore, renal vasculature in the transplanted kidneys and organoids was represented mostly, though not exclusively, by donor-specific cells (**Figure 2B**,**C**,**F**). More mature glomerular vasculature was obtained in kidneys and renal organoids transplanted to CAM than in renal organotypic cultures (**Figure 2D**,**E**).

The FiZD-protocol is a method designed for long-term time-lapse imaging of ex vivo embryonic kidneys and renal organoids using high-resolution confocal microscopy. In the classical organotypic culture protocol designed by Trowell[14], a specimen is placed on a porous filter, supported by a metal grid, and kept at the air-liquid interface (**Figure 3A**). This method is not optimal either for an upright nor inverted type of microscope. The method presented here was specifically designed to function with an inverted microscope for imaging. The sample was immobilized between a glass coverslip from below and a porous membrane of transwell insert (see above) (**Figure 3B**). The optimal distance between the membrane and the coverslip was ~70 µm. Polystyrene beads were used as spacers to set the thickness of the specimen in this range (**Figure 3B**).

There are no commercially available 6 well plates where the glass coverslip is located at the upper surface of the bottom of the well. Therefore, a protocol was developed for the fabrication of custom-designed plates that have this feature (**Figure 3B**). **Figure 4** shows the representative morphology of an embryonic kidney culture (**Figure 4A**,**D**–**F**) and renal organoids (**Figure 4B**,**C**). **Movie 2** represents the results of high-resolution time-lapse imaging of endothelial cells in mouse embryonic kidney cultured in a FiZD setup. The right panel in **Movie 2** demonstrates that both distribution and migration of individual cells could be observed using this method.

FIGURE AND TABLE LEGENDS:

Figure 1: Murine embryonic kidney xenotransplanted to the chicken embryo CAM. Xenotransplantation of E11.5 embryonic mouse kidneys into 8-day-old ex ovo chicken embryo; a murine embryonic kidney was cultivated for 7 days on CAM. Scale bar = 200 µm.

Movie 1: Blood flow in murine embryonic kidney after xenotransplantation to chicken embryo CAM. A murine E11.5 embryonic kidney was xenotransplanted to the CAM of an 8-day-old chicken embryo and cultured for 7 days. The movie shows chicken blood flow in the xenograft at day 7.

Figure 2: Murine embryonic kidney xenotransplantation on chicken embryo CAM. Murine E11.5 embryonic kidneys (m) were cultivated as an organotypic culture (i.e., control) or xenotransplanted to an 8-day-old embryonic chicken CAM (c) (i.e., experiment). After 5 days of

embryonic kidney cultivation, the control and experimental samples were fixed and stained for the endothelial marker CD31 (red) (**A–E**) and nuclei (Hoechst; blue) (**D**, **E**). (**C**) Anastomoses between chicken and mouse blood vessels (arrowheads). (**D**,**E**) A single confocal slice from the middle of the nephron is shown. (**F**) Xenotransplantation of murine E11.5 embryonic kidney to transgenic GFP-chicken CAM for 7 days, stained for CD-31 (red). Scale bars = **A**–**B** 100 µm; **C**-**E** 20 µm; **F** 50 µm.

Figure 3: Fixed Z-direction culture method. (**A**) Traditional culture method where the sample grows on a porous membrane supported by a grid and holding the explant at the air-liquid interface. (**B**) In the new FiZD setup, the sample was gently compressed between the glass coverslip and a transwell porous membrane. Polyester beads controlled the thickness of the sample adjusted in the z-direction. This figure was modified from Saarela et al.[11].

