

## Quantitative Assessment of Neurite Outgrowth in Mouse Retinal Explants

Tom Buyens, Djoere Gaublot, Inge Van Hove,  
Lies De Groef, and Lieve Moons

### Abstract

Despite intensive research efforts over the past years, regeneration of injured axons in the central nervous system (CNS) remains elusive. The discovery of novel neuro-stimulatory agents that promote regeneration is hampered by a gap between high content analysis platforms using neuronal cells and time-consuming preclinical animal models. In this regard, tissue explant cultures, which are easily manageable and more closely resemble the in vivo situation, form an ideal model system to study the effect of compounds on the neuroglial network. Retinal explants have proven to be a useful tool to investigate the effect of molecules on neuronal survival and regeneration. In this chapter, we report a detailed description of how to isolate and culture retinal explants and how to immunolabel the outgrowing neurites. Furthermore, we describe different analysis tools, both manual and automated, to quantify neurite outgrowth from retinal explants.

**Key words** Tissue explant, Retina, Neurite outgrowth, Axonal regeneration, Automated morphometric analysis

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### 1 Introduction

Due to the increasing life expectancy, neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, age-related macular degeneration and glaucoma, are more prevalent than ever before. Therefore, the development of novel therapies for such neurodegenerative diseases and for central nervous system (CNS) injuries in general has become a top priority, as CNS damage drastically diminishes the quality of life and current treatments are inadequate. For many years, the prevailing dogma held that the adult mammalian CNS is unable to regenerate injured or diseased axons. This lack of spontaneous axonal regeneration of adult neurons has been attributed to a loss of neurotrophic support from target neurons, leading to an impaired neuronal survival [1], an inhibitory environment, which prevents axonal regrowth [2], and a decreased growth potential as compared to embryonic neurons [3].

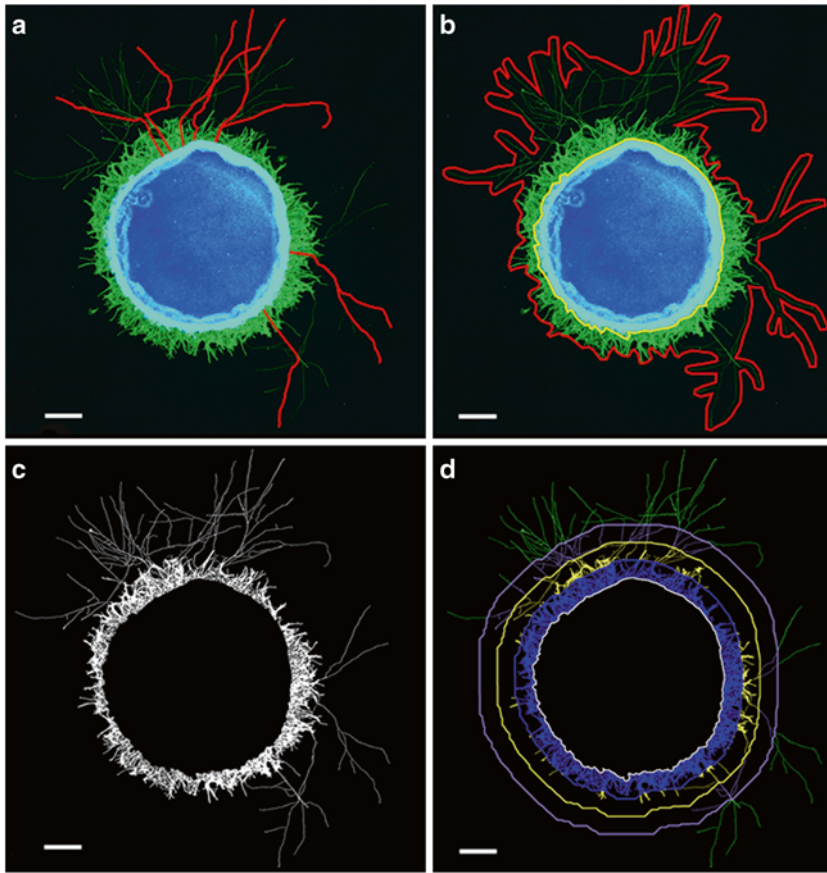
The optical circuit forms an ideal model system to study neuronal survival and axonal regeneration in the diseased or injured CNS, because of its well-characterized morphology and functionality. Furthermore, all axons in the optic nerve (ON) arise from a single neuronal cell population, the retinal ganglion cells (RGCs), which can be experimentally manipulated with minimal surgery. Recent *in vivo* studies have shown that adult RGCs can be stimulated to overcome axonal injury, to extend their axons past the lesion site and to reinnervate their original targets [4, 5]. This discovery has shattered the dogma and formed new hope for patients affected by neurodegenerative disorders or CNS injuries, such as spinal cord injury, and awoken an increased interest of the pharmaceutical industry in the discovery of new therapeutic strategies that stimulate neuroprotection and regeneration [4].

