Journal of Visualized Experiments

An Ex Vivo Choroid Sprouting Assay of Ocular Microvascular Angiogenesis

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61677R1
Full Title:	An Ex Vivo Choroid Sprouting Assay of Ocular Microvascular Angiogenesis
Section/Category:	JoVE Biology
Keywords:	choroid sprouting assay, endothelial cells, retinal pigment epithelium (RPE), choroidal angiogenesis, choroidal neovascularization, age-related macular degeneration (AMD), ex-vivo
Corresponding Author:	Yohei Tomita, M.D., Ph.D. Boston Children's Hospital, Harvard Medical School Bosoton, M.A. UNITED STATES
Corresponding Author's Institution:	Boston Children's Hospital, Harvard Medical School
Corresponding Author E-Mail:	yohei.tomita@childrens.harvard.edu
Order of Authors:	Yohei Tomita, M.D., Ph.D.
	Zhuo Shao
	Bertan Cakir
	Yumi Kotoda
	Zhongjie Fu
	Lois EH Smith
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Boston, MA, USA

TITLE:

An Ex Vivo Choroid Sprouting Assay of Ocular Microvascular Angiogenesis

AUTHORS AND AFFILIATIONS:

Yohei Tomita[1], Zhuo Shao[2], Bertan Cakir[1], Yumi Kotoda[1], Zhongjie Fu[1,3], Lois E.H .Smith[1]

[1]Department of Ophthalmology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

[2]Department of Clinical and Metabolic Genetics, Hospital for Sick Children, University of Toronto, Toronto, Canada

[3]Manton Center for Orphan Disease, Harvard Medical School, Boston Children's Hospital, Boston, MA, USA

Corresponding author:

Lois E. H. Smith (lois.Smith@childrens.harvard.edu)

Email addresses of co-authors:

Yohei Tomita (yohei. tomita@childrens.harvard.edu)

Zhuo Shao (shawn.shao@sickkids.ca)

Bertan Cakir (bertan.Cakir@childrens.harvard.edu)

Yumi Kotoda (yumi.kotoda@childrens.harvard.edu)

Zhongjie Fu (zhongjie.Fu@childrens.harvard.edu)

KEYWORDS:

choroid sprouting assay, endothelial cells, retinal pigment epithelium, RPE, choroidal angiogenesis, choroidal neovascularization, age-related macular degeneration, AMD, ex vivo

SUMMARY:

This protocol presents choroid sprouting assay, an ex vivo model of microvascular proliferation. This assay can be used to assess pathways involved in proliferating choroidal micro vessels and assess drug treatments using wild type and genetically modified mouse tissue.

ABSTRACT:

Pathological choroidal angiogenesis, a salient feature of age-related macular degeneration, leads to vision impairment and blindness. Endothelial cell (EC) proliferation assays using human retinal microvascular endothelial cells (HRMECs) or isolated primary retinal ECs are widely used in vitro models to study retinal angiogenesis. However, isolating pure murine retinal endothelial cells is technically challenging and retinal ECs may have different proliferation responses than choroidal endothelial cells and different cell/cell interactions. A highly reproducible ex vivo choroidal sprouting assay as a model of choroidal microvascular proliferation was developed. This model includes the interaction between choroid vasculature (EC, macrophages, pericytes) and retinal pigment epithelium (RPE). Mouse RPE/choroid/scleral explants are isolated and incubated in growth-factor-reduced basal membrane extract (BME) (day 0). Medium is changed every other day and choroid sprouting is quantified at day 6. The images of individual choroid explant are

taken with an inverted phase microscope and the sprouting area is quantified using a semi-automated macro plug-in to the ImageJ software developed in this lab. This reproducible ex vivo choroidal sprouting assay can be used to assess compounds for potential treatment and for microvascular disease research to assess pathways involved in choroidal micro vessel proliferation using wild type and genetically modified mouse tissue.

INTRODUCTION:

Choroidal angiogenesis dysregulation is associated with neovascular age-related macular degeneration (AMD)[1]. The choroid is a microvascular bed present underneath the retinal pigment epithelium (RPE). It has been shown that reduced blood flow in the choroid is associated with progression of AMD[2]. The intricate relationship between vascular endothelium, RPE, macrophages, pericytes and other cells is responsible for the homeostasis of the tissue[3-5]. Therefore, a reproducible assay modeling choroidal microenvironment is critical for the study of neovascular AMD.

Ex vivo angiogenesis assays and in vitro endothelial cell cultures can complement studies of microvascular behavior in vivo, for testing new drugs and for studies of pathogenesis. Endothelial cells such as human retinal microvascular endothelial cells (HRMECs), Human Umbilical Vein Endothelial Cells (HUVEC), isolated primary animal brain or retinal ECs are often used in in vitro studies for ocular angiogenesis research[6-8]. HRMECs in particular have been widely used as a model of in vitro choroidal neovascularization (CNV)[9] by assessing endothelial proliferation, migration, tubular formation, and vascular leakage to evaluate interventions[6,10]. However, ECs in culture are limited as a model of CNV because of the lack of interactions with other cell types found in the choroid and because most EC used in these assays do not originate from choroid. Mouse choroidal ECs are difficult to isolate and maintain in culture.

The aortic ring assay is widely used as a model of macro vascular proliferation. Vascular sprouts from aortic explants include ECs, pericytes and macrophages[11]. The aortic ring assay models large vessel angiogenesis well[12-14]. However, it has limitations as a model of choroidal neovascularization as aortic rings are a macrovascular tissue lacking the characteristic choroidal microvascular environment, and sprouts from large vessels may differ from sprouts from capillary networks involved in microvascular pathology. Recently a group published an ex-vivo retinal assay[15,16]. Although, it is suitable for retinal neovascular disease, it is not as appropriate for choroidal neovascularization as seen in AMD.

The choroidal sprouting assay using mouse RPE, choroid, and scleral explanted tissue was developed to better model CNV. The tissue can easily be isolated from mouse (or other species) eyes[17]. This assay allows reproducible evaluation of pro- and anti-angiogenic potential of pharmacologic compounds and evaluation of the role of specific pathways in choroidal neovascularization using tissue from genetically modified mice and controls[18]. This choroidal sprouting assay has been referenced in many subsequent publications[9,10,18-20]. Here, the method involved in the use of this assay are demonstrated.

PROTOCOL:

All animal experiments described were approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital (ARCH protocol number 19-04-3913R).

1. Preparation

1.1. Add 5 mL of Penicillin/Streptomycin (10000 U/mL) and 10 mL of commercially available mitogen to 500 mL of complete classic medium with serum. Aliquot 50 mL of the medium initially.

NOTE: Do not return any medium back to the stock to avoid contamination.

1.2. Put an aliquot of complete classic medium on ice.

1.3. Use 70% ethanol to clean the dissecting microscope, forceps, and scissors.

1.4. Prepare two cell culture dishes (10 cm), one on the dissection microscope, one on ice; put 10 mL of complete classic medium in each dish.

