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TITLE:

An *Ex Vivo* Tissue Culture Model for Fibrovascular Complications in Proliferative Diabetic Retinopathy

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Ex vivo culture, fibrovascular tissue, proliferative diabetic retinopathy, patient-derived clinical samples, vasculature, disease pathophysiology, three-dimensional, whole-mount immunofluorescence, vitrectomy, neovessel.

SUMMARY:

Here, we present a protocol to study the pathophysiology of proliferative diabetic retinopathy by using patient-derived, surgically-excised, fibrovascular tissues for three-dimensional native tissue characterization and *ex vivo* culture. This *ex vivo* culture model is also amenable for testing or developing new treatments.

ABSTRACT:

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and one of the leading causes of blindness in working-age adults. No current animal models of diabetes and oxygen-induced retinopathy develop the full-range progressive changes manifested in human proliferative diabetic retinopathy (PDR). Therefore, understanding of the disease pathogenesis and pathophysiology has relied largely on the use of histological sections and vitreous samples in approaches that only provide steady-state information on the involved pathogenic factors. Increasing evidence indicates that dynamic cell-cell and cell-extracellular matrix (ECM) interactions in the context of three-dimensional (3D) microenvironments are essential for the mechanistic and functional studies towards the development of new treatment

strategies. Therefore, we hypothesized that the pathological fibrovascular tissue surgically excised from eyes with PDR could be utilized to reliably unravel the cellular and molecular mechanisms of this devastating disease and to test the potential for novel clinical interventions. Towards this end, we developed a novel method for 3D *ex vivo* culture of surgically-excised patient-derived fibrovascular tissue (FT), which will serve as a relevant model of human PDR pathophysiology. The FTs are dissected into explants and embedded in fibrin matrix for *ex vivo* culture and 3D characterization. Whole-mount immunofluorescence of the native FTs and end-point cultures allows thorough investigation of tissue composition and multicellular processes, highlighting the importance of 3D tissue-level characterization for uncovering relevant features of PDR pathophysiology. This model will allow the simultaneous assessment of molecular mechanisms, cellular/tissue processes and treatment responses in the complex context of dynamic biochemical and physical interactions within the PDR tissue architecture and microenvironment. Since this model recapitulates PDR pathophysiology, it will also be amenable for testing or developing new treatments.

INTRODUCTION:

DR is a serious ocular complication of diabetes, a disease that has reached enormous proportions in the last three decades[1]. Twenty years after diagnosis, virtually every patient with type 1 diabetes and 60% of patients with type 2 diabetes present signs of retinopathy, making diabetes *per se* one of the leading causes of blindness in working age adults[2]. According to the level of microvascular degeneration and ischemic damage, DR is classified into non-proliferative DR (non-PDR) and proliferative DR (PDR). The end-stage disease, PDR, is characterized by ischemia- and inflammation-induced neovascularization and fibrotic responses at the vitreoretinal interface. In untreated conditions, these processes will lead to blindness due to vitreous hemorrhage, retinal fibrosis, tractional retinal detachment, and neovascular glaucoma[3,4]. Despite recent advances, current treatment options target only DR stages, including diabetic macular edema and PDR, when retinal damage has already ensued. Moreover, a great proportion of DR patients does not benefit from current treatment armamentarium, indicating an urgent need for improved therapies[4-6].

Multiple other *in vivo* disease/developmental models and diabetic animal models have been developed to date, but none of them recapitulates the full range of pathologic features observed in human PDR[7,8]. Moreover, increasing evidence indicates that treatment responses are tightly connected to the ECM composition as well as the spatial arrangement and interaction between the cellular and acellular microenvironment[9]. We, therefore, set out to develop a clinically relevant model of human PDR by utilizing the FT pathological material that is commonly excised from eyes undergoing vitrectomy as part of the surgical management of PDR[10].

This manuscript describes the protocol for the 3D *ex vivo* culture and characterization of the surgically-excised, PDR patient-derived pathological FT. The method described here has been used in a recent publication that demonstrated successful deconstruction of the native 3D PDR tissue landscape, and recapitulation of features of PDR pathophysiology including angiogenic and fibrotic responses of the abnormal vascular structures[11]. This model also revealed novel features that cannot be easily appreciated from thin histological sections, such as spatially confined

apoptosis and proliferation as well as vascular islet formation[11]. Vitreous fluid has been successfully used by others on 3D endothelial spheroid cultures to evaluate its angiogenic potential and the efficacy of angiostatic molecules[12]. When combined with an *in vitro* 3D lymphatic endothelial cell (LEC) spheroid sprouting assay using PDR vitreous as stimulant, our model revealed the contribution of both soluble vitreal factors as well as local cues within the neovascular tissue to the as yet poorly understood LEC involvement in PDR pathophysiology[3,11]. In the management of PDR, vitreoretinal surgery is a routinely performed yet challenging procedure. As surgical instrumentations and techniques are seeing continuous advancement and sophistication, timely and conservative removal of fibrovascular proliferative specimen not only improves vision outcome but also provides invaluable tissue material for the investigation of PDR pathophysiology and treatment responses in the complex translational aspects of the live human tissue microenvironment.

PROTOCOL:

This research was approved by the Institutional Review Board and Ethical committee of Helsinki University Hospital. Signed informed consent was obtained from each patient.

1. Preparation of Solutions, Media and Equipment

1.1 Prepare the following equipment prior to collection of the fibrovascular tissue (FT) to ensure rapid processing.

1.1.1. Sterile-autoclave two microdissection tweezers.

1.1.2. Prepare 1x phosphate-buffered saline (PBS) by dissolving 1 pre-weighed PBS tablet (0.14 M NaCl, 0.0027 M KCl, 0.010 M PO_4^{-3}) in 900 mL of deionized water and stir to dissolve. Adjust the water volume up to 1 L and sterile-autoclave the ready solution. Store at RT.

1.1.3. Gather the above mentioned solution and equipment in a basket, together with a single-use sterile scalpel, a sterile 6-cm cell culture dish and five sterile 12-well cell culture plates.

1.2. Prepare a 6 mg/mL fibrinogen solution in Hanks Balanced Salt Solution (HBSS) by dissolving 30 mg of plasminogen-depleted human fibrinogen into 5 mL of HBSS, using a 50 mL tube immersed into a water bath at 37 °C, for at least 1 hour.

NOTE: This solution can be kept in the water bath (37 °C) for several hours (maximum 10 hours) before use.

1.3. Prepare the *ex vivo* culture medium by adding 5% fetal calf serum (FCS) or 5% human serum, 0.4% endothelial cell growth supplement, 10 ng/mL recombinant human epidermal growth factor, 1 µg/mL hydrocortisone, and 50 µg/mL gentamycin to commercially available endothelial cell growth medium and keep in water bath at 37 °C until use. Store at 4 °C for up to a month.

2. Fibrovascular Tissue Dissection

2.1. Collect the fibrovascular tissue (FT) that has been excised from human subjects undergoing trans-conjunctival microincision vitreoretinal surgery, by 23 or 20 gauge three-port pars plana vitrectomy as part of the vitreoretinal surgical management of PDR.

NOTE: The FT is excised using segmentation and delamination, cut if needed with microscissors, and removed from the vitreous cavity with intraocular end-gripping microforceps by experienced vitreoretinal surgeon. Extreme care needs to be taken to remove intact, undamaged FT without causing damage to ocular structures including the optic nerve or temporal vascular arcade where the pathological neovascular tissue is most commonly located. The size of the excised tissue is variable, usually ranging between 3 and 50 mm^2.

2.2. Keep the FT in a 6 cm cell culture dish or 1.5 mL tube containing 1 mL of sterile PBS placed on wet ice, and transfer it immediately to research laboratory for processing.

NOTE: It is essential that the research unit (lab facilities) are physically close to the hospital operating room (OR) to minimize the transfer times of the small excised FT. In this case the transfer takes less than 5 minutes and the explants are embedded within fibrin usually in 1 - 1.5 hours (maximum 2 hours) after surgical removal. The impact of longer transfer times on the 3D culture would need to be tested.

2.3. Place the FT in a 6-cm cell culture dish containing 1 mL of PBS at room temperature (RT, 25 °C) and acquire images of the tissue using an inverted epifluorescence microscope with a 5x objective.

NOTE: Images are acquired for qualitative assessment and documentation. It will be possible to observe the tissue size, density and general composition (*e.g.,* vascular structures).

2.4. Cut the FT into ~1 mm^2 pieces under an upright dissection stereomicroscope, using a sterile scalpel and microdissection tweezers. Hold the tissue, well submerged in PBS, in place using a microdissection tweezer, and perform clear cuts using a sterile scalpel. Avoid tearing FT, as this would alter the native fibrovascular structures.

2.5. Place each individual piece into a well of a 12-well cell culture plate containing 1 mL of sterile PBS.

NOTE: If needed, gently spread the FT onto the plate, using microdissection tweezers, prior to performing cuts.

3. Casting Upright Fibrin Gel Droplets for the Characterization of Native FT and *Ex Vivo* Culture

3.1. Sterile-filter the ready fibrinogen solution prepared in step 1.2 with a 0.22 µm filter mounted into a 10 mL syringe.