Efforts to identify novel compounds for regenerative treatments are limited by a gap between high-throughput screening models and validation in preclinical animal models. High-content and high-throughput screening methods are mostly based on dissociated cells to assess the effect of a compound, thereby enabling the screening of huge compound libraries and the rapid identification of novel lead compounds. Unfortunately, dissociated cells can show an artificial behavior and they are often only used to elucidate a direct effect on the cells in culture. Preclinical animal models, on the other hand, maintain the intercellular relationship, thereby closely resembling the actual pathology. However they are less efficient and time consuming, often because it can take several weeks before the actual disease manifests. Together with the high degree of variability between animals, this results in a costly and labor intensive experimental setup. Organotypical culture models, such as retinal explants, form an ideal bridge to close this gap, as they provide the necessary compromise between the two model systems. The major advantage of explant cultures is that cells are kept within their normal environment, thereby preserving cell-to-cell interactions, while maintaining a higher level of experimental control as in animal models [6]. Organotypic cultures thereby provide an ideal platform for the identification and validation of novel neuroprotective or pro-regenerative substances. As retinal explants have previously been used to study various neural processes such as retinal differentiation [7, 8], neurodegeneration [9, 10], and neurite outgrowth [11–13], they provide an ideal *ex vivo* system to screen promising molecules.

Neurite outgrowth can be visualized by fluorescent immunohistochemistry (IHC) and optical microscopy, such as phase contrast and differential interference contrast (DIC) microscopy. These optical microscopic techniques are often used in combination with manual analyses. As no prior treatment is required, explants can be studied while they are still in culture, thereby allowing time-lapse experiments. A disadvantage is that no objective distinction can

be made between glial and neuronal processes, which may result in a false representation of neurite outgrowth. This distinction can be made using antibodies targeting neuronal markers, such as  $\beta$ -tubulin or neurofilament. Neurofilament is frequently used as a neurite marker in retinal cell and explant cultures of adult animals, resulting in a clear and uniform staining. In neonatal cultures, however, a neurofilament immunostaining results in a patchy expression pattern along RGC neurites, making it unsuitable for neurite quantification [14].  $\beta$ -Tubulin, on the other hand, is commonly used as a marker for RGCs and their axons, providing a uniform staining for neurite outgrowth both in adult and in neonatal cultures. The explant body can easily be visualized using the nuclear marker DAPI. The main disadvantage of using immunological stainings is that time-lapse experiments are no longer possible afterwards. Of note, transgenic animals, that express a fluorescent protein in RGC axons, such as the thyl-YFP mice [15], might allow for time-lapse experiments. However, axonal injury leads to a large shift in the expression pattern of RGCs, e.g., a decreased expression of thyl, thereby resulting in a large decrease in GFP expression in newly formed neurites and thus an underestimation of neurite outgrowth (personal observations and refs. [16, 17]).

Neurite outgrowth assessment in explant cultures is typically done using manual or semiautomated analyses. Manual tracing of “neurite length” (Fig. 1a) and quantification of the “neurite outgrowth area” (NOA) (Fig. 1b) are two of the most common methods to quantify neurite outgrowth [12, 18]. However, these analyses are time consuming, labor intensive, and prone to user-biased errors. Therefore, we recently developed a custom made analysis script to automatically quantify the “immunodetected neurite area” (INA) (Fig. 1c) [19]. Dual-colored pictures are split into two channels, one containing the explant body as visualized by DAPI staining and the second channel representing the network of immunolabeled neurites. Morphological features in both channels are detected by local thresholding and subsequent filtering steps, resulting in a clear detection of, respectively, the explant and its outgrowing neurites. The INA is then quantified as the area that is occupied by the detected neurites, normalized to the perimeter of the explant. Furthermore, the INA can also be categorized into four segments by drawing three concentric rings, generated by successive 100  $\mu\text{m}$  increments from the explant body edge and categorizing neurite outgrowth in three inner ring segments and an outer segment with the remaining detected neurites (Fig. 1d). This categorization can be used to study compound-induced effects on axonal extension, by comparing the neurite area in the outer segment, which only contains long axons, relative to that of the inner segment, representing all axons. Furthermore, we recently included an automated NOA analysis based on the