2. Experimental steps (Figure 1)

2.1. Sacrifice C57BL/6J mice around postnatal (P) 20 using 75-100 mg/kg ketamine and 7.5 -10 mg/kg xylazine injected intraperitoneally. Keep the eyes in complete classic medium on ice before dissection.

2.2. Remove the connective tissue (muscle and fatty tissue) and optic nerve on the eye.

2.3. Use a micro-scissor to circumferentially cut 0.5 mm posterior to the corneal limbus. Remove the cornea/iris complex, vitreous and the lens.

2.4. Use forceps to peel off the retina from RPE/choroid/sclera complex. Make a 1 mm incision perpendicular to the cut edge towards the optic nerve and cut a circumferential band of 1 mm width. Separate the central and peripheral regions of the complex.

2.5. Keep the peripheral choroid band in complete classic medium on ice; isolate the other eye and repeat the process to cut a second band.

2.6. Cut the circular band into 6 ~equal square pieces (~1 mm x 1 mm).

NOTE: Never touch any edge.

2.7. Thaw the basal membrane extract (BME) per manufacture's instruction. Add 30 µL/well of BME into the center of each well of a 24 well tissue culture plate. Make sure the droplet of BME forms a convex dome at the bottom of the plate without touching the edges.

2.8. Take each piece of choroid and remove excess medium from the choroid with wipes then place the tissue in the middle of the BME.

NOTE: Do not flatten the choroid explant; generally, let the tissue expand within the BME. The orientation of the tissue (scleral side up or down) does not impact the experimental outcome.

2.9. Incubate the plate at 37 °C for 10 min to let the gel solidify.

2.10. Add 500 µL of the complete classic medium/well.

2.11. Change the classic medium every other day (500 µL). Choroid sprouting can be observed after 3 days with a microscope.

NOTE: For growth factor treatment, starve the tissue for 4 h. Dilute a trial compound in growth factor-reduced medium (1:200 boost instead of 1:50).

3. SWIFT-Choroid computerized quantification method[17] (Figure 2)

NOTE: A computerized method to measure the area covered by growing vessels was used. A macro plugin to ImageJ software is needed prior to quantification (see **Supplemental Information** for further detail).

3.1. Open the choroid sprouting image with ImageJ and check "**Image | Type| 8-bit**" with gray scale.

3.2. Go to "**Image | Adjust | Brightness/Contrast (Ctrl/shift/C)**" and optimize the contrast.

3.3. Use the magic wand function to outline and remove from the image the choroid tissue which are present in the center of the sprouts (using shortcut key "**F1**") (**Figure 2A,B**).

NOTE: Set the tolerance rate of the magic wand to 20-30%.

3.4. Remove the background of the image with the free selection tools (**Figure 2C**). Go to "**Image | Adjust | Threshold (Ctrl/shift/T)**". Use the threshold function to define the microvascular sprouts against the background and periphery (**Figure 2D**).

3.5 Click "**F2**" and a summary will appear. Save an image of the selected area by clicking "**Save**". Save in the same folder as the original image for future reference.

3.6. After a group of samples is measured, copy the recorded for data analysis.

NOTE: It is also possible to measure the area (µm²) by "**Analyze | Set Scale**" using images with scale bars.

REPRESENTATIVE RESULTS:

Comparison of choroid sprouting growth per day

We dissected the choroid with sclera, embedded in BME and cultured them for 6 days (**Figure 1**). The choroid sprouting in C57BL/6J mice from day 3 to day 6 were examined with a microscope and quantified with SWIFT-Choroid a semi-automated quantification method in ImageJ. In a representative case, the choroidal sprouting area (the vessels extending from the explant, excluding the explant itself) was 0.38 mm^2 at Day 3 (**Figure 3A**), 1.47 mm^2 at Day 4 (**Figure 3B**), 5.62 mm^2 at Day 5 (**Figure 3C**), and 10.09 mm^2 at Day 6 (**Figure 3D**).

Free fatty acid receptor (FFAR)4 suppression exacerbates choroidal neovascularization ex vivo.

The effects of loss of FFAR4 (also known as G -protein coupled receptor 120) on choroidal vascular sprouting were evaluated using the choroid sprouting assay[18]. The choroid, RPE, and sclera complex were dissected from *Ffar4 knock out (-/-)* and *Ffar4+/+* mice and cultured as described above. The sprouting area in *Ffar4-/-* increased choroidal vascular growth compared to *Ffar4+/+* at day 6 ($p = 0.004$) (**Figure 4A,B**). The treatment of FFAR4 agonist (1 µM) reduced the choroidal sprouting area compared to untreated mice at day 6 ($p = 0.03$) (**Figure 4C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic illustration showing choroid sprouting assay. Eyes were first enucleated and cut circumferentially about 0.5 mm posterior to the limbus. The cornea, iris, lens and vitreous were removed. Then a 1 mm cut was made from the edge of the eye cup towards the optic nerve. A band was then cut circumferentially about 1.0 mm posterior to the cut edge and the band and the peripheral regions of the complex were separated. The band was cut into approximately 1 mm x 1 mm pieces and embedded in BME. Then using a microscope, the microvascular sprouts from the choroid were visualized.

Figure 2: SWIFT-Choroid computerized quantification method. (**A,B**) Magic wand function was used to outline the choroid tissue (white arrow) in the center of the sprouts and it was removed digitally (Shortcut key "F1"). **(C, D)** The background of the image was removed with the free selection tools (black arrow). Microvascular sprouts were then defined by using the threshold function against the background and periphery.

Figure 3: Mouse peripheral choroid sprouting. (A-D) Representative images of choroid sprouts with a C57BL/6J mouse and demonstrations of SWIFT-Choroid method quantifying the area of the sprouts. The choroidal sprouting area was 0.38 mm^2 at Day 3 (**A**), 1.47 mm^2 at Day 4 (**B**), 5.62 mm^2 at Day 5 (**C**), and 10.09 mm^2 at Day 6 (**D**). Scale bar; 500 µm.

Figure 4: Free fatty acid receptor (FFAR) 4 suppression exacerbate choroidal neovascularization. (A) Representative images of the sprouting assay with choroid from *Free fatty acid receptor* (*Ffar)4+/+* compared with *Ffar4 knock out (-/-)* mice: upper image shows the choroid of *Ffar4+/+* while lower image shows *Ffar4-/-* choroid. **(B)** *Ffar4-/-* showed increased

choroid sprouting compared to *Ffar4 +/+* mice (n = 6-8). **(C)** Representative images of choroid sprouting: upper image demonstrates vehicle treatment (control); lower image demonstrates FFAR4 agonist treatment. **(D)** FFAR4 agonist suppressed choroidal sprouting compared to control (n = 10–12). Scale bars = 500 µm. The data were analyzed by Student's t-test and were expressed as mean ± SE. *p < 0.05; **p < 0.01. This figure has been modified from Tomita et al.[18].

Supplemental File 1: How to create plugins and shortcuts for the choroid sprouting assay program.