Figure 4: Embryonic kidneys and kidney organoid development in the new FiZD culture method. Embryonic kidneys (**A**, **D–F**) and kidney organoids (**B–C**) were cultivated using the new FiZD setup. (**A**) Brightfield image of an intact embryonic mouse kidney on day 7 of the FiZD culture. (**B**) Brightfield image of a mouse kidney organoid on day 7 of the FiZD culture. (**C**) MtmG (red) mouse kidney organoid was cultivated for 4 days using the new FiZD setup. Snapshot of the time-lapse image stack shows *Wnt4Cre*-activated GFP (green) expression in the developing nephrons. (**D**) Mouse embryonic kidney was cultivated for 7 days using the new FiZD setup. Six2 (green) and Troma-1 (Krt8, red) staining showed nephron precursors and ureteric bud (UB) bifurcations, respectively. (**E**) Mouse embryonic kidney rudiment was cultured for 12 days using the new FiZD setup. It was stained with Troma-1 (red) and Nephrin (green), and showed UB bifurcations and podocytes, respectively. (**F**) The same sample as in (**E**) with high-power magnification of Troma-1+ (red) UB and the Nephrin (green) + podocytes. This figure was modified from Saarela et al.[11]. Scale bars = **A**-**D**, **F** 100 µm; **E** 1,000 µm.

Movie 2: Renal endothelial cells labelled with GFP grow in the new FiZD setup and can be observed with high-resolution confocal microscopy. Embryonic kidneys from E11.5 *mTmG; Tie1Cre* mice were dissected and grown 6 days using the new FiZD setup. The movie shows *Tie1Cre*-induced GFP expression in endothelial cells. A stack of 20 z-layers was taken using a 10x/0.45 objective, with images obtained every 15 min. In the movie, five GFP z-layers and one bright field focal plane are merged. The panel on the right shows a close-up of a developing kidney. Endothelial cells migrating into the vascular cleft of S-shape stage nephrons can be seen. In the panel on the right, GFP z-layer and one bright field focal plane are merged. The rapidly moving cells are likely macrophages[15]. GFP signal was also expressed by some blood cells. Voxel size 0.69 µm x 0.69 µm x 4.13 µm. This movie has been modified from Saarela et al.[11].

DISCUSSION:

Two detailed protocols are presented that refine the classical renal organotypic culture method, and enable vascularization, extended development, and optimal 4D (i.e., 3D image and time) imaging of ex vivo embryonic kidneys and organoids. This section highlights the critical steps in the methods and discusses troubleshooting.

The significant difference between other CAM culture methods and this improved chicken CAM culture method is the use of custom-made minireservoirs in the xenografting of embryonic kidneys and kidney organoids to the CAM. The membrane bottom of the modified transwell insert presses the samples against the chicken CAM and keeps them in place. The sides of the insert serve as a reservoir for culture medium that protects the graft from drying. Xenografting to ex ovo cultured embryonic chicken CAM is strongly recommended, as this method exposes a larger area of the CAM where the minireservoir can be placed and makes it easy to visually follow the vascularization process[12,13].

A drawback of the chicken CAM culture method is that the time window for performing the xenotransplantation is limited to the 8–9 days when the CAM of the chicken embryo functions before it starts degrading. Grafts needing a longer time to develop should therefore be vascularized by xenotransplantation to a different tissue (e.g., mouse kidney capsule)[6]. Serial xenotransplantation of donor tissue might be a solution to this long incubation time problem. The method could be adapted for time-lapse imaging of the vascularization and development of the graft, but first the requirement for stabilization of the minireservoir and CAM for long-term imaging must be addressed. The main advantage of this improved renal xenotransplantation protocol to chicken CAM is that the method provides conditions for donor-derived endothelial cells to thrive.

The FiZD-protocol describes a method designed for long-term time-lapse imaging of ex vivo embryonic kidneys and renal organoids using high-resolution confocal microscopy. For the setup it is critical that the dimensions of the well and insert match. When a coverslip is glued to the bottom of a 6 well plate and the insert is placed on the well, the membrane of the insert should touch the glass. Then, when the culture is set up, the spacer beads will determine the position of the membrane and consequently the thickness of the cultured organ or organoid. For this reason, it is critical to remove the excess glue and check that no material is restricting the insert from touching the cover glass. It is also important to use nontoxic tissue glue to attach the cover glass. Please note that the glue does not attach very strongly, so it is critical to handle the plates carefully. The plates can be reused, and if the cover glass detaches during washing steps, it can be glued back on.