**Fig. 1** Overview of quantification methods determining neurite outgrowth in retinal explants. **(a)** In the “maximum neurite length” analysis method, the 15 longest neurites are manually traced as shown in *red*. **(b)** In the “Neurite outgrowth area” or NOA analysis method, the total neurite area is outlined, as shown in *red*. The area of the explant body, outlined in *white*, is then subtracted. **(c, d)** The automated analysis method automatically detects the “immunopositive neurite area” or INA (*white*) **(c)** and segments it into four concentric rings, shown in different colors (*blue, purple, yellow, and green*) **(d)**. Figure modified from ref. [19]

detected neurites from the INA assay. In this analysis, the detected neurites are dilated and any remaining gaps in the dilated area are filled, thereby marking the neurite outgrowth area, similar as in the manual NOA analysis. The use of this automated script offers many advantages, such as increased objectivity and reproducibility. Most importantly, the speed of analysis is significantly higher compared to the manual methods, which take several hours hands-on time per experiment.

Taken together, retinal explants form an interesting model to efficiently evaluate neurite outgrowth in an *in vivo*-like situation. This chapter precisely describes retinal explant dissection, culture, immunostaining, and analysis methods. Of note, all analysis tools are described based on Axiovision software, but other analysis programs, such as ImageJ, can also be used after mild adaptations.

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## 2 Materials

### 2.1 Tissue Dissection and Retinal Explant Culture

All dissection tools should be sterilized with 70 % ethanol or autoclaved and all solutions should be prepared with ultrapure water and autoclaved.

1. Dissection microscope with light source, e.g., Leica S6E.
2. Dissection instruments: Blunt standard scissors, curved Dumont #7 forceps, 2 Dumont #5 forceps, 23G needle, Vannas spring scissor, Blunt straight forceps, and Versi handle with 750  $\mu$ M FUE standard punch needle (Mediquip Surgical).
3. P200 micropipette.
4. Ice-cold Neurobasal-A medium (Invitrogen).
5. Phosphate-buffered saline (PBS) consisting of 8 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 2 mM  $\text{KH}_2\text{PO}_4$ , 150 mM NaCl, and 3 mM KCl, pH 7.4.
6. Poly-L-lysine hydrobromide ( $M_w$  70,000–150,000, Sigma) diluted in PBS to a concentration of 0.25 mg/mL.
7. Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane (Sigma) diluted in PBS to a concentration of 2  $\mu$ g/mL.
8. Nunc round 4-well culture plates (Thermo Scientific).
9. Explant culture medium consisting of Neurobasal-A medium supplemented with 1 mM L-glutamine, 0.25  $\mu$ g/mL fungizone, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 % B27 supplement (all from Invitrogen), and 0.4 % methylcellulose (Sigma). Culture medium is stored at 4 °C in the dark (*see Note 1*).
10. Mice: 6–8 littermate pups (at 0–3 days after birth (*see Note 2*)).

### 2.2 Immunohisto- chemistry

1. Fixation solution consisting of 4 % paraformaldehyde in PBS (*see above*).
2. Washing buffer containing 50 mM Tris, 150 mM NaCl, and 0.1 % Triton X-100, pH 7.6.
3. Incubation buffer containing 50 mM Tris, 150 mM NaCl, and 0.5 % blocking reagent (Perkin Elmer), pH 7.6.
4. Blocking solution containing 20 % pre-immune serum of goat (Invitrogen) in incubation buffer.
5. Primary antibody solution containing monoclonal mouse anti- $\beta$ -tubulin antibody (Sigma, clone SDL3D10) diluted 1/500 in incubation buffer.
6. Secondary antibody solution containing goat anti-mouse IgG labeled with Alexa Fluor 488 (Invitrogen) diluted 1/500 in incubation buffer (*see Note 2*).

7. DAPI solution consisting of 1  $\mu\text{g}/\text{mL}$  DAPI (Applichem) in PBS.
8. Storage buffer consisting of 0.01 % sodium azide in PBS.

### **2.3 Image Acquisition and Analysis**

1. Image acquisition is performed using an inverted confocal microscope, e.g., FV1000 IX-82 microscope (Olympus).
2. Image analysis is performed using Axiovision analysis software (Zeiss).