DISCUSSION:

The choroidal sprouting assay aids research in neovascular AMD[9,10,18-20]. Choroid explants can be isolated from mice as well as rats and humans[17,21]. The choroid explant includes ECs, macrophages, and pericytes[17]. In this assay the interaction between choroidal ECs and adjacent cells such as RPE cells, help elucidate the mechanisms involved in choroidal vascular growth[17]. Furthermore, this reproducible and semi-automated assessment method decreases interobserver variability[17].

The first published study of ex vivo choroidal tissue used isolated choroid to test pharmacological interventions with the potential to treat DR and AMD[22-25]. The assay counted the number and length of sprouting vessels, which can be subject to interobserver variability. In contrast, the quantification method described here has been standardized[17]. This assay of microvascular choroid angiogenesis includes interactive partner cells and extracellular matrix. ECs in culture may lose many of their physiological properties, such as the ability to form vascular tubes[26] which may be due to the loss of interaction with other cells such as RPE. Therefore, EC in vitro cultures may not reflect all aspects of choroidal neovascularization. The aortic ring assay includes large vessel endothelial cells and interactive cells to evaluate sprouting from large vessels. But large vessel sprouts may not accurately reflect choroidal microvascular disease[27]. The choroidal sprouting assay is a closer representation of choroidal microvascular responses.

There are important caveats. First, the peripheral choroid complex explant sprouts are more consistent and grow much faster than explants from the central sections[17]. Second, the RPE from choroid was not removed because choroid sprouts with RPE grow much faster than without RPE[17]. To understand the impact of a drug on choroid alone, the assay without RPE may be used. Finally, when outlining the image of choroid tissue and sprouting area by using the magic wand function, it is sometimes difficult to trace the area of choroid sprouting because of high background noise. There can be variation among images. Therefore, for consistency, it is important to digitally create sufficient contrast between choroid and background as seen in **Figure 2**.

In summary, ex vivo choroid sprouting assay has been characterized and semi-automated. This method provides an experimental tool for AMD research. This assay can be used to screen compounds as potential treatments or to assess pathways involved in proliferating choroidal micro vessels using wild type and genetically modified mouse tissue.

ACKNOWLEDGMENTS:

The work was supported by Grants from the Manpei Suzuki Diabetic Foundation (YT), Boston Children's Hospital OFD/BTREC/CTREC Faculty Career Development Grant, Boston Children's Hospital Ophthalmology Foundation, BCH Pilot Award, BCH Manton Center Fellowship, and Little Giraffe Foundation (ZF), The German Research Foundation (DFG; to BC [CA1940/1-1]), NIH R24EY024868, EY017017, R01EY01717-13S1, EY030904-01, BCH IDDRC (1U54HD090255), Massachusetts Lions Eye Foundation (LEHS).

DISCLOSURES:

The authors have no financial disclosures. The computerized method is available free of charge to academic institutions through the authors.

REFERENCES:

1. Zarbin, M. A. Current concepts in the pathogenesis of age-related macular degeneration. *Arch Ophthalmol.* **122** (4), 598-614 (2004).
2. Pemp, B., Schmetterer, L. Ocular blood flow in diabetes and age-related macular degeneration. *Canadian Journal of Ophthalmology.* **43** (3), 295-301 (2008).
3. Murakami, Y., Ishikawa, K., Nakao, S., Sonoda, K. H. Innate immune response in retinal homeostasis and inflammatory disorders. *Progress in Retinal and Eye Research.* **74**, 100778 (2020).
4. Fu, Z. et al. Dyslipidemia in retinal metabolic disorders. *EMBO Molecular Medicine.* **11** (10), e10473 (2019).
5. Daruich, A. et al. Mechanisms of macular edema: Beyond the surface. *Progress in Retinal and Eye Research.* **63**, 20-68 (2018).
6. Tomita, Y. et al. Long-Acting FGF21 Inhibits Retinal Vascular Leakage in In Vivo and In Vitro Models. *International Journal of Molecular Sciences.* **21** (4), e21041188 (2020).
7. Maisto, R. et al. ARPE-19-derived VEGF-containing exosomes promote neovascularization in HUVEC: the role of the melanocortin receptor 5. *Cell Cycle.* **18** (4), 413-424 (2019).
8. Mazzoni, J. et al. The Wnt Inhibitor Apcdd1 Coordinates Vascular Remodeling and Barrier Maturation of Retinal Blood Vessels. *Neuron.* **96** (5), 1055-1069 (2017).
9. Fu, Z. et al. Adiponectin Mediates Dietary Omega-3 Long-Chain Polyunsaturated Fatty Acid Protection Against Choroidal Neovascularization in Mice. *Investigative Ophthalmology and Visual Sciences.* **58** (10), 3862-3870 (2017).
10. Gong, Y. et al. Cytochrome P450 Oxidase 2C Inhibition Adds to omega-3 Long-Chain Polyunsaturated Fatty Acids Protection Against Retinal and Choroidal Neovascularization. *Arteriosclerosis, Thrombosis and Vascular Biology.* **36** (9), 1919-1927 (2016).
11. Nicosia, R. F., Zorzi, P., Ligresti, G., Morishita, A., Aplin, A. C. Paracrine regulation of angiogenesis by different cell types in the aorta ring model. *International Journal of Developmental Biology.* **55** (4-5), 447-453 (2011).
12. Bellacen, K., Lewis, E. C. Aortic ring assay. *Journal of Visulaized Experiments.* (33), e1564 (2009).
13. Masson, V. V. et al. Mouse Aortic Ring Assay: A New Approach of the Molecular Genetics of Angiogenesis. *Biological Procedures Online.* **4**, 24-31 (2002).
14. Katakia, Y. T. et al. Ex vivo model for studying endothelial tip cells: Revisiting the classical