3.2. Prepare aliquots of the fibrinogen solution (25 µL per 1.5 mL tube) and keep at RT. Prepare an aliquot for fibrin gel/every FT piece obtained after the dissection, and a couple of extra aliquots for testing the fibrin gel formation.

3.3. Prepare a solution of HBSS containing 4 units/mL of thrombin and 400 µg/mL of aprotinin, called hereafter TA solution, and keep at RT. Prepare as much TA solution as needed, depending on the number of pieces obtained after dissection (25 µL of TA solution is used for each FT/fibrin gel), and an extra amount (*e.g.,* 250 µL) for testing the fibrin gel formation and ensuring that enough solution is available throughout casting of the fibrin gel droplets.

NOTE: Thrombin is required for fibrin polymerization while aprotinin is added to prevent degradation of the fibrin gel by cellular proteases expressed by the FTs[13,14].

3.4. Test the timing of fibrin gel formation: add 25 µL of TA solution to the aliquoted 25 µL fibrinogen solution prepared in step 3.2, mix in the tube by pipetting and dispense into a 6 cm cell culture dish, forming an upright droplet.

NOTE: The fibrin gel formation should take place in ~1 min. If this takes over 1.5 min, the concentration of thrombin in the TA solution prepared in step 3.3 can be increased by adding more thrombin stock solution. One tenth of the initially used volume of thrombin stock solution can be added, repeatedly, until the fibrin gel formation occurs within 1.5 min. The time of fibrin gel formation is critical for the three dimensionality of the culture, as quick gel formation (within 1.5 minutes) prevents the FT piece from sinking to the bottom of the fibrin gel, and thus coming in contact with the 2D plastic surface of the cell culture plate, which can lead to 2D cell adhesion and outgrowth.

3.5. Place one FT piece at the center of one well of a 24-well plate using a sterile tweezer and remove any excess PBS by pipetting with a 0.5-10 µL pipette to avoid suction force on the FT. In the case the tissue sticks to the tweezer, use an additional tweezer to aid the placement of the tissue piece on the plate.

NOTE: FT/fibrin gels can also be cast on 48-well plate to reduce the usage of reagents. However, this is more challenging as the space is more limited and fibrin will spread if it comes into contact with the well wall.

3.6. Add 25 µL of TA solution to the 25 µL fibrinogen solution aliquoted in step 3.2, and mix in the tube by pipetting. Dispense onto the FT piece placed on the plate well, and pipette up and down 2- 3 times in order to include the FT piece within the upright droplet, providing a 3D surrounding matrix to the FT.

3.6.1. Ensure that the FT is not aspirated during pipetting and that no air bubbles are formed. Ensure also that mixing, dispensation and pipetting are performed quickly as the fibrin gel will form within 1.5 min, as tested in step 3.4.

NOTE: If casting fibrin gels on 48-well plate, use instead 20 µL of fibrinogen solution and 20 µL of TA solution.

3.7. Repeat steps 3.5 and 3.6 for all the FT pieces.

3.8. Allow the fibrin gels to solidify by incubating the plates in a cell culture incubator at 37 °C in a humidified atmosphere with 5% CO_2.

3.9. After 0.5-1 h, check that the fibrin gel is completely formed by carefully tilting the plate. Proceed to step 3.10 when the gel does not move as the plate is tilted, indicating that the fibrin gel formation is complete.

3.10. Overlay the FT/fibrin gels with *ex vivo* culture medium (600 µL/well in 24-well plate or 300 µL/well in 48-well plate) supplemented with 100 µg/mL aprotinin. Pipette the medium gently by drop-wise dispensation.

NOTE: Here, aprotinin is used to prevent degradation of the fibrin gel by cellular proteases expressed by the FTs[13,14]. The *ex vivo* culture medium can additionally be supplemented with recombinant growth factors, such as VEGFA, VEGFC, bFGF, TGFβ (see **Table of Materials**)[11].

3.11. Place the FT *ex vivo* culture plate back into the 37 °C, 5% CO_2 cell culture incubator and culture for the desired time period (*e.g.*, 2- 18 days, longer culture periods will need to be tested). Replace 50% of the medium with fresh *ex vivo* culture medium every three days.

4. "In Matrix" Imaging

4.1. Acquire images of the FT *ex vivo* cultures on the same day (day 0) and at set intervals (*e.g.,* every other day), using an inverted epifluorescence microscope, in order to follow the 3D growth.

NOTE: The images obtained can be used for the quantification of the FT outgrowth as the total length of two perpendicular diameters across the explant[11].

5. Native FT and *Ex Vivo* Culture End-Point

NOTE: The FT/fibrin gels can be cultured *ex vivo* for the desired time period (FT *ex vivo* cultures) or fixed on the same day (native FT) for native FT characterization[11].

5.1. Fix the native FT or the FT *ex vivo* cultures by replacing the culture medium with 1 mL/well of 4% paraformaldehyde in PBS solution, for 1 hour at RT. For the native FT/fibrin gels, fix 0.5-1 hour after addition of the *ex vivo* culture medium (if the fibrin gels have not been soaked in medium, fixation will damage them).

NOTE: Perform this step in fume hood as paraformaldehyde is corrosive, toxic and carcinogenic.

5.2. Rinse the FT/fibrin gels with three 5 min washes in PBS (2 mL/well).

5.3. Replace the PBS with 2 mL/well of PBS containing 0.02% sodium azide and store the fixed fibrin gels at 4 °C until further processing. Seal the plates with paraffin film to ensure that the FT/fibrin gels do not dry in storage.

NOTE: Perform this step in fume hood as sodium azide is toxic.

6. Whole-Mount Immunofluorescence Staining

NOTE: This protocol lasts 5 days and can be interrupted with overnight incubations where indicated. Do not let the fibrin gels dry at any point. All steps are performed at RT unless otherwise indicated.

6.1. Prepare the following solutions before starting the staining protocol.

6.1.1. Post-fixation solution: prepare 50:50 acetone:methanol solution by mixing equal amounts of acetone and methanol (*e.g.,* 50 mL). Store at -20 °C. Prepare this solution latest on the day before the staining. This solution can be stored back at -20 °C and be used for subsequent stainings.

NOTE: Prepare this solution in fume hood as acetone and methanol are flammable and hazardous to health.

6.1.2. Blocking solution: prepare a 15% fetal bovine serum (FBS), 0.3% octyl phenol ethoxylate solution in PBS by first adding 15% FBS to PBS. Add octyl phenol ethoxylate and stir to dissolve. Store at 4 °C.

NOTE: This solution can be prepared in large amount in advance, stored in 15 mL aliquotes at -20 °C and thawed before use, on the day when the staining protocol is started. Use a freshly prepared or freshly thawed aliquote for each staining protocol.

6.1.3. Washing solution: Prepare 1 L of PBS supplemented with 0.45% octyl phenol ethoxylate by adding 4.5 mL of octyl phenol ethoxylate to 995.5 mL of PBS. Stir to dissolve. Store at RT.

6.2. Lift the droplets carefully from the plate by using a stainless steel square-edged spatula. Detach the droplet first from the edges before lifting the center, and transfer to a well of 12-well plate containing 1 mL of PBS.

NOTE: Perform post-fixation, washes and blocking steps in this plate format.

6.3. Post-fix the droplets with 1 mL of ice-cold 50:50 acetone:methanol solution for ~1 min (the border of the droplets will turn white).

NOTE: Perform this step in fume hood as acetone and methanol are hazardous to health.

6.4. Rinse with 3 - 5 washes in 2 mL of PBS (the droplets will sink in the PBS solution when rehydration is complete).

NOTE: Avoid touching the droplets with the pipette tip as, after post-fixation, they are sticky to plastic. Throughout the protocol, avoid aspirating post-fix, antibody, blocking and washing solutions by suction, as this may damage or completely destroy the droplets. Instead, tilt the plate and carefully remove solutions using a 500-5000 µL pipette.

6.5. Centrifuge the blocking solution for 15 min at 21,000 x g and 4 °C in order to remove debris.

NOTE: The solution can be aliquoted in 1.5 mL tubes for centrifugation. The unused solution can be stored at 4 °C and used for all relevant subsequent steps.

6.6. Incubate the droplets in 500 µL blocking solution for 2 h at RT.

NOTE: To reduce the volume of blocking solution used, the plate can be tilted on a support and as little as 300 µL of solution/droplet can be used.

6.7. Prepare the primary antibody mixture (at least 30 µL/droplet) at appropriate dilution in blocking solution and centrifuge for 15 min at 21,000 x g and 4 °C.

6.8. Transfer the droplets into round (U)-bottom 96-well plate by using a round-edged spatula, and incubate with at least 30 µL/droplet of the primary antibody mixture overnight at 4 °C.

6.9. On the following day, transfer the droplets into 12-well plate containing 2 mL of washing solution/well, rinse with three 5 min washes first and then with nine 30 min washes. Leave the last wash (2 mL) overnight at 4 °C.

6.10. On the following day, rinse three times with PBS and transfer the droplets into round (U)-bottom 96-well plate.

6.11. During the washes, prepare the appropriate fluorophore-conjugated secondary antibody mixture (diluted 1:500, at least 30 µL/droplet) in blocking solution and centrifuge for 15 min at 21,000 x g and 4 °C.