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## **3 Methods**

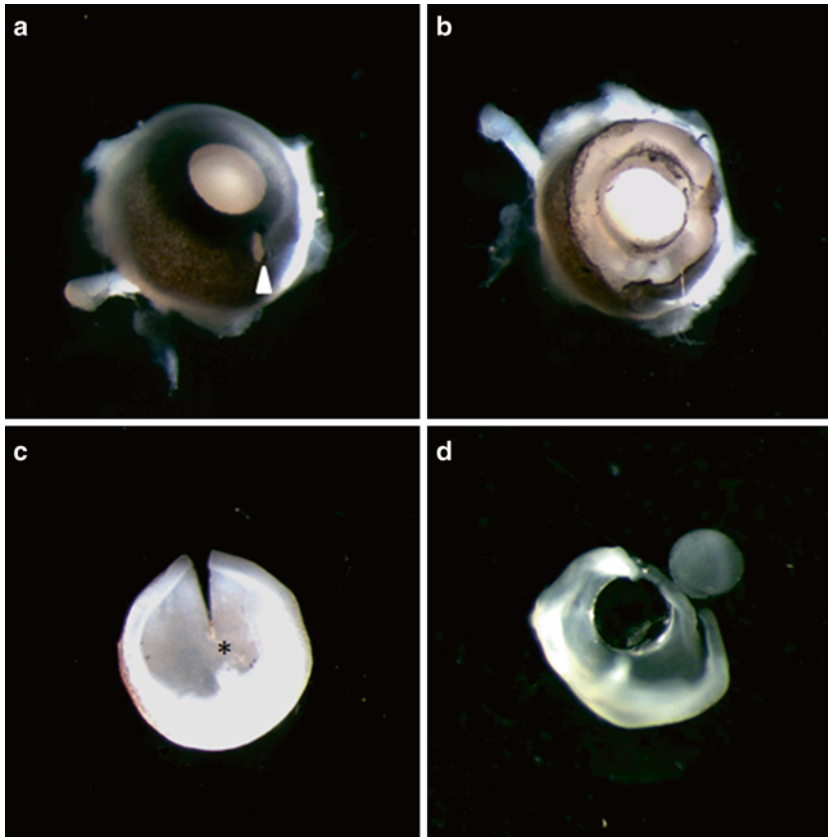
The following procedures are all performed under a horizontal laminar flow to prevent contamination.

### **3.1 Coating of Cell Culture Plates**

1. The day before dissection, culture plates are coated with poly-L-lysine. Fill each well with 300  $\mu\text{L}$  of 0.25  $\text{mg}/\text{mL}$  poly-L-lysine solution and place the plates in the incubator at 37 °C for overnight incubation (*see Note 3*).
2. On the day of dissection, aspirate the poly-L-lysine solution and rinse the plates three times with sterile PBS.
3. Fill the wells with 300  $\mu\text{L}$  laminin solution and incubate for 2 h in the incubator at 37 °C.
4. Aspirate the laminin solution and rinse twice with PBS.
5. Add 250  $\mu\text{L}$  of explant culture medium to each well and store in the incubator at 37 °C till use.

### **3.2 Explant Dissection**

1. Euthanize mouse pups by decapitation (*see Note 4*).
2. Disinfect the head with 70 % ethanol spray.
3. Peel off the skin covering the eye using Dumont #5 forceps, carefully dissect the eyeball using the Dumont #7 curved forceps, and place it in a drop of ice-cold Neurobasal medium in a plastic petri dish (*see Note 5*). Store one eye on ice, while dissecting the other under a dissection microscope.
4. Punch a hole in the limbus with the 23 G needle, while holding the eye by the ocular muscles (Fig. 2a, arrow). Cut along the limbus using the spring scissors to remove the anterior chamber (Fig. 2b). Remove the lens, inner limiting membrane, and blood vessels from the retinal cup using Dumont #5 forceps. Finally remove the retinal pigment epithelium/sclera and pinch off the optic nerve. Make a perpendicular cut from the edge of the retina to the optic nerve head (Fig. 2c).
5. Fix the retinal cup by placing the blunt forceps at the optic nerve head. Place the punch needle at the periphery of the



**Fig. 2** Overview of the dissection of retinal explants. A small hole (*arrow*) is punched at the limbus of an isolated eye using a 23 G needle (**a**). The anterior chamber is cut off at the limbus (**b**), next the lens and pigmented sclera are removed and an incision is made from the edge of the retina to the optic nerve head as indicated by (*asterisk*) (**c**). Explants are excised one by one at a similar peripheral position, while fixing the retina by placing a blunt forceps at the optic nerve head (**d**)

retina and gently press down to excise the explant (Fig. 2d) (*see* **Notes 6** and **7**). Most of the time, the punched explants will immediately attach to the petri dish, however if the explant sticks in the punch needle, gently dislodge it using fine forceps.