The FiZD imaging method allows study of nephrogenesis at a single-cell level. The high quality of high-resolution time-lapse images allows the application of automated cell segmentation to study cellular behavior in renal organoids and kidney cultures. Several samples located in different wells of a 6 well plate can be studied simultaneously in different culture conditions. The technique can be easily modified for different sizes and types of tissue and organoids by changing the size of the spacer beads; this was demonstrated with ovarian cell cultures[14]. As a further optimization of this method, combining the FiZD setup with recently published microfluidic approaches[16] might be highly beneficial for high-resolution microscopic analysis of cellular morphogenesis at later stages of kidney development.

One advantage of the FiZD method is that there is plenty of culture medium available and it is not necessary to change it during the 1 week of imaging. Still, if a media change is needed, the

medium can easily be changed from an opening in the wall of the insert. The FiZD method is also superior to other organ culture methods in that it brings the specimen closer to the microscope objective. In other widely used culture methods there is a membrane or filter and a glass plate bottom between the imaged sample and the objective, prohibiting accurate high-resolution imaging[17]. With the FiZD method it is possible to acquire high-resolution images of living tissues.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Saxen, L., Wartiovaara, J. Cell contact and cell adhesion during tissue organization. *International Journal of Cancer*. **1** (3), 271-290 (1966).
2. Saxen, L., Toivonen, S., Vainio, T., Korhonen, P. Untersuchungen über die tubulogenese der niere. *Zeitschrift Für Naturforschung.* **B 20** (4). (1965).
3. Saxen, L. *Organogenesis of the Kidney*, 1st ed. Cambridge University Press, Cambridge (1987).
4. Takasato, M. et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nature*. **536** (7615), 238 (2016).
5. Halt, K. J. et al. CD146+ cells are essential for kidney vasculature development. *Kidney International*. **90**, 311-324 (2016).
6. van den Berg, C. W. et al. Renal Subcapsular Transplantation of PSC-Derived Kidney Organoids Induces Neo-vasculogenesis and Significant Glomerular and Tubular Maturation In Vivo. *Stem Cell Reports.* **10** (3), 751-765 (2018).
7. Sariola, H., Ekblom, P., Lehtonen, E., Saxen, L. Differentiation and vascularization of the metanephric kidney grafted on the chorioallantoic membrane. *Developmental Biology*. **96** (2), 427-435 (1983).
8. Sariola, H. Incomplete fusion of the epithelial and endothelial basement membranes in interspecies hybrid glomeruli. *Cell Differentiation*. **14** (3), 189-195 (1984).
9. Ekblom, P., Sariola, H., Karkinen-Jääskeläinen, M., Saxén, L. The origin of the glomerular endothelium. *Cell Differentiation*. **11** (1), 35-39 (1982).

10. Short, K. M. et al. Global Quantification of Tissue Dynamics in the Developing Mouse Kidney. *Developmental Cell*. **29**, 188–202 (2014).
11. Saarela, U. et al. Novel fixed z-direction (FiZD) kidney primordia and an organoid culture system for time-lapse confocal imaging. *Development*. **144** (6), 1113-1117 (2017).
12. Yalcin, H. C., Shekhar, A., Rane, A. A., Butcher, J. T. An ex-ovo Chicken Embryo Culture System Suitable for Imaging and Microsurgery Applications. *Journal of Visualized Experiments*. **44**, e2154 (2010).
13. Schomann, T., Qunneis, F., Widera, D., Kaltschmidt, C., Kaltschmidt, B. Improved method for ex ovo-cultivation of developing chicken embryos for human stem cell xenografts. *Stem Cells International.* **2013**, 960958 (2013).
14. Prunskaite-Hyyryläinen, R. et al. Wnt4 coordinates directional cell migration and extension of the Müllerian duct essential for ontogenesis of the female reproductive tract. *Human Molecular Genetics*. **25** (6), 1059-1073 (2016).
15. Gustafsson, E., Brakebusch, C., Hietanen, K., Fässler, R. Tie-1-directed expression of Cre recombinase in endothelial cells of embryoid bodies and transgenic mice. *Journal of Cell Science.* **114**, 671–676 (2001).
16. Homan, K.A. et al. Flow-enhanced vascularization and maturation of kidney organoids in vitro. *Nature Methods*. **16** (3), 255-262 (2019).
17. Trowell O.A. A modified technique for organ culture in vitro. *Experimental Cell Research.* **6** (1), 246-248 (1954).