6.12. Transfer the droplets into a round (U)-bottom 96-well plate by using a round-edged spatula and incubate with at least 30 µL of the secondary antibody mixture, for 4 hours at RT, protected from light.

NOTE: Perform all subsequent incubations and washing steps protected from light.

6.13. Transfer the droplets into 12-well plate containing 2 mL of washing solution/well using a round-edged spatula, rinse with three 5 min washes first and then with four 30 min washes. Leave the last wash (2 mL) overnight at 4 °C.

6.14. On the following day, rinse the gels with five 30 min washes and then three times with PBS. Leave the last wash (2 mL) overnight at 4 °C.

6.15. On the following day, transfer the droplets into a round (U)-bottom 96-well plate by using a round-edged spatula and counterstain nuclei by incubating with 10 µg/mL Hoechst nuclear stain (at least 30 µL/droplet) for 30 minutes at RT.

NOTE: Mounting medium supplemented with 4',6-diamidino-2-phenylindole (DAPI) for nuclei counterstaining can alternatively be used. In which case, this step and step 6.16 are skipped, proceeding directly to step 6.17.

6.16. Transfer the droplets into 12-well plate containing 2 mL of PBS/well using a round-edged spatula and rinse three times with PBS.

6.17. Mount the droplets onto microscope slide as follows:

6.17.1. Apply a narrow layer of quick-hardening mounting medium on the edges of a square-shaped 22 mm x 22 mm coverglass and let dry for ~1 minute. Perform this step for each drop individually, to avoid excessive hardening of the mounting medium.

NOTE: Perform this step in fume hood as the quick-hardening mounting medium is hazardous to health.

6.17.2. Rinse the droplet by dipping in a well of a 12-well plate containing 2 mL of deionized water and transfer onto a microscope slide, using a round-edged spatula. Remove excess water by using a small piece of absorbent paper.

6.17.3. Dispense 15 µL of non-hardening antifade mounting medium onto the droplet.

NOTE: Alternatively, if Hoechst nuclear stain has not been used, mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) can be used.

6.17.4. Gently position the cover glass, with the quick-hardening mounting medium facing the microscopic slide, over the droplet and let settle.

NOTE: The quick-hardening medium will stick to the microscopic slide and keep the droplet in place.

6.18. Repeat step 6.16 for all droplets. Mount two droplets on each microscope slide.

6.19. Let the slides air-dry at RT for ~2 h and store at 4 °C overnight. Ensure that the slides are positioned horizontally and protected from light.

6.20. Image the stained native or end-point FT *ex vivo* cultures using a confocal microscope or an upright epifluorescence microscope equipped with an optical sectioning function and 20x or 40x objective. Use tile function to capture a greater area of the tissue at once.

REPRESENTATIVE RESULTS:

Deeper understanding of the PDR fibrovascular tissue properties and protein expression has relied mainly on vitreous samples and thin histological FT sections[3,15-17]. To develop a method for thorough investigation of the 3D tissue organization and multicellular physiopathological processes of PDR, we set out to utilize the surgically excised, patient-derived pathological FTs for 3D characterization and *ex vivo* culture. The fresh FTs are transferred to the research laboratory and processed as illustrated in the schematic workflow in **Figure 1**.

The FTs can be imaged prior to dissection for qualitative assessment and documentation (**Figure 2**). As shown in **Figure 2**, the FTs display great inter-patient variation in size, density and abundance of vascular structures. In some tissues, loose cells, presumably inflammatory/immune cells or red blood cells, as well as pervious vascular structures of different caliber can also be easily distinguished (**Figure 2**). After dissection, a decision needs to be made on the downstream usage of the limiting number of the obtained explants. A portion of the explants is embedded within fibrin matrix and fixed after fibrin gel formation while the remaining explants can be subjected to *ex vivo* culture on a separate plate (**Figure 1**). The fresh FTs have also been successfully utilized for ultrastructural characterization by electron microscopy. The samples can be either processed for conventional transmission electron microscopy (TEM) of ultrathin sections or for serial block face-scanning electron microscopy (SBF-SEM)[3,11].

The fixed fibrin gels can be stored for several weeks and stained by whole-mount immunofluorescence[18,19]. The stained samples are likewise stable when stored at 4 °C in the dark. The thick FT/fibrin gels can be imaged time-efficiently with epifluorescence microscope equipped with an optical sectioning function, useful for removing scattered out-of-focus light. Imaging with this method provides fine visualization of the structures. Alternatively, confocal microscopy, though more time-demanding, can allow the capture of finer details. The characterization of the freshly fibrin-embedded and fixed, uncultured, native FTs by whole-mount immunofluorescence allows the characterization of vascular structures, multiple cell types and their reciprocal arrangement within the 3D PDR tissue landscape[11]. For example, CD31 antibodies can be used to display the endothelium and NG2 to display pericytes (**Figure 3**) while Lyve1 antibodies visualize newly discovered lymphatic-like endothelial structures (**Figure 4**)[11]. Multiple combinations of antibodies can be used to visualize several structures and cell types (see **Table of Materials**); for example, ERG can also visualize the endothelium, which has a discontinuos expression pattern in the abnormal PDR neovasculature. The vascular structures stained with a membrane marker (*e.g.*, CD31 and Lyve1) can be quantitatively analysed for preservation and density by using Angiotool, a free-source software developed by the NIH National Cancer Institute[11,20]. 3D volumes can also be rendered from the dataset obtained (see **Video 1**). If an antibody is not

suitable for whole-mount immunofluorescence, the fibrin droplets can alternatively be embedded in paraffin, cut to thin sections and analyzed by immunohistochemistry. In this case the droplets benefit from overnight fixation at 4 °C instead of the 1h at RT.

Ex vivo culture sustains the growth of the fibrin-embedded FTs, developing cellular outgrowths already after two days (**Figure 5**). The *in vitro* fibrin gel is used as the matrix for *ex vivo* culture, since it typifies the *in vivo* provisional ECM, formed after thrombin cleavage of serum fibrinogen at the sites of vascular leakage, in direct contact with the leaky PDR neovessels in the inflamed fibrotic milieu[15,21,22].

PDR is a microvascular complication characterized by an angiogenic and fibrotic response, and the FT explants retain this cellular repertoire in culture, as well as respond efficiently to exogenous stimuli added to the culture media[11]. The representative data in **Figure 6** shows that the PDR *ex vivo* cultures induced sprouting of the CD31-positive endothelium and vasculature preservation in response to VEGFA, while TGFβ induced a fibrotic response reflected by the outgrowth on NG2-positive pericytes/SMCs. Several exogenous stimuli can be tested and, when informed by measurements of their *in vivo* vitreal abundance, the herein developed PDR *ex vivo* culture model can reveal novel microenvironment and context dependent PDR pathological mechanisms that cannot be visualized in fixed tissue material.

FIGURE AND TABLE LEGENDS:

Figure 1. *Ex vivo* PDR fibrovascular tissue culture model. Schematic representation of the workflow from the vitreoretinal surgery (pars plana vitrectomy) for the excision of the FT to its dissection into explants and embedding into fibrin for three-dimensional characterization and *ex vivo* culture.

Figure 2. Freshly excised PDR fibrovascular tissues prior to dissection. Phase contast micrographs of freshly excised FTs taken prior to dissection. The FTs are highly variable in size, density and abundance of vascular structures. Arrowheads indicate vascular structures of different caliber. The red partially transparent line highlights two individual vessels of different caliber. The images were taken using an inverted epifluorescence microscope with a 5x, 0.15 numerical aperture (NA), objective.

Figure 3. Whole-mount immunofluorescence of a native PDR fibrovascular tissue. Epifluorescence micrograph of a native PDR FT stained by whole-mount immunofluorescence. CD31 (**A**, green) visualizes the endothelium while NG2 (**B**, red) visualizes the pericytes within the irregular neovascular structures. Merged images are shown in (**C**). DAPI (blue) counterstain visualizes nuclei. The image was taken using an upright epifluorescence microscope with optical sectioning function and combined with a computer-controlled 1.3 megapixel monochrome CCD camera and image acquisition software, using a 40x, 1.4 NA, oil objective. Nine optical sections were combined by using image processing software ImageJ.

Figure 4. Whole-mount immunofluorescence of a native PDR fibrovascular tissue. Epifluorescence micrograph of a native PDR FT stained by whole-mount immunofluorescence.

CD31 (**A**, green) visualizes the endothelium while Lyve1 (**B**, red) visualizes the newly discovered lymphatic-like endothelial structures. Merged images are shown in (**C**). Hoechst-33342 (blue) counterstain visualizes nuclei. The image was taken using an upright epifluorescence microscope with optical sectioning function and combined with a computer-controlled 1.3 megapixel monochrome CCD camera and image acquisition software, using a 20x, 0.8 NA, objective. Four optical sections were combined by using image processing software ImageJ.

Figure 5. *Ex vivo* growth of the PDR fibrovascular tissues. Phase contrast micrographs of two FT *ex vivo* cultures at indicated time points (day). The FTs grow upon *ex vivo* culture, developing cellular outgrowths already after two days. The images were taken using an inverted epifluorescence microscope with a 5x, 0.15 NA, objective.