aortic-ring assay. *Microvascular Research.* **128**, 103939 (2020).
15. Rezzola, S. et al. In vitro and ex vivo retina angiogenesis assays. *Angiogenesis.* **17** (3), 429-442 (2014).
16. Rezzola, S. et al. A novel ex vivo murine retina angiogenesis (EMRA) assay. *Experimental Eye Research.* **112**, 51-56 (2013).
17. Shao, Z. et al. Choroid sprouting assay: an ex vivo model of microvascular angiogenesis. *PLoS One.* **8** (7), e69552 (2013).
18. Tomita, Y. et al. Free fatty acid receptor 4 activation protects against choroidal neovascularization in mice. *Angiogenesis.* **23**, 385-394 (2020).
19. Li, J. et al. Endothelial TWIST1 promotes pathological ocular angiogenesis. *Investigative Ophthalmology and Vision Science.* **55** (12), 8267-8277 (2014).
20. Liu, C. H. et al. Endothelial microRNA-150 is an intrinsic suppressor of pathologic ocular neovascularization. *Proceedings of the National Academy of Science U. S. A.* **112** (39), 12163-12168 (2015).
21. Zhou, Q. et al. LncEGFL7OS regulates human angiogenesis by interacting with MAX at the EGFL7/miR-126 locus. *Elife.* **8**, e40470, (2019).
22. Kobayashi, S., Fukuta, M., Kontani, H., Yanagita, S., Kimura, I. A quantitative assay for angiogenesis of cultured choroidal tissues in streptozotocin-diabetic Wistar and spontaneously diabetic GK rats. *Japanese Journal of Pharmacology.* **78** (4), 471-478 (1998).
23. Kobayashi, S. et al. Inhibitory effects of tetrandrine and related synthetic compounds on angiogenesis in streptozotocin-diabetic rodents. *Biological and Pharmaceutical Bulletin.* **22** (4), 360-365 (1999).
24. Kobayashi, S., Shinohara, H., Tsuneki, H., Nagai, R., Horiuchi, S. N(epsilon)-(carboxymethyl)lysine proliferated CD34(+) cells from rat choroidal explant in culture. *Biological and Pharmaceutical Bulletin.* **27** (9), 1382-1387 (2004).
25. Kobayashi, S. et al. Overproduction of N(epsilon)-(carboxymethyl)lysine-induced neovascularization in cultured choroidal explant of streptozotocin-diabetic rat. *Biological and Pharmaceutical Bulletin.* **27** (10), 1565-1571 (2004).
26. Bergers, G., Song, S. The role of pericytes in blood-vessel formation and maintenance. *Neuro-Oncology.* **7** (4), 452-464 (2005).
27. Browning, A. C., Stewart, E. A., Amoaku, W. M. Reply to: Phenotypic plasticity of human umbilical vein endothelial cells. *British Journal of Ophthalmology.* **96** (9), 1275-1276 (2012).

Figure1

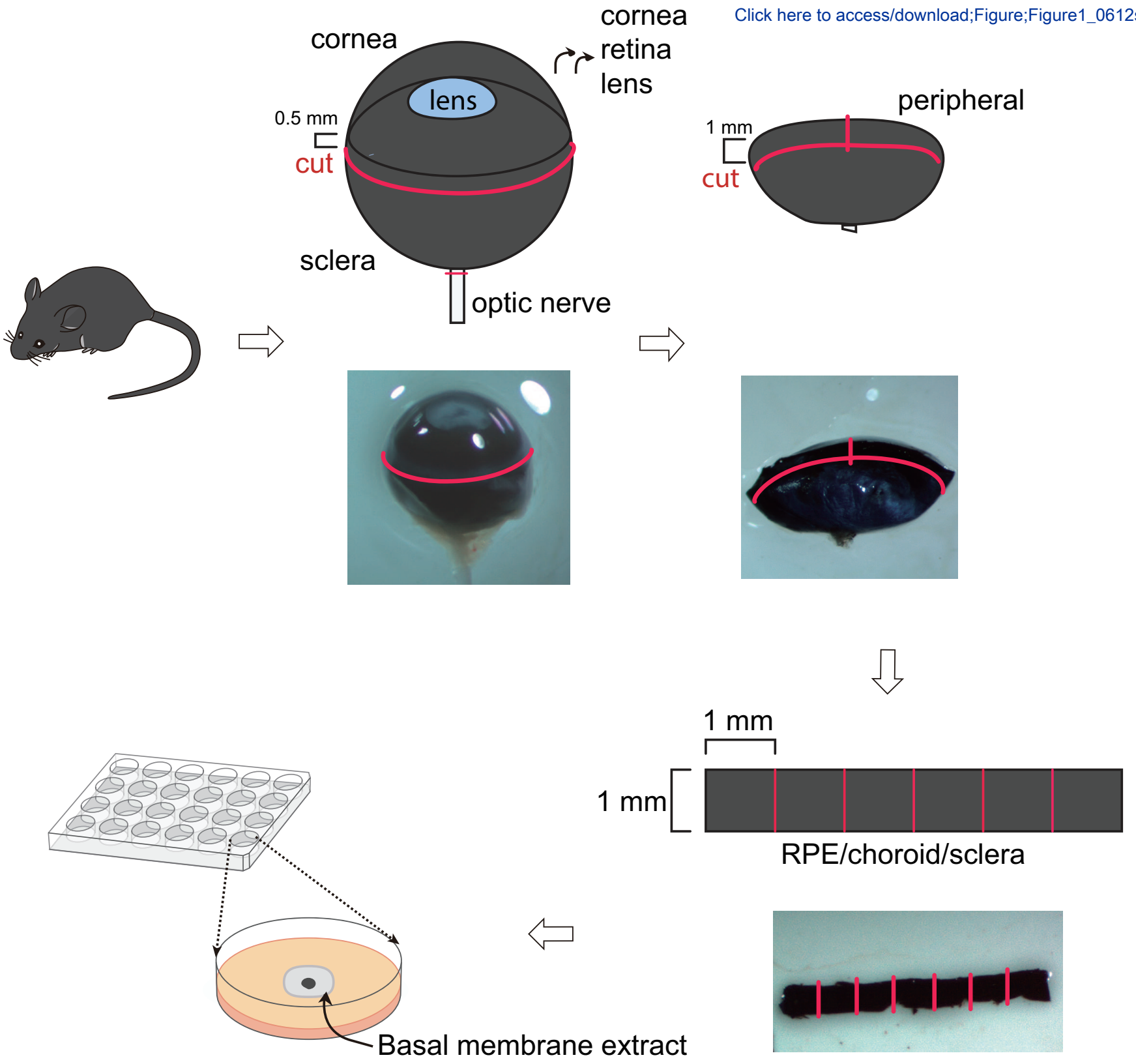
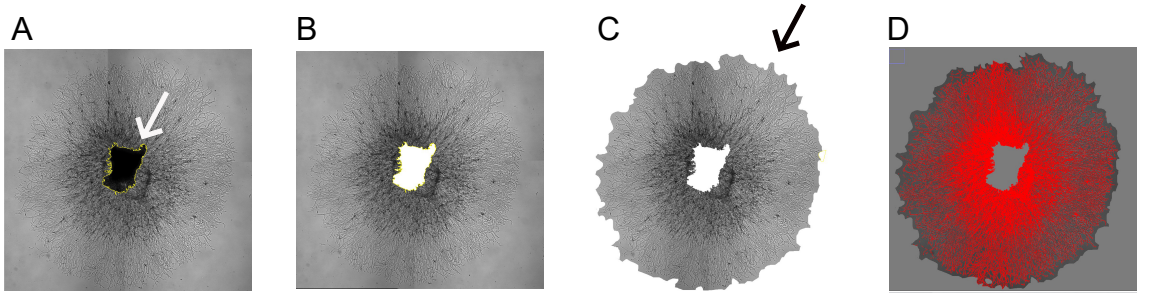