Figure 1

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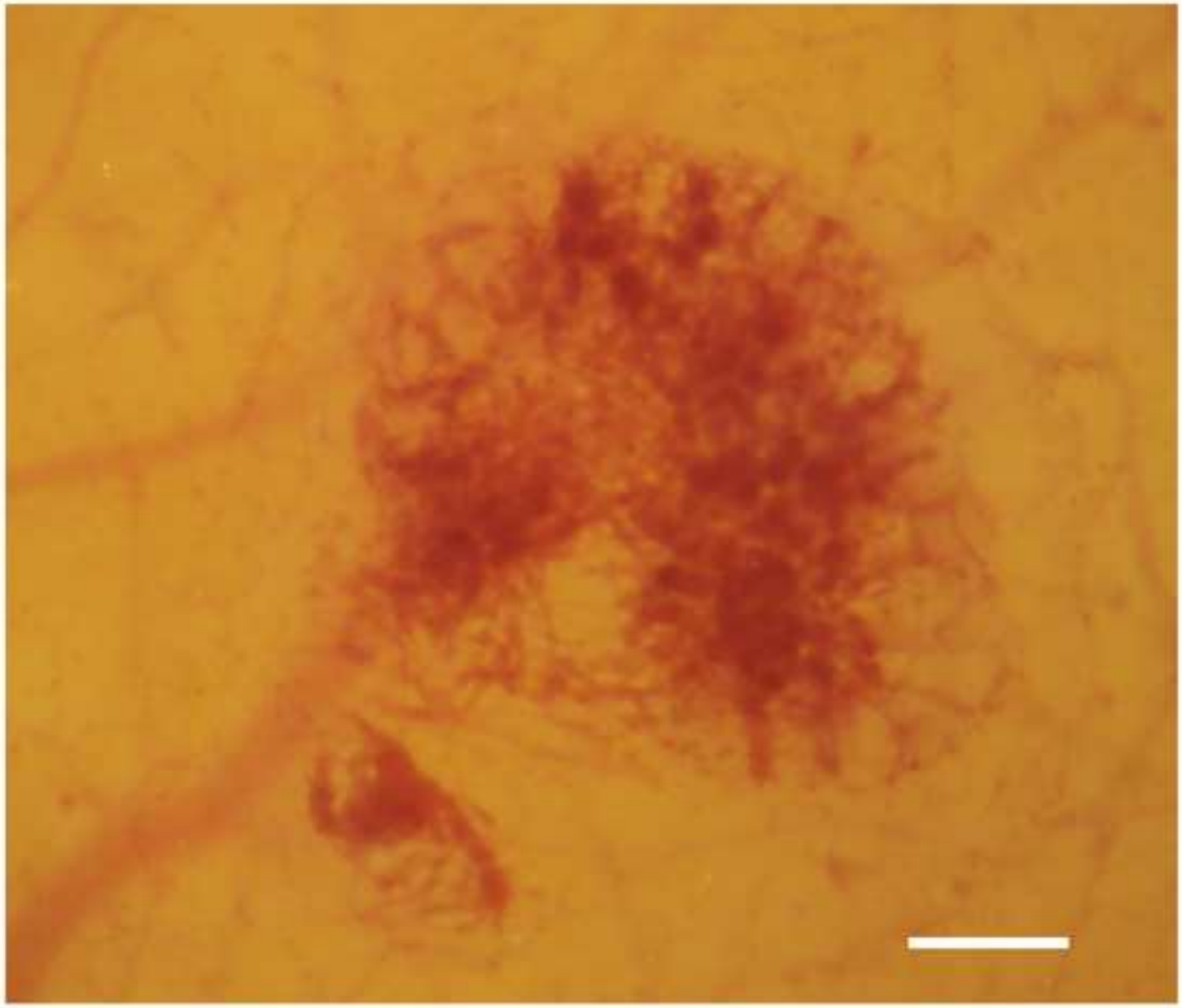