Figure 6. Whole-mount immunofluorescence of PDR fibrovascular tissues cultured *ex vivo* untreated (CTRL), or in presence of VEGFA or TGFβ. Epifluorescence micrograph of FT cultured *ex vivo* untreated (CTRL) or in presence of VEGFA or TGFβ for 9 days and subsequently stained by whole-mount immunofluorescence. CD31 (green) visualizes the endothelium while NG2 (red) visualizes the pericytes. DAPI (blue) counterstain visualizes nuclei. VEGFA stabilized the vasculature while TGFβ induced a fibrotic response. The images were taken using an upright epifluorescence microscope with optical sectioning function and combined with a computer-controlled 1.3 megapixel monochrome CCD camera and image acquisition software, using a 20x, 0.8 NA, objective. Nine optical sections were combined by using image processing software ImageJ.

Video 1. 3D volume reconstruction of a native FT stained by whole-mount immunofluorescence. CD31 (green), Lyve1 (red). Hoechst-33342 counterstain (blue) visualizes nuclei. The image was taken using an upright epifluorescence microscope with optical sectioning function and combined with a computer-controlled 1.3 megapixel monochrome CCD camera and image acquisition software, using a 20x, 0.8 NA, objective. 3D volume reconstruction and video rendering was performed using a commercial software. A portion of the video is reprinted with permission from Gucciardo *et al.*[11].

DISCUSSION:

Considering the importance of relevant tissue microenvironment for reliable functional cell and molecular mechanistic results, it is imperative to find appropriate experimental models that provide this tissue environment. The herein described *ex vivo* PDR culture model for the fibrin-embedded FTs allows the investigation of the mechanisms of PDR pathophysiology in the native, complex and multicellular context of the PDR clinical samples.

Critical steps within the protocol are the proper fibrin gel formation, the positioning of the FT and adequate washing during the staining. Since fibrin gel formation depends mostly on the thrombin activity, affected by the concentration and temperature, the amount of thrombin needs to be adjusted for every batch of fibrin gel preparation. Fibrin gel formation should be quick enough to prevent 2D growth of the FT explants but also slow enough to allow positioning of the FT to the center of the droplets vertically and horizontally. In the case the FT happens to locate at the edge

of the droplet, additional pipetting could aid the placement of the FT to the center. FTs that still remain at the edge of the droplets or that grow in 2D will need to be excluded. Adequate washing during staining and avoiding drying of the droplets are in turn critical in order to optimize staining and minimize background signal. Transfer times may also be critical. We have embedded the FTs up to two hours after vitrectomy and this did not affect the *ex vivo* growth. The impact of longer transfer times on the culture success would need to be tested.

This protocol could be modified by using alternative matrices for FT embedding. *In vivo*, the PDR neovessels grow towards the vitreous cortex rich in collagen[23]. However, upon vascular leakage serum components, including fibrinogen, dissolve into vitreous in close proximity to the vessels whereby the fibrotic response is initiated. Therefore, the *in vitro* fibrin clot was utilized here for the *ex vivo* culture in order to typify the provisional ECM formed in the inflamed fibrotic milieu of PDR[15,21,22]. Alternatively, the fibrin clots could be supplemented with laminin and fibronectin to alter the stability of sprouting capillaries, or the explants could be embedded within type I collagen and other mixed matrices to typify the most fibrotic microenvironments in neovascular tissues. These matrices are generally suitable for 3D culture and whole-mount immunofluorescence but their effects on the PDR FTs remain to be tested and considerations on the timing of 3D matrix formation will need to be made in order to stay within the limits for preventing 2D growth[18,19]. When the physical proximity of the research unit to the hospital represents a limitation, longer transfer times could be compensated with team work; TA solution preparation, fibrinogen sterile-filtration and fibrin gel formation testing can be performed during FT transfer. If the fibrin gels do not form even after 1 hour, a problem with the fibrinogen or TA solution preparation might have occurred. In this case, the FT/fibrin gels cannot be used.

Limitations of this approach, as with every *ex vivo* approach, are the variable quality of primary tissue specimen across individuals as well as intra-patient and inter-patient tissue heterogeneity. In the case of diabetic patients, part of this variability includes the extent of vascularization and fibrosis which depend on numerous factors like duration of the disease, metabolic condition, genetic/epigenetic factors, type of diabetes and systemic therapy. The size of the surgical PDR FT recovered is also a limiting factor in determining the number of conditions that can be investigated for each specimen. While providing reciprocal spatial information, whole-mount immunofluorescence allows the investigation of only a limited amount of features/markers per droplet. As a compromise, the fibrin droplets can alternatively be embedded in paraffin, cut to thin sections and analysed by immunohistochemistry.

Existing diabetic mouse models develop many features of early stage DR but fail to comprehensively recapitulate the progressive changes occurring in human PDR, thus hindering the studies of the PDR disease mechanisms[7,8]. Moreover, the murine eye is fundamentally different from the human eye, in that it lacks the macula, further emphasizing the importance of studying the human disease[24]. The surgical PDR FTs have previously either been discarded, or used for paraffin sections, transmission electron microscopy, serial block face-scanning electron microscopy, bulk RNA sequencing or 2D culture of dissociated cells[3,25]. The herein described model allows the 3D characterization of this precious surgical material, as well as the investigation of PDR pathophysiology in the native diseased tissue microenvironment. When

combined with the use of vitreous fluid, this model also allows the investigation of the contribution of both the cellular and acellular PDR microenvironment. Since the cultures respond efficiently to growth factors detected in the vitreous, such as VEGFA, bFGF, VEGFC and TGFβ, thus recapitulating features of PDR pathophysiology, this PDR *ex vivo* culture model is amenable for testing or developing new PDR treatments[11]. In this model, anti-VEGFA prevented capillary sprouting and induced signs of capillary regression, responses that are consistent with the expected clinical outcomes of anti-VEGFA treatment[26,27]. Therefore, this model can also be used for better understanding the effects of current therapies, including anti-VEGFA and corticosteroid treatments.

With live-cell imaging instrumentation, the herein described *ex vivo* culture model could be subjected to time-lapse microscopy to allow real-time investigation of processes such as vascular regression, sprouting and cellular plasticity. When combined with *in vitro* and *in vivo* models, as well as clinical data, this *ex vivo* PDR model will help in investigating patient responses based on particular sets of identifying markers, a step closer to the avenue for personalized medicine. Identifying patient-specific responses and/or response-specific markers is especially relevant in the case of PDR, which is a multifactorial disease with a complex interplay of microvascular, neurodegenerative, metabolic, genetic/epigenetic, immunological, and inflammation-related factors, thus requiring increasingly multidisciplinary efforts for development of improved therapeutic targeting and disease management. Besides whole-mount immunofluorescence, the *ex vivo* cultured FTs could also be retrieved from the fibrin by plasmin/nattokinase treatment and subjected to transcriptomic and proteomic analyses[28]. The suitability of the herein described model for *ex vivo* studies of fibrovascular tissues developed in other ocular conditions, such as in severe cases of sickle cell retinopathy, could also be explored in the future.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