6. Turn the eye cup somewhat to the right and continue the dissection until all explants are harvested. Do the same with the other eye. In total 12–16 explants can be harvested from one mouse depending on its age (*see* **Note 7**).
7. Collect the punches using a P200 micropipette with a cut off tip and transfer to a coated 4-well plate with explant medium. All explants are divided over the 4 wells of the plate, thereby seeding 3–4 punches per well, depending on the age of the mouse.

8. Gently orientate the punches using blunt forceps. Explants should be positioned with the RGC layer facing the coated surface (*see Note 8*). Do not scratch the coated well bottom as this will interfere neurite outgrowth. Apply gentle pressure to allow good adhesion to the coated surface.
9. Gently add an additional 250  $\mu\text{L}$  of explant medium to each well.
10. Place the culture plate in the incubator at 5 %  $\text{CO}_2$  at 37  $^{\circ}\text{C}$ .
11. During the 72 h incubation period, 250  $\mu\text{L}$  of the medium is changed every 24 h (*see Note 9*).

### **3.3 Immunohisto-chemistry and Microscopy**

1. Collect the explants after 3 days of culture.
2. Gently remove the culture medium.
3. Carefully rinse once with cold PBS.
4. Fix the explants with 4 % PFA for 1 h.
5. Rinse the explants three times for 5 min with washing buffer.
6. Aspirate the washing buffer and add 300  $\mu\text{L}$  of blocking solution. Incubate for 45 min at room temperature (*see Note 10*).
7. Remove the blocking solution and add the primary antibody solution. Incubate for 2 h at room temperature.
8. Aspirate the primary antibody solution and rinse the plates three times with washing buffer.
9. Add 300  $\mu\text{L}$  of secondary antibody solution in each well and incubate for 2 h at room temperature. Protect from light by covering the plates with tin foil.
10. Rinse the plates three times for 5 min with washing buffer, while protecting them from light.
11. Remove the washing buffer and incubate for 30 min with DAPI solution at room temperature. Protect from light by covering the plates with tin foil.
12. Remove the DAPI solution and add 500  $\mu\text{L}$  of storage buffer. Wrap the plates in tin foil to protect from light and store at 4  $^{\circ}\text{C}$  until microscopic analysis.
13. The 4-well plates with the retinal explants are allowed to warm to room temperature before microscopic analysis. Explants are visualized under an inverted confocal fluorescence microscope with a motorized stage using a 20 $\times$  objective (*see Note 11*). DAPI, used to visualize the explant body, is excited by a 405 nm laser, and the Alexa 488 fluorophore, used to label the outgrowing neurites, by a 488 nm laser. The explant and its neurites are photographed as a two colored picture using a mosaic acquisition function. All pictures are saved as tiff files.



### 3.4 Computer-Assisted Quantification of Neurite Outgrowth

Although all analysis methods are described using the Axiovision software, alternative morphometric analysis programs, such as ImageJ, can be applied as well.

#### 3.4.1 Computer-Assisted Quantification of Maximum Neurite Length

1. The picture file is opened in Axiovision software. Maximum neurite length is determined by manual tracing of the length of the neurites using the “curve” function in the Axiovision software (*see* **Notes 12** and **13**). Neurites are traced starting from the growth cone to the explant body, visualized by the DAPI staining.
2. Start with the neurite that appears to be the longest and work your way down until the 15 longest neurites are traced (Fig. 1a).
3. Use the “create table” function to collect all measurements in a table. Save this table as an Excel sheet.
4. Repeat **steps 1–3** till all explants are analyzed.
5. The length of the 10 longest neurites is averaged per explant. This value can now be used to compare the average neurite length under different conditions.