Figure 2

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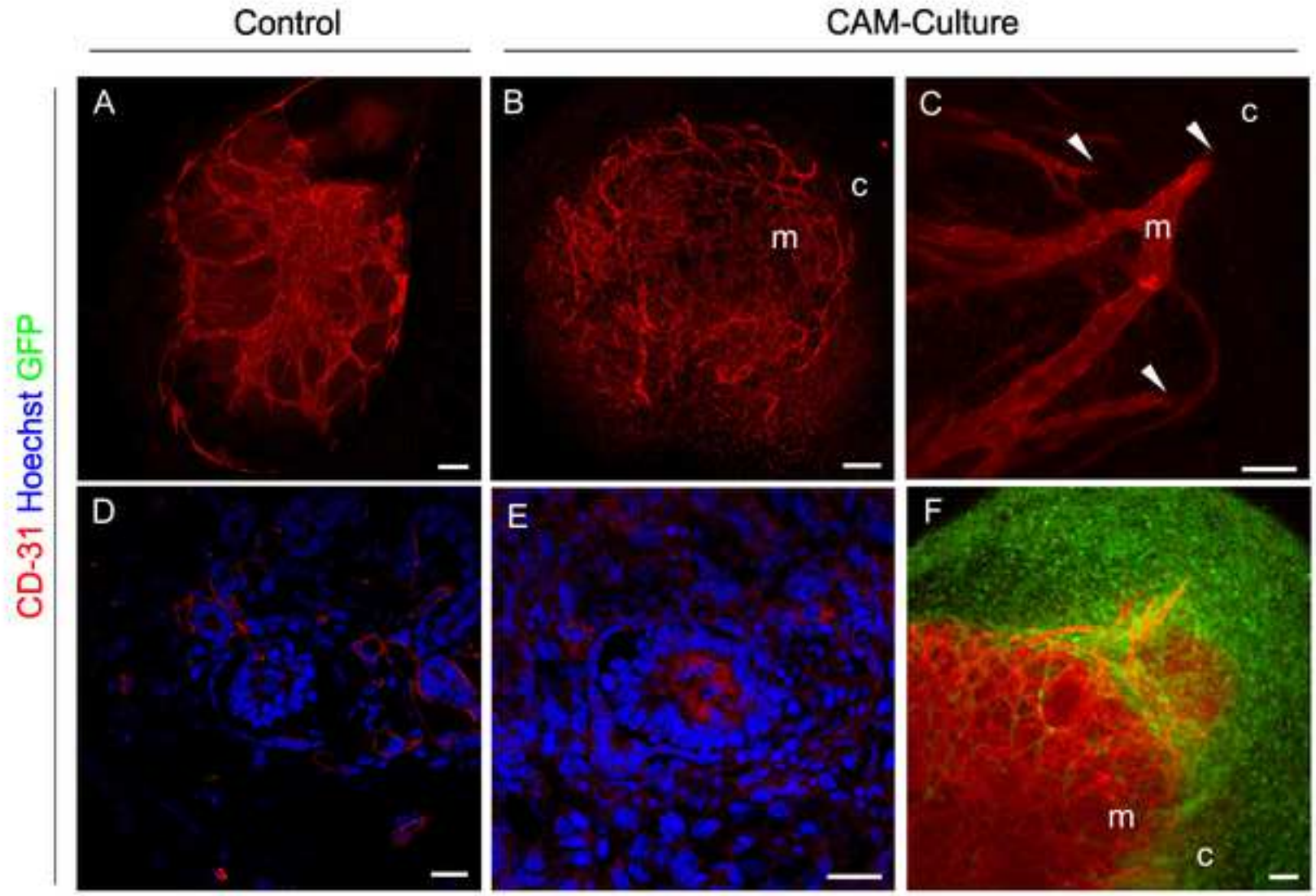