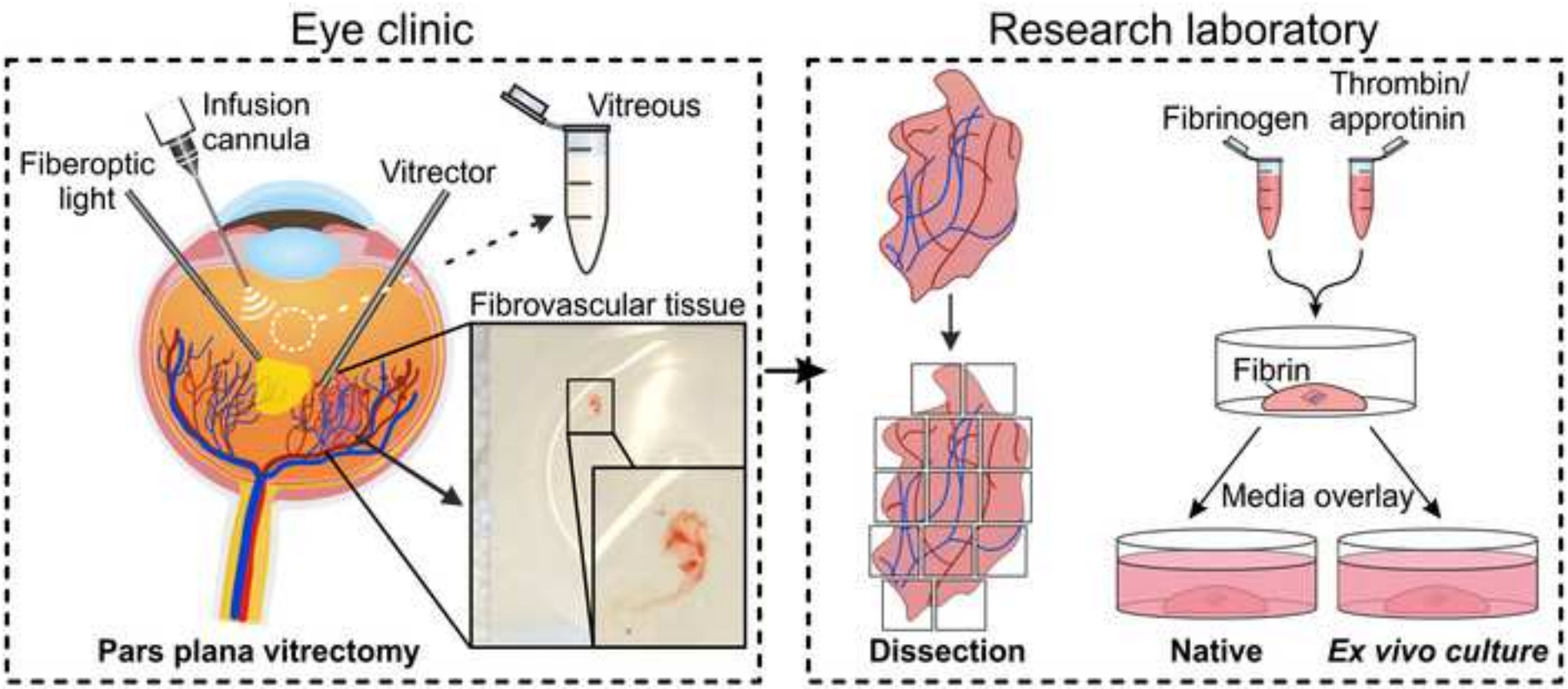
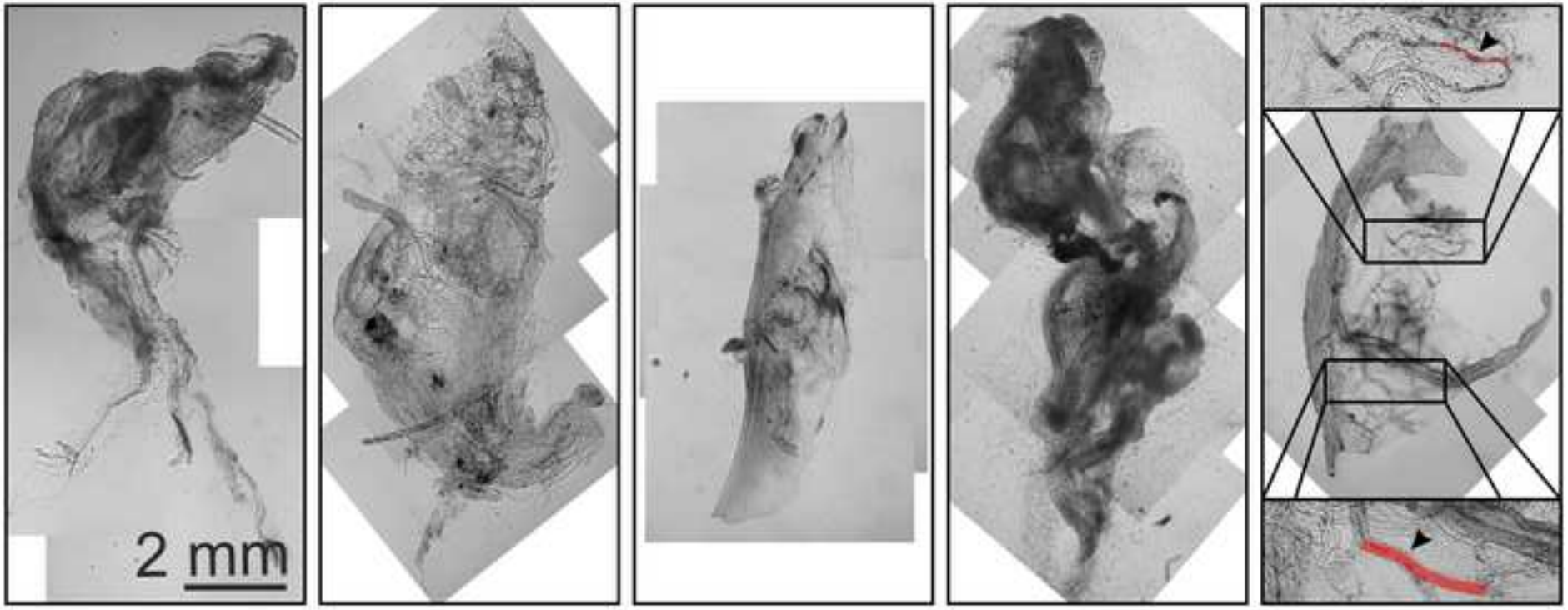


Figure 2



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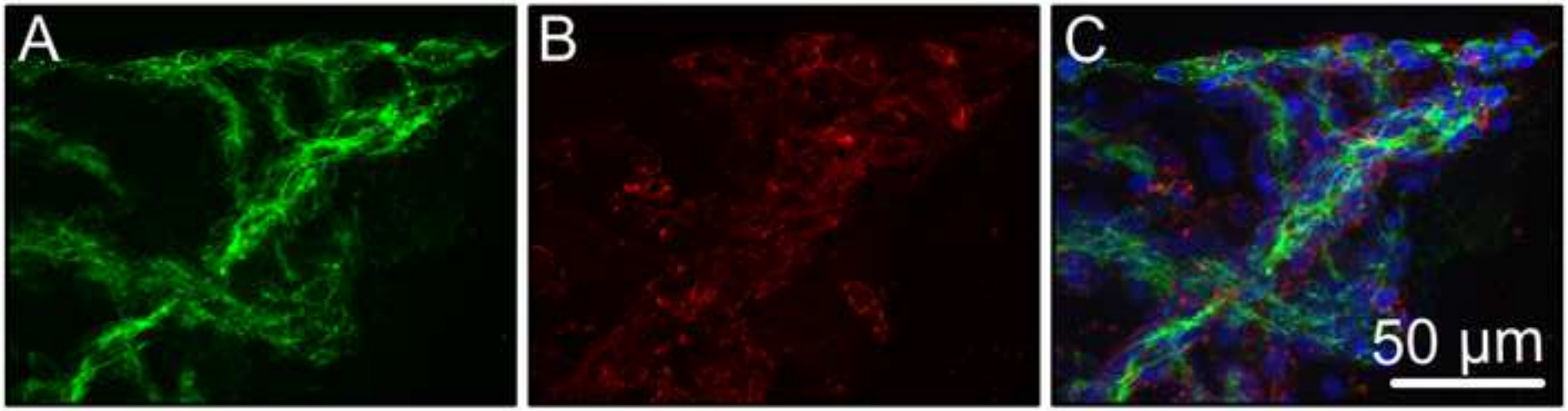

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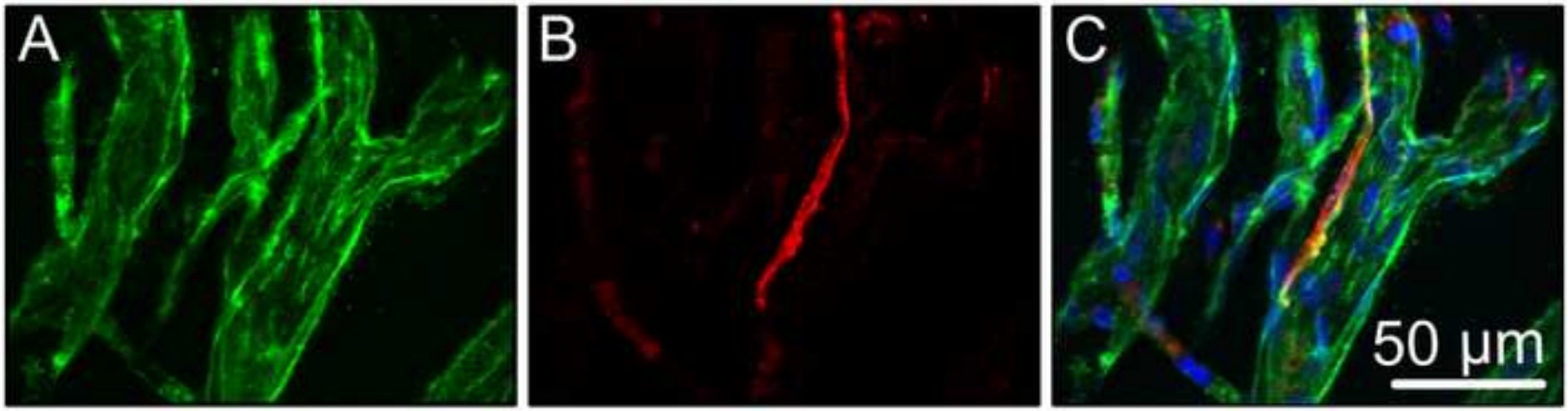