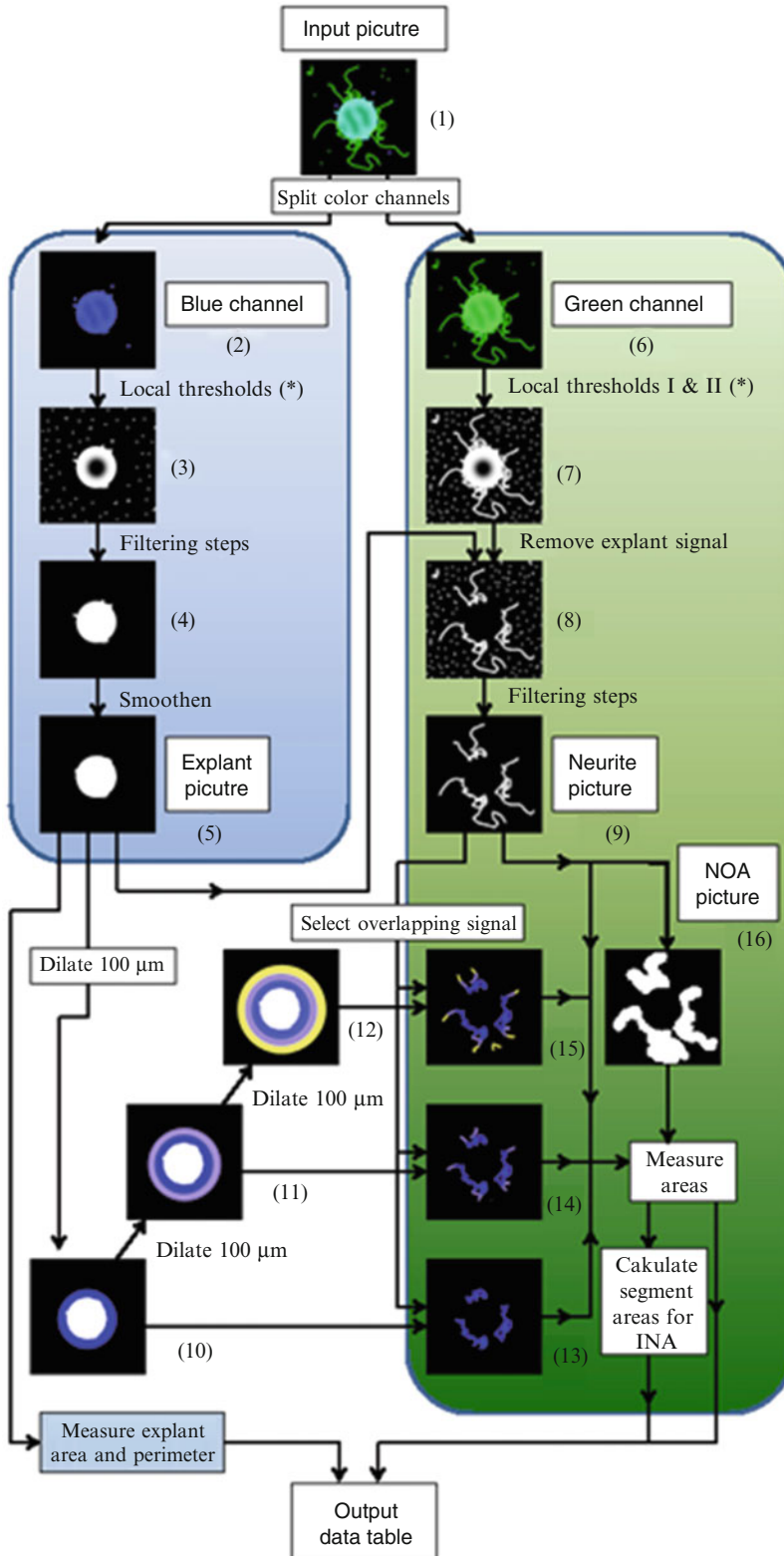
#### 3.4.2 Computer-Assisted Quantification of Neurite Outgrowth Area (NOA)

1. Open the picture file in Axiovision software. Use the “outline” tool to quantify the area and perimeter of the explant body visualized by the DAPI staining.
2. Use the “outline” tool to quantify the neurite area by outlining the neurites as visualized by the  $\beta$ -tubulin staining (Fig. 1b) (*see* **Notes 12** and **13**).
3. Use the “create table” function to collect all measurements in a table. Save this table as an Excel sheet.
4. Repeat **steps 1–3** till all explants are analyzed.
5. The NOA is obtained by subtracting the explant body area from the neurite area and subsequently dividing that value by the explant body perimeter. This value can now be used to compare NOA in different conditions.