Figure2

Click here to access/download;Figure;figure 2.pdf



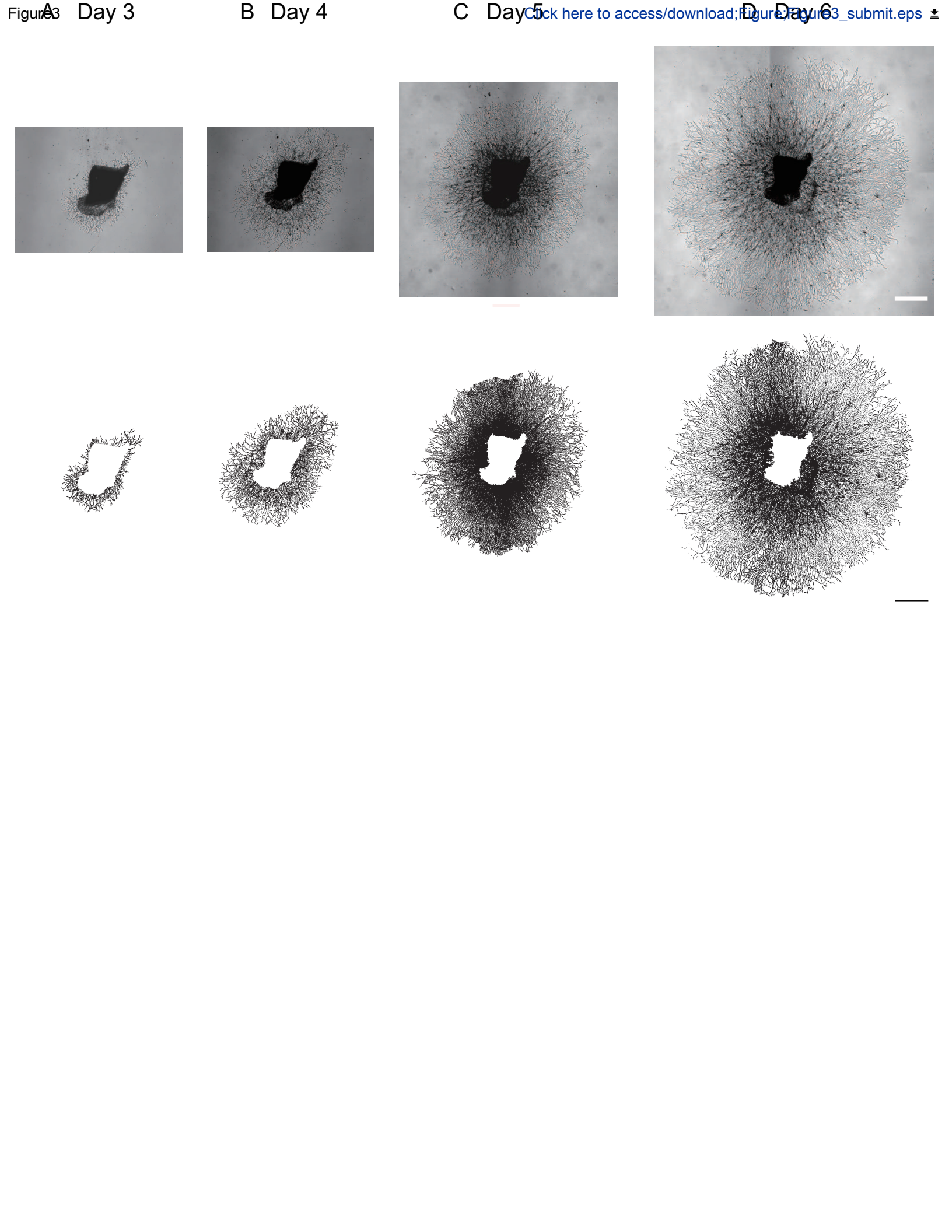
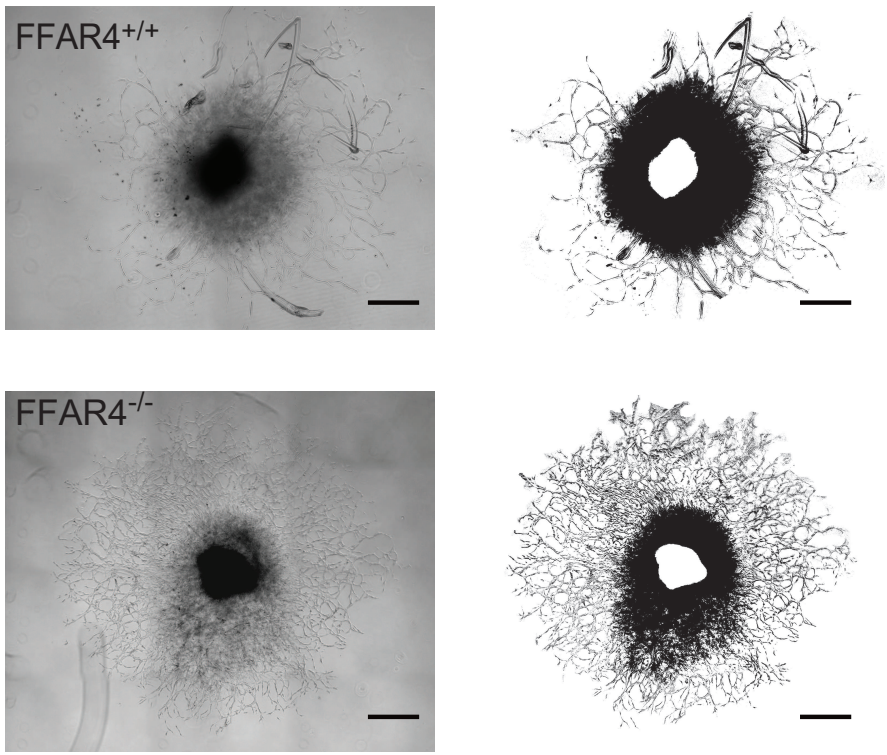
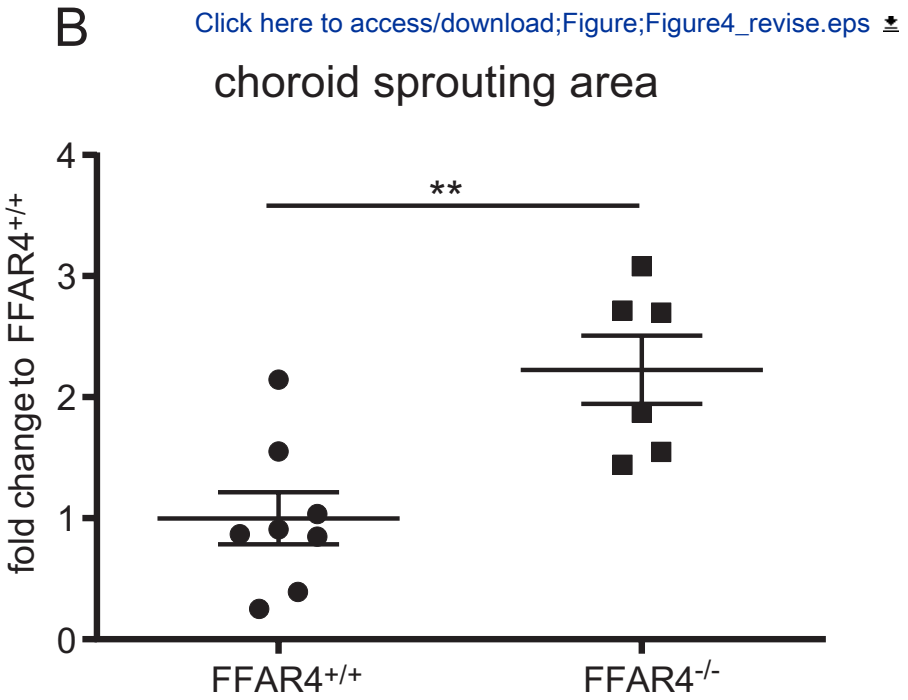
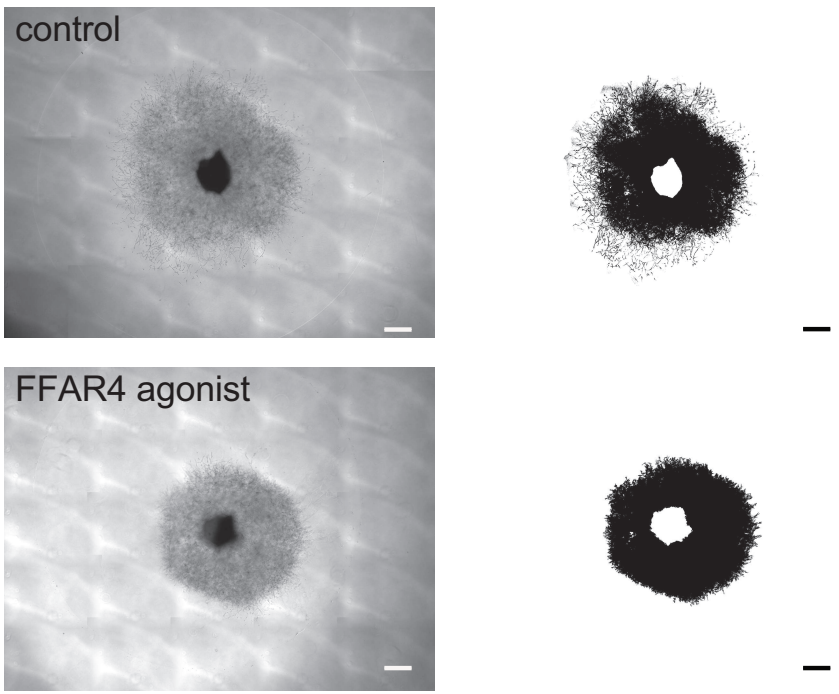
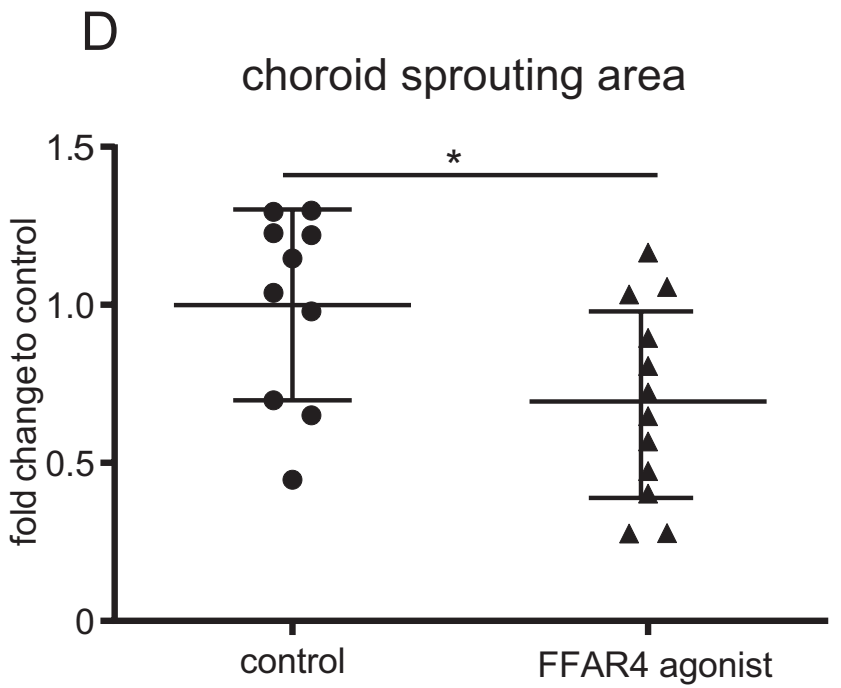