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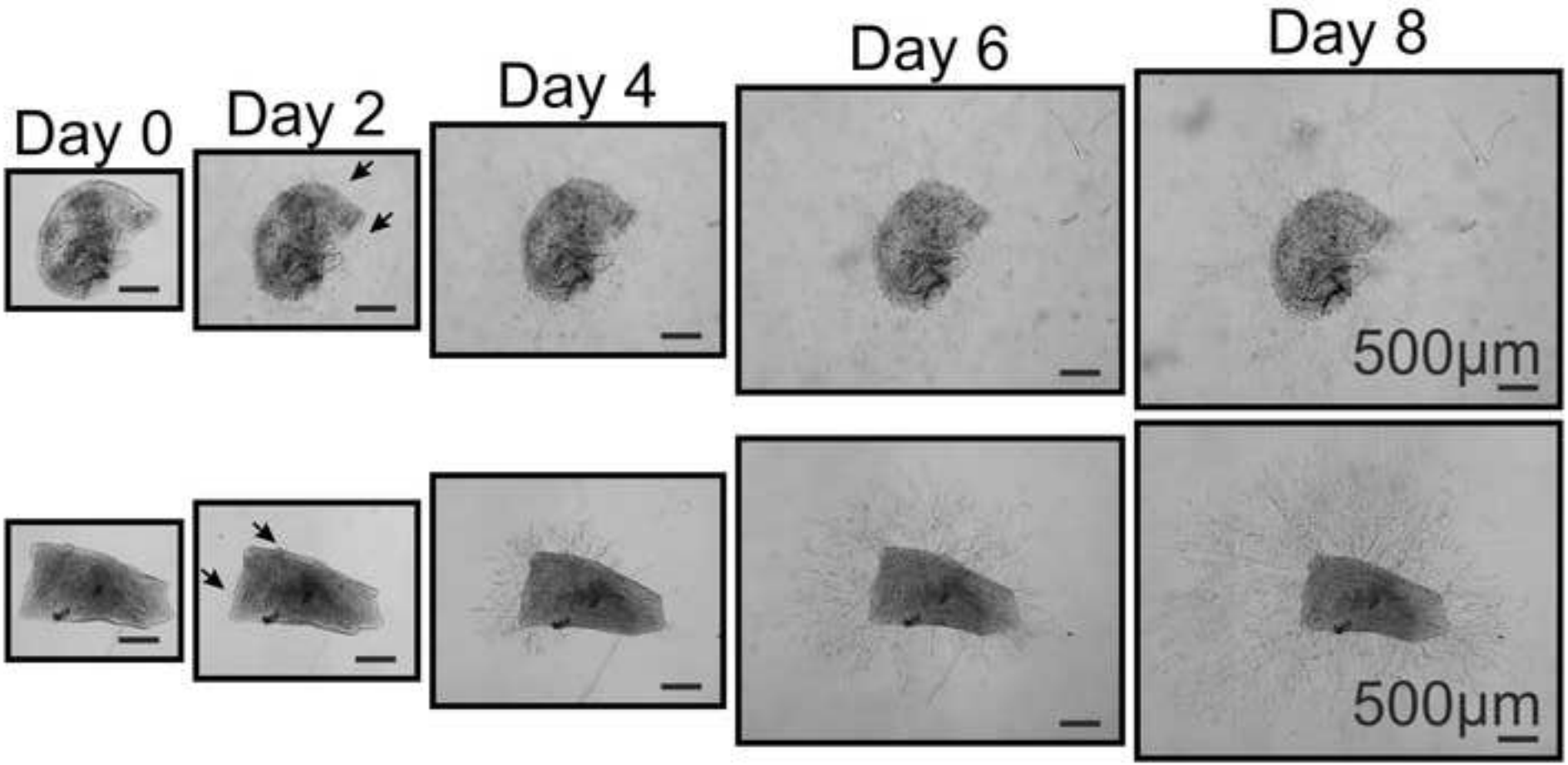

Figure 6

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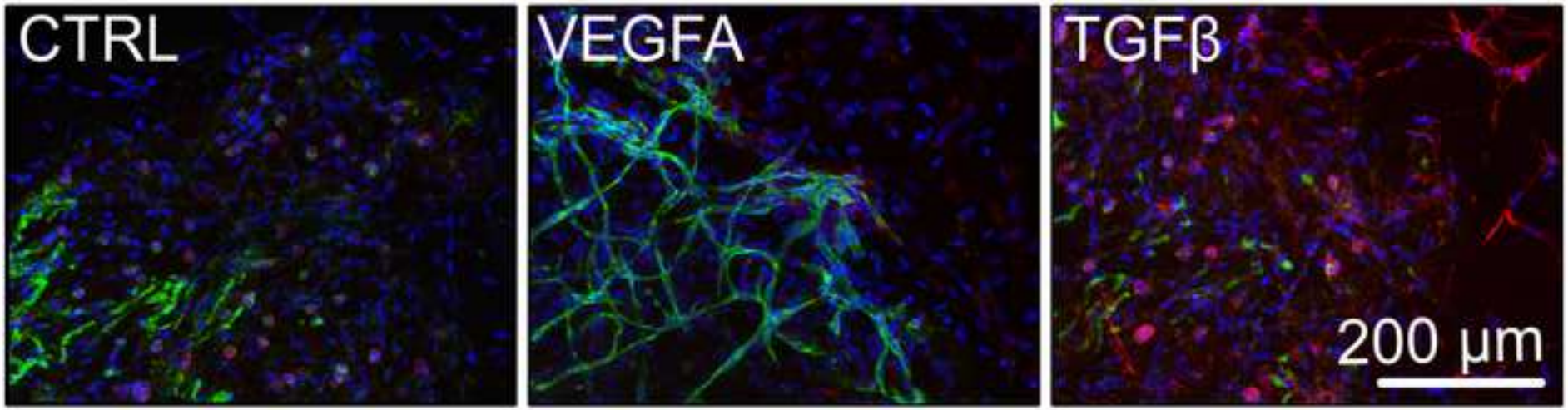