#### 3.4.3 Automated Assessment of Neurite Outgrowth Area

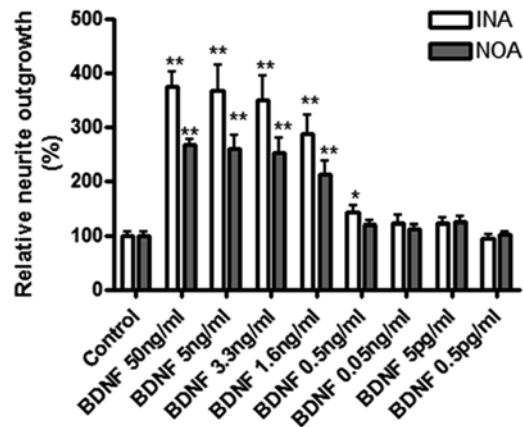
A detailed overview of all the steps in the automated analysis script is represented in Fig. 3. The analysis relies on three input values that determine the thresholding parameters. These parameters are obtained on the first picture of an entire experiment. All following explants will be automatically quantified using these input values. The setup of the analysis is described in the following steps.

1. Open the Axiovision software.
2. Use the “batch processing” function to select all the pictures of a single experiment. Select the custom made explant analysis script, as described in Gaublonne et al. [19]. Press start.



3. Set the local threshold offset and size for the nuclear staining in the dialog screen (*see Note 14*) (Fig. 3, **step 3**) and press OK.
4. Set the threshold values for the neurite signal. Set the local threshold offset and size in the first dialog screen so that thin neurites are selected (*see Note 15*). Press OK. Set the local threshold offset and size in the second dialog screen so that neurites in dense areas are detected (Fig 3, **step 6**) (*see Note 16*). Press OK.
5. The analysis script will now automatically analyze all selected pictures from the experiment, as illustrated in Fig. 3. After thresholding a series of filtering steps is applied to generate the explant and neurite picture (Fig. 3, **steps 5 and 9**). Categorization of the INA is obtained by enlarging the explant body three times with 100  $\mu\text{m}$  increments (Fig. 3, **step 10–12**) and selecting the overlapping signal with the neurite picture

**Fig. 3** Schematic representation of “immunodetected neurite area” script. In the script measuring the “immunodetected neurite area,” three input values, determining local thresholding parameters, are required. These values are determined on the first picture of a set of photographs from an entire experiment (at steps noted with *asterisk*). Once these parameters are set, all other pictures from the same experiment can be analyzed automatically, using the sequence of steps described below. Each input picture (1) (saved as a .tiff file) is split into separate color channels. The *blue* channel (2) visualizes the DAPI signal and is used for explant detection. The image is binarized with local thresholding (3). Next, in a series of subsequent filtering steps, all objects are dilated to close any gaps in the explant edge and afterwards filled up. Nuclei or tissue fragments that are slightly in contact with the explant are automatically separated and removed, together with any noise produced by local thresholding (4). Finally, the explant is slightly enlarged and smoothened and an explant picture (5) is generated, from which the explant area and perimeter are measured. The *green* channel (6) visualizes the  $\beta$ -tubulin signal and is used for neurite detection. Two local thresholds (I and II) are performed in parallel, differing in the size of the local area. Most neurites are detected using a threshold with a small local area (I). However, this setting may generate black gaps in dense neurite areas. To alleviate this problem, a second thresholding step with a larger local area (II) is used. Both output pictures are then combined in order to detect the entire neurite network (7). Next, the signal from the explant body itself is removed (8), followed by two subsequent filtering steps eliminating any remaining noise and artifacts. The end result is a binarized image of the undilated neurites without artifacts, the neurite picture (9). To categorize the total neurite area in different segments, the explant picture is dilated by 100  $\mu\text{m}$  three times (10–12). Then, the overlapping signal between the neurite picture (9) and the dilated explant pictures (10–12) is selected, yielding pictures with neurites extending from the explant to the edge of various segments (13–15). Finally, from these resulting pictures (13–15) and from the total neurite picture (9) the neurite area is measured. From these measures, the immunodetected areas per segment are calculated by subtraction. The automated NOA measurement of the neurite outgrowth starts from the previously generated neurite picture. The detected neurites are dilated and all remaining holes in the neurite network are filled (16). From this NOA picture the neurite outgrowth area is measured. All values from one input picture are put, together with the image name, in a single row in an output data table, which will ultimately contain the data from all input pictures from one experiment after completion. The script also saves a jpeg file of the neurite picture (9) and the NOA picture (16) to quickly perform a quality control. Figure modified from ref. [19]