Figure4


A
FFAR4+/+
FFAR4-/-


B
choroid sprouting area
fold change to FFAR4+/+
4
3
2
1
0
**
FFAR4+/+
FFAR4-/-


C
control
FFAR4 agonist


D
choroid sprouting area
fold change to control
1.5
1.0
0.5
0
*
control
FFAR4 agonist

Name of Material/Equipment	Company	Catalog Number	Comments/Description
AnaSed (Xylazine)	AKORN	59339-110-20	
Basal membrane extract (BME) Matrigel	BD Biosciences	354230	
Cell culture dish	NEST	704001	10cm
Complete classic medium with serum and CultureBoost	Cell systems	4Z0-500	
Ethyl alcohol 200 Proof	Pharmco	111000200	use for 70%
Kimwipes	Kimberly-Clark	06-666	
Microscope	ZEISS	Axio Observer Z1	
Penicillin/Streptomycin	GIBCO	15140	10000 U/mL
Tissue culture plate (24-well)	Olympus	25-107	
VetaKet CIII (Ketamine)	AKORN	59399-114-10	

June 18th, 2020

Vineeta Bajaj, PhD

Review Editor

JoVE,

Dear Dr. Bajaj,

We appreciate your thorough review of our manuscript entitled '**Choroid Sprouting Assay: An Ex Vivo Assay of Ocular Microvascular Angiogenesis'**. We have revised our manuscript according to the reviewers' suggestions. We feel that the article has been much improved with the revisions. New text added to the manuscript is in red, and the reviewers' original questions are in blue text in this document. We believe that the revision fully addresses the comments raised by you and the reviewers and hope that it will be published in *JoVE.*

Sincerely yours,

Corresponding author:

Lois E.H. Smith, MD, PhD

Institution: Boston Children's Hospital/ Harvard Medical School

Address: Children's Hospital, 300 Longwood Ave, Boston, MA 02115

Phone number: 617-919-2529, Email: Lois.Smith@childrens.harvard.edu

Editorial comments:

Changes to be made by the Author(s):

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

Thank you for your comments. We checked it again and modified it.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

I have checked it and revised it.

3. Unfortunately, there are a few sections of the manuscript that show overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 60-62, 77-81, 86-88, 89-93, 187-188, 196-201, 239-242,

Thank you very much for pointing out them. We have changed them all.

4. Figure 1: Please include a space between all numbers and the corresponding unit. Please avoid the use of commercial terms (Matrigel). Please use genetic terms whenever possible.

I have changed them. We describe Matrigel as Basal membrane extract (BME).

5. Please remove the figure labeling from the figures. Figure 4 has a label for Figure 3.

I removed the figure label in the figures.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

I have changed it.

7. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

I have changed it.

8. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager

account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have changed and uploaded the approval document.