Figure 3

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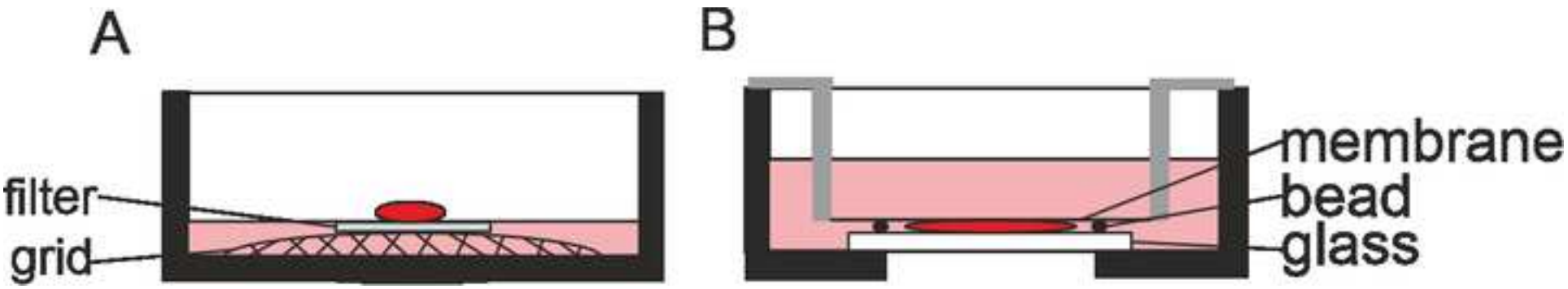