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Table of Materials

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Name	Company	Catalog Number	Comments/Description
Material			
Microforceps	Medicon	07.60.03	Used for handling the FTs
Disposable Scalpels - Sterile	Swann-Morton	0513	Used for FT dissection
Culture dish, vented, 28 ml (60mm)	Greiner Bio-One	391-3210	Used for dissection and for testing fibrin gel formation
Cell culture plates, 12-well	Greiner Bio-One	392-0049	Used for FT dissection and whole-mount immunofluorescence
Reagent/centrifuge tube with screw cap, 15 mL	Greiner Bio-One	391-3477	
Reagent/centrifuge tube with screw cap, 50 mL	Greiner Bio-One	525-0384	
Millex-GV Syringe Filter Unit, 0.22 µm, PVDF	Millipore	SLGV033RS	Used to sterile-filter the fibrinogen solution
Syringe, 10 mL	Braun	4606108V	Used to sterile-filter the fibrinogen solution
Polypropylene Microcentrifuge Tubes, 1.5 mL	Fisher	FB74031	
Cell-Culture Treated Multidishes, 24-well	Nunc	142475	Used for casting the FT/fibrin gels for native FT characterization and *ex vivo* culture
Cell culture plates, 96-well, U-bottom	Greiner Bio-One	392-0019	Used for whole-mount immunofluorescence
Round/Flat Spatulas, Stainless Steel	VWR	82027-528	Used for whole-mount immunofluorescence
Coverslips 22x22mm #1	Menzel/Fisher	15727582	Used for mounting
Microscope slides	Fisher	Kindler K102	Used for mounting
Absorbent paper	VWR	115-0202	Used for mounting
Reagents			
PBS tablets	Medicago	09-9400-100	Used for preparing 1x PBS
Fibrinogen, Plasminogen-Depleted, Human Plasma	Calbiochem	341578	
Hanks Balanced Salt Solution	Sigma-Aldrich	H9394-500ML	Used for preparing the fibrinogen and TA solution
Fetal bovine serum	Gibco	10270106	Used for preparing the blocking solution
Human Serum	Sigma-Aldrich	H4522	Aliquoted in -20 °C, thaw before preparing the *ex vivo* culture media
Gentamicin Sulfate 10mg/ml	Biowest	L0011-100	
Endothelial cell media MV Kit	Promocell	C-22120	Contains 500 ml of Endothelial Cell Growth Medium MV, 25 mL of fetal calf serum, 2 mL of endothelial cell growth supplement, 500 µL of recombinant human epidermal growth factor (10 µg/ mL) and 500 µL of hydrocortisone (1 g/ mL)
Sodium azide	Sigma-Aldrich	S2002	Used for storage of the native and *ex vivo* cultured FTs. TOXIC: wear protective gloves and/or clothing, and eye and/or face protection. Use in fume hood.
Acetone	Sigma-Aldrich	32201-2.5L-M	Used to prepare the post-fixation solution. HARMFUL: wear protective gloves and/or clothing. Use in fume hood.
Methanol	Sigma-Aldrich	32213	Used to prepare the post-fixation solution. TOXIC: wear protective gloves and/or clothing. Use in fume hood.
Triton X-100 (octyl phenol ethoxylate)	Sigma-Aldrich	T9284	Used for whole-mount immunofluorescence. HARMFUL: wear protective gloves and/or clothing.
Hoechst 33342, 20mM	Life Technologies	62249	For nuclei counterstaining. HARMFUL: wear protective gloves and/or clothing, and eye and/or face protection.
VECTASHIELD Antifade Mounting Medium	Vector Laboratories	H-1000	Wear protective gloves and/or clothing, and eye protection. Use in fume hood.
VECTASHIELD Antifade Mounting Medium with DAPI	Vector Laboratories	H-1200	Mounting medium with nuclei counterstaining. Wear protective gloves and/or clothing, and eye protection. Use in fume hood.
Eukitt Quick-hardening mounting medium	Sigma-Aldrich	03989-100ml	TOXIC: Wear protective gloves and/or clothing, and eye protection. Use in fume hood.
Thrombin from bovine plasma, lyophilized powder	Sigma-Aldrich	T9549-500UN	Dissolve at 100 units/ mL, aliquote and store at -20 °C, avoid repeated freeze/ thaw
Aprotinin from bovine lung, lyophilized powder	Sigma	A3428	Dissolve at 50 mg/ mL, aliquote and store at -20 °C, avoid repeated freeze/ thaw
Growth factors			
Recombinant human VEGFA	R&D Systems	293-VE-010	50 ng/ mL final concentration
Recombinant human VEGFC	R&D Systems	752-VC-025	200 ng/ mL final concentration
Recombinant human TGFβ	Millipore	GF346	1 ng/ mL final concentration
Recombinant human bFGF	Millipore	01-106	50 ng/ mL final concentration
Primary antibodies			
CD31 (JC70A)	Dako	M0823	Used at 1:100 dilution, Donkey anti Mouse Alexa 488 Secondary Ab
CD34 (QBEND10)	Dako	M716501-2	Used at 1:100 dilution, Donkey anti Mouse Alexa 488 Secondary Ab
CD45 (2B11+PD7/26)	Dako	M070129-2	Used at 1:100 dilution, Donkey anti Mouse Alexa 488 Secondary Ab
CD68	ImmunoWay	RLM3161	Used at 1:100 dilution, Donkey anti Mouse Alexa 488 Secondary Ab
Cleaved caspase-3 (5A1E)	Cell Signalling	9664	Used at 1:200 dilution, Goat anti Rabbit Alexa 594 Secondary Ab
ERG (EP111)	Dako	M731429-2	Used at 1:100 dilution, Goat anti Rabbit Alexa 594 Secondary Ab
GFAP	Dako	Z0334	Used at 1:100 dilution, Goat anti Rabbit Alexa 594 Secondary Ab
Ki67	Leica Microsystems	NCL-Ki67p	Used at 1:1500 dilution, Goat anti Rabbit Alexa 594 Secondary Ab
Lyve1	R&D Systems	AF2089	Used at 1:100 dilution, Donkey anti Goat Alexa 568 Secondary Ab
NG2	Millipore	AB5320	Used at 1:100 dilution, Goat anti Rabbit Alexa 594 Secondary Ab
Prox1	ReliaTech	102-PA32	Used at 1:200 dilution, Goat anti Rabbit Alexa 568 Secondary Ab
Prox1	R&D Systems	AF2727	Used at 1:40 dilution, Chicken anti Goat Alexa 594 Secondary Ab
VEGFR3 (9D9F9)	Millipore	MAB3757	Used at 1:100 dilution, Donkey anti Mouse Alexa 488 Secondary Ab
α-SMA (1A4)	Sigma	C6198	Used at 1:400 dilution, Cy3 conjugated
Secondary antibodies			
Alexa Fluor488 Donkey Anti-Mouse IgG	Life Technologies	A-21202	Used at 1:500 dilution
Alexa Fluor594 Goat Anti-Rabbit IgG	Invitrogen	A-11012	Used at 1:500 dilution
Alexa Fluor568 Donkey anti-Goat IgG	Thermo Scientific	A-11057	Used at 1:500 dilution
Alexa Fluor568 Goat anti-Rabbit IgG	Thermo Scientific	A-11036	Used at 1:500 dilution
Alexa Fluor594 Chicken Anti-Goat IgG	Molecular Probes	A-21468	Used at 1:500 dilution
Microscopes			
Axiovert 200 inverted epifluorescence microscope	Zeiss		For imaging of the fresh and fibrin-embedded FT
SZX9 upright dissection stereomicroscope	Olympus		For FT dissection
LSM 780 confocal microscope	Zeiss		For imaging of whole-mount immunostained FT
AxioImager.Z1 upright epifluorescence microscope with Apotome	Zeiss		For imaging of whole-mount immunostained FT

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Date:	11.09.2018

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The Journal of Visualized Experiments

Dear Editor Vineeta Bajaj,

I am pleased to submit a revised version of our manuscript entitled "*Ex vivo* tissue culture model for fibrovascular complications in proliferative diabetic retinopathy" (manuscript # JoVE59090).

We appreciate the careful review of our manuscript by you as the editor and by the expert referees. The numerous points raised were constructive and helpful for revising and improving the manuscript. We have now addressed all the comments, and provide detailed point-by-point answers in the attached document for the comments and responses. We hope that the manuscript now meets the criteria for publication in JoVE.

All authors are aware of and agree to the content of the paper and concur with the submission of this revised version.

Thank you for considering our revised work for publication in The Journal of Visualized Experiments.

Yours sincerely,

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Editorial comments:

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Re: We have now thoroughly proofread the entire manuscript.

2. Please provide an email address for each author.

Re: An email for each author has now been added in the "AUTHORS & AFFILIATIONS" paragraph.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

Re: Thank you for this comment, we have now rephrased the Short Abstract/Summary accordingly.

4. Please do not cite any literature in the abstract section. Please move the citation 1 to the introduction instead.

Re: We have now removed the citation 1 and included it in Introduction (now citation 11).

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Re: We have now removed all company names and substituted them with generic terms.

6. 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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Re: We have now reviewed the entire protocol and written all steps in the imperative tense and added all other remarks as notes. We have also mentioned the use of safety procedures where needed.

7. Please leave a single line space between the sub step and the note following it.

Re: We have now added a single line space between every sub step and the note following it.

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. e.g., Line 211-219.

Re: Thank you for this comment. We have now thoroughly checked the protocol and included numbered steps where necessary, including lines 211-219. We have made the text in line 165-166 "Repeat steps 3.5 and 3.6 for all the FT pieces." as step 3.7.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Re: Thank you for this comment which helps us to improve the protocol. More details have now been provided.

10. 1.1. Prepare how?

Re: See comment #9.

11. 1.3: Please remove the quoted from the name of the media.

Re: We have now removed the statement "This ready medium is hereafter called "ex vivo culture medium" and instead named the medium already in the first sentence of section 1.3.

12. 2.1: How much tissue is excised?

Re: The size of the excised tissue is very variable, ranging between 3 and 50 mm^2. This information has now been added as a note to step 2.1.

13. 2.3: Image and look for what? Please explain in a one-liner note.

Re: We have now added in a one-line note the purpose of the imaging and some properties of the tissue that can be visualized.

14. 2.4. How much PBS is present in one well. Please provide volumes throughout the protocol.

Re: We have now revised the entire protocol and added volume information where relevant.

15. 3.3: Volume?

Re: See comment #14.

16. 3.4. Please provide the step number for the aliquoted fibrinogen solution to bring out clarity.

Re: We have now added this notion in the text.

17. Lines 157-161: We cannot have two notes section together. Please consider moving some part to the discussion and make one note only.

Re: We have now added the first note to the protocol step since this is most commonly the case when handling the FTs.

18. 3.6: How long do you incubate this for? Does the droplet forms immediately?

Re: Thank you for this comment. We have now added the following statement to step 3.6 "Ensure also that mixing, dispensation and pipetting are performed quickly as the fibrin gel will form within 1.5 minutes, as tested in step 3.4.".

19. Line 188: Please make it a substep. Image how? Please provide citations if any.

Re: We have now made it a sub step and added notion on the quantification, as performed in Gucciardo *et al., The Journal of Pathology*, 2018. The citation has also been added.

20. 5.1: The difference between native and ex vivo culture is not very clear in section 3. Please bring out the difference.

Re: Thank you for this comment. We have now added a statement concerning the native FTs and the FT *ex vivo* cultures at the beginning of the protocol step 5 as "The FT/fibrin gels can be cultured ex vivo for the desired time period (FT *ex vivo* cultures) or fixed on the same day (native FT) for native FT characterization[11]". In order to better clarify this difference we have also substituted the term "fresh", sometimes referring to native FTs, with the term "native" throughout the manuscript. The term "fresh" was instead used only where it referred to the FTs prior to dissection and fibrin embedding.

21. Once formatted, please ensure that the highlight is no more than 2.75 pages including heading and spacings and forms a cohesive story. This is our hard-cut limit for filming.

Re: We have now revised the highlighted text to fit within the limit.

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Re: Thank you for this note. According to Wiley, publisher of the article in The Journal of Pathology, permission is not required for reusing a part of a figure while appropriate acknowledgement and citation must be given (please see https://onlinelibrary.wiley.com/page/journal/10969896/homepage/permissions.html). I uploaded this link to the editorial policy as a .docx document in our Editorial Manager account. The Video 1 of the current manuscript shows the 3D volume reconstruction of a native FT stained by whole-mount immunofluorescence. A snapshot/frame is included in Figure 1B of the above mentioned

published article (Gucciardo et al., 2018). Citation as “Snapshot/frame in Gucciardo et al., The Journal of Pathology, Volume 245/ Issue 2 Copyright © 2018 Pathological Society of Great Britain and Ireland, first published by John Wiley & Sons Ltd.” is included in the Video 1 legend.

23. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Re: Thank you for this comment. We have now revised all Figure legends and added all missing information.