9. Please do not abbreviate journal titles.

We are not now using abbreviate in the journal titles.

Reviewers' comments:

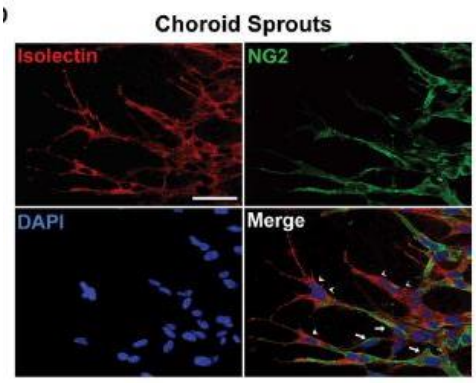
Reviewer #1:

Manuscript Summary:

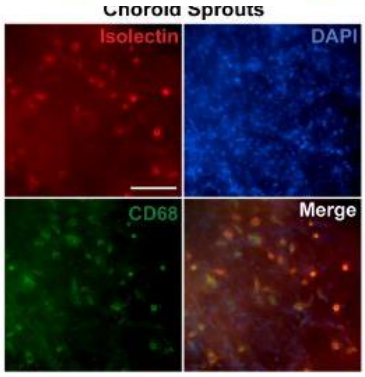
The manuscript by Tomita et al described the experiments for mouse choroid sprouting assay. Over it is well presented, and the paper was well written. Major Concerns:

Some comments are as follows:

(1) Can the authors show some sections and staining pictures, so that the identity and organization of the cells that are sprouting can be confirmed.

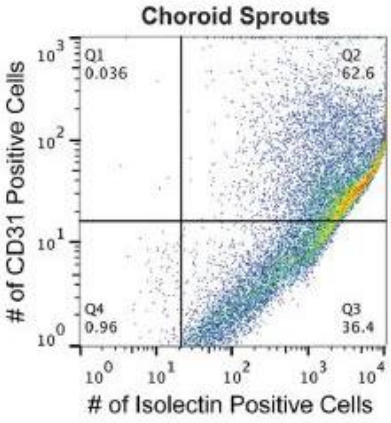


Thank you for your valuable comment. It is very difficult to make cross sections of embedded Matrigel but we have (in whole mounts) previously stained with isolectin and NG2, microvascular marker for macrophages and endothelial cells and for pericytes respectively, see left figure [1]. This figure showed that the extending growth cone resembles vascular tube formation in vivo and stains positively with isolectin GS (arrow head) surrounded by chondroitin sulfate proteoglycan neuron-glial antigen 2 (NG2) positive pericytes (arrow).

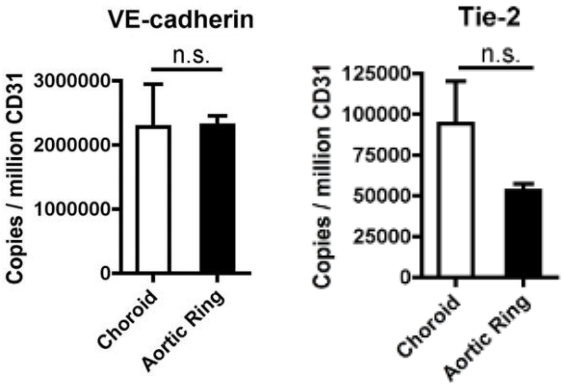


We also showed that a population of isolectin and CD68-positive cells was also detected in the sprouts adjacent to planted choroidal tissue as left figure. These isolectin and CD68-positive cells exhibited monocyte/macrophage-like cell morphology [1].

(2) If not all the sprouting cells are EC cell, how can the sprouting of the ECs be quantified?



We previously examined CD 31, a marker of ECs, with flow cytometry [1]. The left figure showed flow cytometry analysis of choroid sprouting cell populations. About 60% of the cell population from the choroid sprouts stained positive for both CD-31 and isolectin, indicating ECs/macrophages. 36% of the cell population is isolectin-positive but did not stain for CD-31.



Furthermore, compared to the aortic ring assay, the choroidal sprouts expressed similar quantities of endothelial markers (i.e. VE-cadherin and Tie-2) when normalized to CD31 as left figure [1].

(3) The code for the macro image J plug-in should be listed in the paper, or available for download from public domain.

The macros are available to academic institutions through the authors. We added the comments in the manuscript as below.

P3 line 149-151

A computerized method to measure the area covered by growing vessels was developed which is available free to academic institutions through the authors. A macro plugin to ImageJ software is needed prior to quantification (see supplemental information for further detail)

References

1. Shao Z, Friedlander M, Hurst CG, Cui Z, Pei DT, Evans LP, Juan AM, Tahiri H, Duhamel F, Chen J, Sapieha P, Chemtob S, Joyal JS, Smith LE (2013) Choroid sprouting assay: an ex vivo model of microvascular angiogenesis. PLoS One 8 (7):e69552. doi:10.1371/journal.pone.0069552

Reviewer #2:

This manuscript is well-written and the study is well-designed. The choroid sprouting assay are performed correctly and the results are presented well without identifiable errors.

Great work and excellent manuscript. I recommend proceeding with publication.

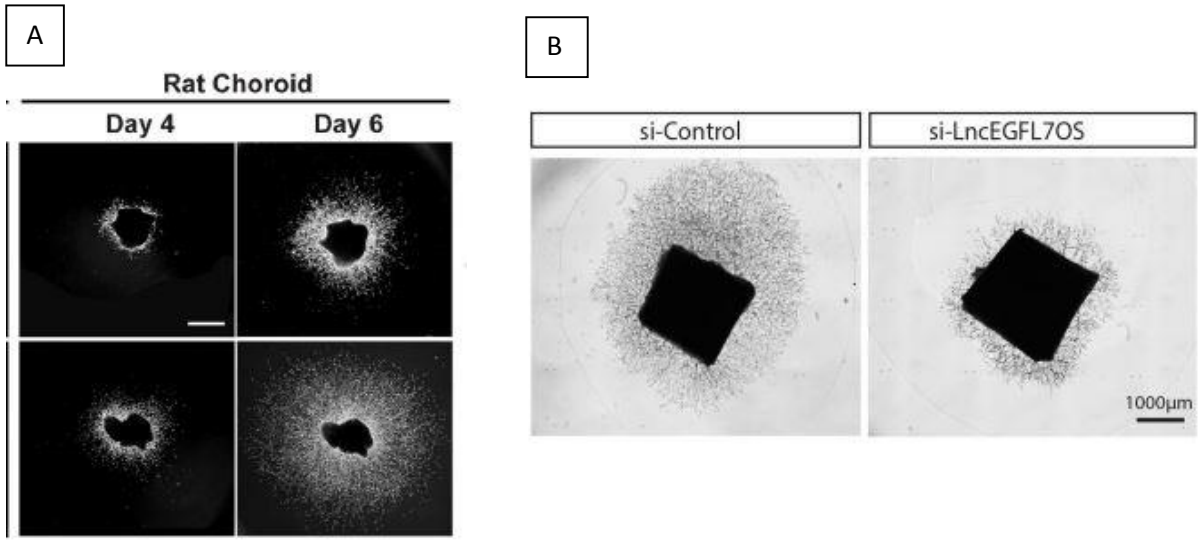
Following is only one suggestion to improve the manuscript,

1) Is it possible to perform the choroid sprouting assay using rabbit and even human eye?