Figure 4

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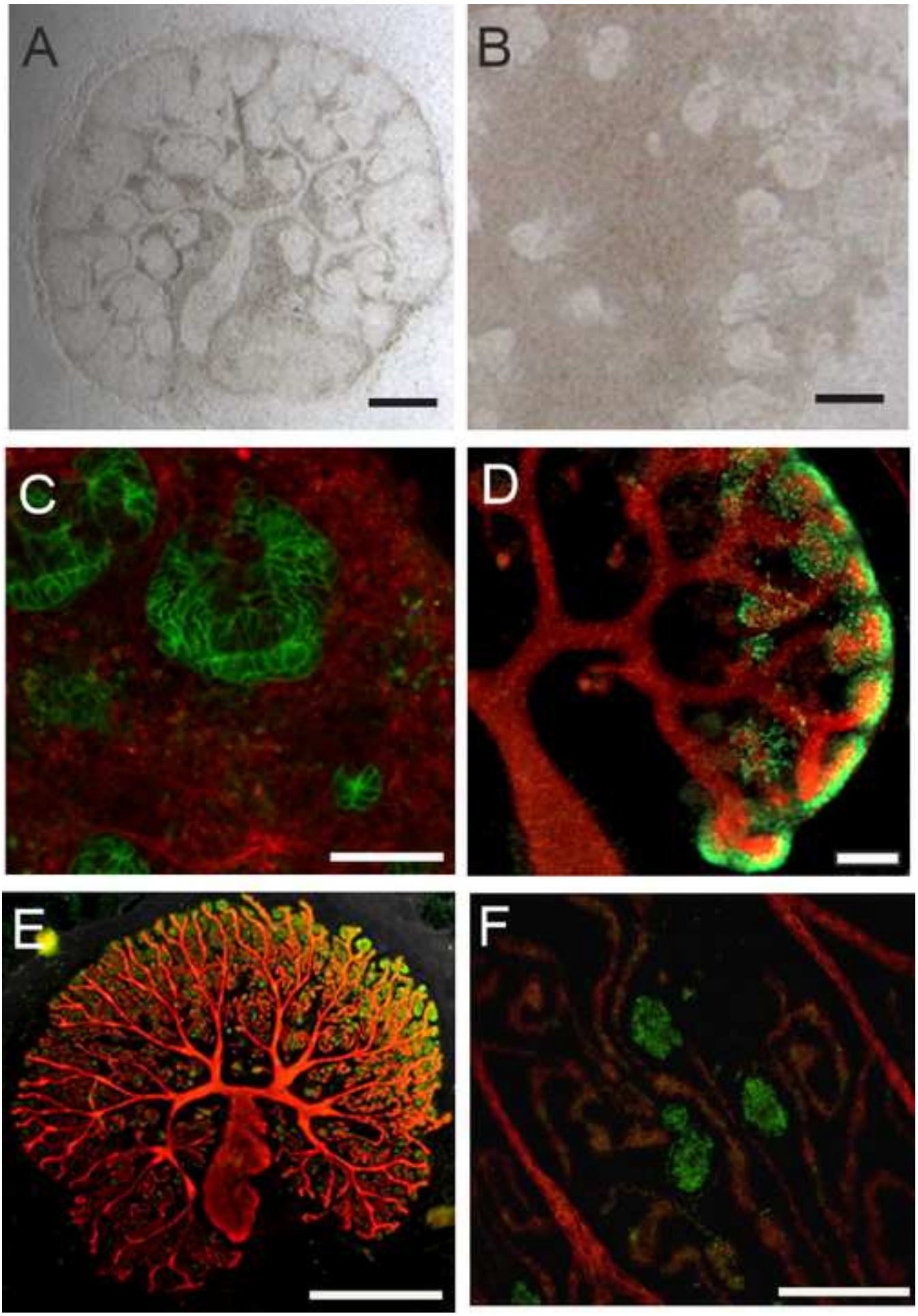

Movie 1

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Video or Animated Figure

Kaisto et al Movie1.mov

Movie 2

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Name of Material/ Equipment

Adjustable Spade Drill Bit
Automatic Egg Turner
Cell Incubator
Cell Incubator
Cellstar 6-well Plate
Circular Saw Blade for Dremel Rotary Tool
Confocal Microscope
Corning Transwell Multiple Well Plate with Permeable Polycarbonate Membrane Inserts
Countersink Drill Bit
Disposable Glass Capillary Tube
Disposable Scalpel
Dissecting Microscope
Drilling Machine
Dulbecco's Modified Eagle's Medium
Egg Incubator Compact S84
Ethanol (70%)
Fertilized Eggs
Fetal Bovine Serum
Forceps DUMONT #5
Glass Coverslips
Histoacryl Glue
Matrigel
On-stage Incubator
PBS -/-
PBS +/+
Penicillin and Streptomycin
Polystyrene Beads
Rotary Multi Tool System
Soldering Iron
Thincert 12-well Cell Culture Inserts With 0.4 µm-pore Polystyrene Membrane
Thincert 6-well Cell Culture Inserts With 0.4 µm-pore Polystyrene Membrane