24. Table 1 and 2: Please remove the manufacture’s detail and move it to the materials table. If possible please merge table 1,2 with the Table of Materials.

Re: We have now merged Tables 1 and Table 2 with the Table of Materials. We also included additional information in the comments/description field.

25. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Re: We have now revised the entire discussion to include the above-mentioned topics and transferred the removed information to the protocol section.

26. Please do not abbreviate the journal title in the reference section.

Re: We have now revised the references format to include full journal titles.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The manuscript by Gucciardo et al described a new protocol for 3D culture of fibrovascular tissue explants obtained from the diabetic patients suffering proliferative retinopathy. The protocol is very well-described and detailed in all the steps and it will for sure allow the researcher set it up straightforwardly. This 3D culture of diseased diabetic retinal tissue may facilitate the understanding of the pathogenesis of this long-term diabetes complication and also open avenues to try new inhibitors and therapies aimed at stopping cell proliferation and clear up the fibrotic tissue to improve/restore visual capacity.

Major Concerns:

None.

Minor Concerns:
1. Point 2.2., line 128. The authors should mention as a guide the longest time the explant is still viable after vitrectomy since it will be very difficult for most of researchers to get the tissue in such a short time (1-2 minutes).

Re: This is a valid point. We have tested as long as 2 hours after vitrectomy and this did not affect the *ex vivo* growth. However, we cannot provide a different estimate, as longer transfer times remain to be tested. We have now modified point 2.2 of the protocol to also include this info as well as info that was in discussion (see comment #9 of this reviewer) as "In our case the transfer takes less than 5 minutes and the explants are embedded within fibrin usually in 1 - 1.5 hours (maximum 2 hours) after surgical removal. The impact of longer transfer times on the 3D culture would need to be tested.". Nevertheless, we think that when team work is involved, longer transfer times can still be compensated by shortening the times *e.g.* for TA solution preparation, fibrinogen sterile-filtration and fibrin gel formation testing. We have added this notion in Discussion. Yet, we consider the close physical proximity of the research unit to the hospital instrumental for the use of these small tissue specimens.

2. Point 2.4., line 132. The authors say: 'Cut the FT into ~ 1mm2 pieces…'. Is the FT thick? Should this thickness be taken into account when cutting the pieces?

Re: This is a valid point. The FTs do not usually grow as a thick mass, but rather as a sheet at the vitreoretinal surface. However, they can fold during transfer and handling, prior to dissection. Therefore we added a note to step 2.4 advising to gently spread the FT prior to performin cuts.

3. Point 3.3 (and also 3.9.). Of interest to mention for what purpose is aprotinin added to the thrombin/fibrinogen reaction…to avoid gel degradation by 'proteases'?

Re: This is a good point. We have added this information in step 3.3 and revised step 3.10.

4. Point 3.4., lines 150-151. 'One tenth of the initially used volume of thrombin…'. Just of thrombin or of the TA solution? No HBSS plus only thrombin has been prepared during the protocol.

Re: Thank you for this comment. If needed, we usually add one tenth of the initially used volume of thrombin. If the volume of thrombin used was e.g. 30 µL, we start by adding 3 µL of thrombin stock solution to the ready TA solution (this leads roughly to a 10% increase in the final concentration of thrombin), and continue doing so until the fibrin gel formation occurs within 1.5 minutes. We have now clarified this statement as follows "The fibrin gel formation should take place in ~1 minute. If this takes over 1.5 minutes, the concentration of thrombin in the TA solution prepared in step 3.3 can be increased by adding more thrombin stock solution. One tenth of the initially used volume of thrombin stock solution can be added, repeatedly, until the fibrin gel formation occurs within 1.5 minutes.".

5. Point 3.6. (and also 3.4.), line 164. When pipetting the gel and particularly the FT into the gel which type of pipette should be used (a p100?)?

Re: The upright fibrin gel droplet is cast by dispensing the fibrinogen/TA solution mix onto the FT already placed on the plate well. To better clarify this we have modified this step as "Add 25 µL of TA solution to the 25 µL fibrinogen solution aliquoted in step 3.2, mix in the tube by pipetting, dispense onto the FT piece, placed on the plate well,……." and "Ensure that the FT is not aspirated during pipetting….". The fibrinogen mixed with the TA solution (50 µL in total) is pipetted with a 10–100 µL or 20–200 µL pipette. Same applies if the fibrin gels are cast on a 48-well plate.

6. Point 6.1. Please give an indicative/estimated volume of acetone:methanol and blocking solutions to be prepared.

Re: We have now added indicative volumes of all solutions to be prepared. See also editorial comment #14.

7. Points 6.3. and 6.4 and so on. Indicate the recommended volume for post-fixation and for the washes.

Re: We have now added indicative volumes also for these steps.

8. Point 6.20. The authors may include here the microscope settings/details recommended for image acquisition (although some details are further developed in the Figure legends).

Re: We have now added some details as "……equipped with an optical sectioning function and 20x or 40x objective. Use tile function to capture a greater area of the tissue at once.".

9. Discussion. The reviewer recommends to remove from discussion the second and third paragraphs since they are included mostly in the protocol and refer to M&M (from lines 406 to 421). The comment on longer transfer times to be tested (line 421) can be included as a note in the protocol and also mention if the authors have actually tested longer transfer times and their impact on 3D culture success (see also point 1).

Re: We thank the reviewer for this comment. We have now revised the discussion and included the info, mentioned in lines 406-421, in points 2.2, 2.4, 3.4, 3.5 and 3.6 of the protocol section (see comment #1 of this reviewer).

10. Discussion, line 449. Mention some of the identified growth factors in the vitreous of PDR and include the reference.

Re: We have now included this info and reference. In Gucciardo *et al.,* 2018, we quantified angiogenic and fibrotic growth factors VEGFA, bFGF, VEGFC and TGFβ as well as tested the *ex vivo* culture responsiveness to these growth factors.

11. Discussion, line 452. Include the reference.

Re: Thank you for this comment. We have now added the references.

12. Mention if the authors have tried to embed the FT into Matrigel or COL I and if the 3D culture and staining work as well as in fibrin.

Re: We have now added this notion in the discussion as follows "These matrices are generally suitable for 3D culture and whole-mount immunofluorescence but their effects on the PDR FTs remain to be tested and considerations on the timing of 3D matrix formation will need to be made in order to stay within the limits for preventing 2D growth[18,19]"

13. Mention if the 3D FT culture is amenable for time-lapse microscopy (have they tried?).

Re: This is an interesting point. We think that with the appropriate instrumentation this 3D *ex vivo* culture model is amenable for time-lapse microscopy, even though we have not yet tried performing such analysis. We have now included this notion in the discussion.

14. Of interest to mention that PDR FT displays proliferation (Gucciardo et al, 2018). But what about signs of 'hypoxia/hyper-glucose' signaling? Is it possible/have the authors tried to perform western blot/qPCR analysis from the tissue explants after culture?

Re: This is an interesting point. We have not tested hypoxia even though this should be doable with commercially available hypoxia probes or CA9 staining, for example. Western blot or qPCR analysis of the tissue explants after culture is possible, by first retrieving the FTs from the fibrin gel by fibrinolysis using *e.g.* plasmin/ nattokinase treatment. We have added this notion to Discussion.

15. Maybe of interest to mention whether there are reliable PDR mouse models and if ex vivo eye cultures from these models have been established/are feasible so molecular pathways can also be explored/identified in mice and compared/validated in the human 3D cultured-FT samples.

Re: This is an interesting point. However, mouse models of diabetes do not develop proliferative changes of human PDR, which makes the 3D *ex vivo* culture of patient-derived PDR FTs described in this manuscript, essential for the study of PDR pathophysiology. We have now clarified this concept in the Discussion as follows "Existing diabetic mouse models develop many features of early stage DR but fail to comprehensively recapitulate the progressive changes occurring in human PDR, thus hindering the studies of the PDR disease mechanisms[7,8]. Moreover, the murine eye is fundamentally different from the human eye, in that it lacks the macula, further emphasizing the importance of studying the human disease[24].

Reviewer #2:

Manuscript Summary:

This manuscript reports a method to suspend patient-derived fibrovascular tissues in polymerized fibrin matrices to study vessel outgrowth in the presence of various stimuli.

Major Concerns:

1. The temperature of all solutions should be indicated when handling all reagents.

Re: We have now revised the entire protocol and added this info.

2. The volumes of all solutions should be indicated throughout the protocol.

Re: We have now revised the entire protocol and added this info. See also editorial comment #14.

3. The heterogeneity in response is concerning in Figure 5. Also, have the authors ensured that the tissue is not contacting the bottom surface of the tissue culture plate. This will enhance cell outgrowth and potentially lead to variability in other explant models, such as aortic rings.

Re: We thank the reviewer for this comment. Majority of the *ex vivo* cultures develop outgrowths already after 2 days of culture (72%; 22%, day 4; 6%, days 6–13, Gucciardo et al., 2018). In some cases, the FTs do outgrow in 2D and such explants are excluded from growth quantifications. Most often, these explants outgrowing in 2D reach sooner the fibrin border. Such explants are also excluded. We have included this notion in Discussion.

TITLE:
Ex vivo tissue culture model for fibrovascular complications in proliferative diabetic retinopathy.

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