**Fig. 4** Neurite outgrowth in retinal explants is stimulated after incubation with BDNF and CNTF. The neurotrophic factors BDNF and CNTF, in a 5:1 ratio, were added to the culture medium and explants were cultured for 3 days. Neurite outgrowth was plotted against the concentration of BDNF (the concentration of CNTF was adjusted accordingly). Both automated morphometric analyses, INA and NOA, showed a clear and dose-dependent stimulation of neurite outgrowth. Maximal stimulation was obtained at a concentration of 50 ng/mL BDNF and 10 ng/mL CNTF. Concentrations lower than 0.5 ng/mL BDNF and 0.1 ng/mL CNTF did not result in a significant stimulation of neurite outgrowth (*asterisk*) =  $P < 0.05$ , (*double asterisk*) =  $P < 0.01$

(Fig. 3, step 13–15). Finally, the INA from all these pictures is measured and the INA per segment is calculated by subtraction.

6. For the quantification of the NOA, the detected neurites from the neurite picture are dilated. Next, all remaining holes in the neurite network are filled to generate the NOA picture (Fig. 3, step 16) from which the NOA is measured.
7. The automated analysis generates 2 jpeg files for each input picture, the neurite picture and the NOA picture. A single table, containing all measurements, is automatically saved as a csv file, which can be opened with Excel. For each explant a single row lists the following data: explant area and perimeter, the total and segmented INA values, and the NOA value.

**3.5 Proof of Principle Experiment**

As an example, a dose-response experiment using a combined administration of brain-derived neurotrophic factor and ciliary neurotrophic factor was performed (Fig. 4). Both INA and NOA show a clear and dose-dependent growth factor-induced upregulation of neurite outgrowth, as is described in literature. The combination of both neurotrophic factors can therefore be used a positive control in follow-up experiments.

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## 4 Notes

1. Fresh explant culture medium is prepared for each experiment. Supplemented culture medium should not be stored for more than 7 days to ensure consistent results.
2. The primary and secondary antibodies listed provide excellent results for the immunological staining. There are other commercial anti- $\beta$ -tubulin and secondary antibodies that give similar results.
3. Poly-L-lysine-coated plates can be stored in PBS for up to 2 days at 4 °C. Prolonged storage may result in reduced cell adhesion.
4. Depending on the protocol approved by the institute, mice can be euthanized by other methods.
5. The use of cold dissection medium will facilitate the dissection, as the retina is less prone to curling. Renew the dissection medium after every eye dissection to minimize the contamination with pigment cells.
6. Applying too much pressure will damage the bottom of the petri dish and cause plastic chips to attach to the explant, thereby interfering with the growth and the detection of the neurites. Make sure you excise the punches consistently at the same peripheral level. A large variation in the centro-peripheral position will introduce variability into the model.
7. Depending on the size of the punch needle and the age of dissection, 4–8 punches can be obtained from 1 retina. Using punch needles with a diameter of 750  $\mu$ m, 6 punches can easily be harvested from a P0 retina and 8 punches from a P3 retina.
8. The RGC layer is located at the concave side of the explant. Furthermore, it has a clear crystal-like appearance. The opposite side of the retina frequently still has some dark-colored retinal pigment epithelium cells attached.
9. Depending on the type of experiment and age of mice, incubation can last for 2–7 days. Neurite outgrowth will be visible from 2 days on.
10. Incubation with the blocking solution, primary and secondary antibodies, and DAPI solution should be carried out in a dark and humid chamber to prevent evaporation of the buffers as well as photobleaching of the fluorophores.
11. Other forms of microscopy, such as phase contrast and DIC, may be used. Of note, glial cells also produce processes that extend past the explant body. Neurites can be identified as thin processes growing out of the explant body, whereas glial

processes have a thicker morphology. In our opinion superior results are obtained using immunohistochemical stainings for  $\beta$ -tubulin.

12. Alternatively, NIH image J software can be used. Different plugins, such as simple neurite tracer or NeuronJ, can assist in the tracing of the neurites.
13. Manual analysis should be performed blind to the experimental condition and by an experienced researcher in order to minimize user-biased variation.
14. The threshold value should be set so that the selected signal forms a closed circle, if not, the explant body cannot be detected.
15. The threshold value should be set so that the selected signal forms a continuous line. Individual pixels should not touch the detected neurite as this will result in a faulty measurement. In dense areas the neurite signal may not be detected at this stage, these denser areas will be detected in the following step. Afterwards, individual pixels will be filtered out (Fig. 3, step 8).
16. The threshold value should be set so that the neurite signal is detected in dense areas that are missed in the previous step. Thin neurites, detected in the previous step, may not be detected in this step.

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