Company	Catalog number
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Panasonic	13090543
SANYO	10070347
Greiner	M9062
Dremel	SC690
Zeiss	LSM780
Corning	10301031
Craftomat, Bauhaus	22377902
Blaubrand	7087 33
Swann-Morton	0501
Olympus	SZ61
Bosch	GSR 18 V-EC Professional
Sigma	D777
Grumbach, Germany	8012
VWR	
Haaviston Siitoskanala, Panelia, Finland	
HyClone	SH3007003HI Thermo Scientific
Dumont	#5SF
Menzel-Gläser	Menzel BBAD02200220#A1TBCMNZ#0##
Braun	1050052
Corning	356230
Okolab	
Corning	20-031-CV
Biowest	X0520-500
Sigma	P4333
Corpuscular	1000263-10
Dremel	4000
Weller	TCP S
Greiner Bio-One	665641
Greiner Bio-One	657610

Comments/Description

For drilling 20 mm diameter holes

For incubation of eggs before ex ovo setup.

Temperature set at 37° C, 90% humidity, 5% CO_2

Chicken CAM-culture incubator. Temperature set at 37° C, 90% humidity, 5% CO_2

16 mm depth of wells to match with the inserts

For 6-well plates. 40 µm pore size

For polishing (20.5 mm, 1/4", HSS)

High glucose

For incubation of eggs before ex ovo setup. Temperature set at 38° C with relative humidity se

Hy-Line White and Nick Chick

22x22 mm

Boldline, custom made

Washing the mini reservoirs.

70 µm in diameter

Heated glass capillary can also be used

et above 60%

Response letter to the Editorial and production comments:

We would like to thank the editors for the comments on the manuscripts and the video.

Editorial comments:

1. Figure 4: Please explain the colors in the legend.

The colors are now explained in the figure legend.

2. Movie 1: Is this real-time? Also, please explain when exactly this movie was taken (e.g., after the 7-day culture?).

The movie is shot real-time so the chicken blood flow and movement of blood cells to the grafted organ can be seen. The exact time of the filming was added to the movie legend in the manuscript.

3. Video Glitches

a. @12:43: There is a black flash frame here

b. @03:27: Black flash frame

c. @05:57: The shot of the purple glue applicator fades out with a flash into the next shot. Is there an editing glitch here that can be fixed?

The black flash frames were caused by the editing program and they disappeared. The flash was probably caused because of same reason as we cannot see it anymore.

4. Audio Track Issues

a. The interview segments' video and audio are not synchronized. The audio is coming in 2 to 4 frames early (i.e., the video is coming in 2 to 4 frames late.) Try shifting the audio forward 2 to 4 frames see what looks best.

The interviews were re-filmed and new synchronization was done carefully.

b. There are several audio pops throughout the video. If they are the result of a editing issue that can be easily resolved, do try to remove them. If they cannot be easily removed, then they can be left as-is. Examples, but there may be many more throughout:

i. @00:47 Audio pop on the word "cam".

ii. @01:15 on "confocal"

iii. @01:40 on "made"

iv. @01:46 on "publication"

v. @01:54 on "organogenesis"

vi. @02:45 on "polish"

vii. @03:00 on "mini reservoirs"

viii. @04:45 on "cultivate"

ix. @05:40 on "dry them"

x. @05:43 on "wash"

xi. @05:53 on "glue"

xii. @07:32 on "kidney"

xiii. @10:36 on "membrane"

We have found out that the audio pops are caused by the editing program (Corel Video Studio X9). The sounds are not present in the original video or audio tracks and they appear when editing to random places. We were able to remove some of them but unfortunately not all.

c. @07:59: Some extra audio on the narration track (lip smacks, objects moving on the table, or mouth sounds) that can be removed

These sounds have been removed.

5. Editing Style & Pacing

a. @00:14: For the "punch-in" style edit on Vainio's face, his head seems to fall too perceptively low in the frame. Use a common eye-line (line up the eyes from shot to shot) to better achieve this edit.

We have re-filmed this part and paid attention that the eye-line is almost the same in both frames.

6. Text Placement, Size, and Style

a. 02:14: Fade this name tag in, as you did with Vainio's

This has been modified accordingly.

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