Thank you very much for addressing this important point. We previously published rat choroidal explant assay as below (figure A) [1]. In addition, Zhou, et al. performed human choroid sprouting assay as below (figure B) [2]. We added passage in the manuscript.

Page 5 line 231-232

The reproducible choroidal sprouting assay aids research in neovascular AMD. Choroid explants can be isolated from mice as well as rats and humans.



References

1. Shao Z, Friedlander M, Hurst CG, Cui Z, Pei DT, Evans LP, Juan AM, Tahiri H, Duhamel F, Chen J, Sapieha P, Chemtob S, Joyal JS, Smith LE (2013) Choroid sprouting assay: an ex vivo model of microvascular angiogenesis. PLoS One 8 (7):e69552. doi:10.1371/journal.pone.0069552
2. Zhou Q, Yu B, Anderson C, Huang ZP, Hanus J, Zhang W, Han Y, Bhattacharjee PS, Srinivasan S, Zhang K, Wang DZ, Wang S (2019) LncEGFL7OS regulates human angiogenesis by interacting with MAX at the EGFL7/miR-126 locus. Elife 8. doi:10.7554/eLife.40470

Reviewer #3:

Manuscript Summary:

In this method article, Tomita et al. describe an ex vivo choroid sprouting assay. Overall, the study has the merit to look at an interesting topic. Indeed, ex vivo cultures represent an interesting tool that allow the maintenance of the complexity of the tissue structure. In addition, the model implemented by Tomita and colleagues, allow the study of the pathological mechanisms of choroidal neovascularization and may be exploited for the testing of drug candidates. I found the protocol clear and well described. However, I have few concerns, listed here below.

Major Concerns:

1) Introduction: the authors make a comparison between their choroid assay and the aortic ring model. I understand that aortic ring assay represents the most widely used ex vivo angiogenesis model, but since here we are speaking about ocular diseases, I think it would be more interesting to compare their model with the ex vivo retina models actually used.

I really appreciate your comments. Although several studies about ocular disease have used aortic ring model [3,4], ex vivo retina model [5,6] is also used as ocular disease model as you said. We added the comments in the introduction.

Page1 line 75-77

Recently a group published an ex-vivo retinal assay. Although, it is suitable for retinal neovascular disease, it is not as appropriate for choroidal neovascularization seen in AMD.

2) Representative results: in the text the authors refer to Figure 1, 2 and 3. Anyway, there are 4 figures. I am also confused regarding the concordance between Fig 2 and Fig 3 and their legends. Are they correct? Please, fix these discrepancies.

We are sorry for this discrepancy. I have edited them.

Minor Concerns:

I would suggest the authors to choose better images for Figure 4 a-c (first column)

Thank you very much for your suggestion. We have added better images.

References

3. Gong Y, Fu Z, Edin ML, Liu CH, Wang Z, Shao Z, Fredrick TW, Saba NJ, Morss PC, Burnim SB, Meng SS, Lih FB, Lee KS, Moran EP, SanGiovanni JP, Hellstrom A, Hammock BD, Zeldin DC, Smith LE (2016) Cytochrome P450 Oxidase 2C Inhibition Adds to omega-3 Long-Chain Polyunsaturated Fatty Acids Protection Against Retinal and Choroidal Neovascularization. Arterioscler Thromb Vasc Biol 36 (9):1919-1927. doi:10.1161/ATVBAHA.116.307558
4. Gong Y, Shao Z, Fu Z, Edin ML, Sun Y, Liegl RG, Wang Z, Liu CH, Burnim SB, Meng SS, Lih FB, SanGiovanni JP, Zeldin DC, Hellstrom A, Smith LEH (2016) Fenofibrate Inhibits Cytochrome P450 Epoxygenase 2C Activity to Suppress Pathological Ocular Angiogenesis. EBioMedicine 13:201-211. doi:10.1016/j.ebiom.2016.09.025

5. Rezzola S, Belleri M, Ribatti D, Costagliola C, Presta M, Semeraro F (2013) A novel ex vivo murine retina angiogenesis (EMRA) assay. Exp Eye Res 112:51-56. doi:10.1016/j.exer.2013.04.014
6. Rezzola S, Belleri M, Gariano G, Ribatti D, Costagliola C, Semeraro F, Presta M (2014) In vitro and ex vivo retina angiogenesis assays. Angiogenesis 17 (3):429-442. doi:10.1007/s10456-013-9398-x

Supplemental information

Creating plugins for this program.

1. Go to "**C drive | Program Files | ImageJ | plugins**" and create folder titled "SWIFT_Choroid".

2. Copy "Steps 1" and "Step 2" which are provided into this newly created folder.

3. Restart Image J and click "**Plugins| SWIFT_Choroid| Step 1, Step 2"**.

Creating shortcuts for this program.

1. To create shortcuts for these steps, go to "**Plugins | Shortcuts | Add Shortcut | Shortcut**". Type out the shortcut key to use (i.e.,"**F1**") | Command: "**Step1**".

NOTE: Make sure to have a shortcut key available. If all of shortcuts keys are already assigned, then one needs to first remove a shortcut using "**Plugins | Shortcuts | Remove**" a shortcut before using that key for Swift_Choroid.

2. Quit ImageJ after creating shortcuts; the new function will not work or show up as an option in the menu until doing so.

SPRINGER NATURE LICENSE
TERMS AND CONDITIONS

Jun 15, 2020

This Agreement between Boston Childrens Hospital/ Harvard medical scool -- Yohei Tomita ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4821980959712
License date	May 04, 2020
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Angiogenesis
Licensed Content Title	Free fatty acid receptor 4 activation protects against choroidal neovascularization in mice
Licensed Content Author	Yohei Tomita et al
Licensed Content Date	Mar 5, 2020
Type of Use	Journal/Magazine
Requestor type	academic/university or research institute
Is this reuse sponsored by or associated with a pharmaceutical or a medical products company?	no

Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Will you be translating?	no
Circulation/distribution	500 - 999
Author of this Springer Nature content	yes
Title of new article	Choroid Sprouting Assay: An Ex Vivo Assay of Ocular Microvascular Angiogenesis
Lead author	Yohei Tomita
Title of targeted journal	Journal of Visualized Experiments
Publisher	MyJove Corp
Expected publication date	Sep 2020
Portions	Figure 3A and B
Requestor Location	Boston Childrens Hospital/ Harvard medical scool 3 Blackfan Circle 18030-9B BOSTON, MA 02115 United States Attn: Boston Childrens Hospital/ Harvard medical scool

Total	0.00 USD

Terms and Conditions

Springer Nature Customer Service Centre GmbH
Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use, subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping,

adapting, omitting material that affect the meaning, intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:*** Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT]** (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.