



Jan 23, 2025

Automated Neurite Outgrowth Measurement of Explants

 [PLOS One](#)

✓ Peer-reviewed method

DOI

<https://dx.doi.org/10.17504/protocols.io.6qpvr853olmk/v1>

Christin Geißler¹, Monika Orsolic¹, Marc Diensthuber¹

¹Department of Otolaryngology, University Hospital Frankfurt, Germany

PLOS ONE Lab Protocols

Tech. support email: plosone@plos.org



Christin Geißler

Department of Otolaryngology, University Hospital Frankfurt

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.6qpvr853olmk/v1>

External link: <https://doi.org/10.1371/journal.pone.0318613>

Protocol Citation: Christin Geißler, Monika Orsolic, Marc Diensthuber 2025. Automated Neurite Outgrowth Measurement of Explants. protocols.io <https://dx.doi.org/10.17504/protocols.io.6qpvr853olmk/v1>

Manuscript citation:

Geißler C, Orsolic M, Guchlerner L, Diensthuber M, Stöver T (2025) Technical report: Efficient and accurate assessment of neurite outgrowth in spiral ganglion explants using Sholl analysis and repeated measurement ANOVA. PLOS One 20(6). doi: [10.1371/journal.pone.0318613](https://doi.org/10.1371/journal.pone.0318613)

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: September 26, 2024

Last Modified: January 23, 2025

Protocol Integer ID: 108434

Keywords: Spiral ganglion, Neuronal explants, Neurite outgrowth, Sholl analysis, automated neurite outgrowth measurement of explant, automated neurite outgrowth measurement, dimensional spiral ganglion explant, neurite outgrowth, sholl analysis quantifies neurite outgrowth, explants from postnatal rat, neuronal survival, gray value analysis, fluorescence intensity, outlining primary neurite, neurotrophic potential, primary neurite, neurotrophic potential of compound, using immunohistochemical staining, outgrowth, immunohistochemical staining, average brightness

Abstract

Three-dimensional spiral ganglion explants are cultivated in an in-vitro assay to assess the neurotrophic potential of compounds. The protocol presents both manual and automated methods, including Sholl analysis and Gray Value analysis, based on image evaluation. Explants from postnatal rats can be cultured with varying concentrations of growth factors and visualized using immunohistochemical staining to assess neuronal survival and neurite outgrowth. Manual tracing involves outlining primary neurites and branches to measure neurite number and length. Sholl analysis quantifies neurite outgrowth by counting intersections with concentric circles drawn at regular intervals around the explant. Gray Value analysis measures fluorescence intensity to infer neuronal survival and outgrowth. The explant's outline is expanded incrementally to assess the average brightness in the surrounding ring-shaped areas. This protocol describes how to evaluate Sholl and Gray Value analysis raw data across multiple measurement points at varying distances from the explant. Statistical analysis is conducted using repeated measures ANOVA (rmANOVA).

Materials

Animals:

- o Sprague Dawley rats, aged 3-5 days, Janvier

Reagents and Solutions:

- o HBSS (Hank's Balanced Salt Solution), Gibco/Thermo Fisher
- o DPBS (Dulbecco's Phosphate-Buffered Saline), Gibco/Thermo Fisher
- o Laminin (10 µg/ml), Corning/Merck
- o Ornithine (0.01% w/v), Sigma/Merck
- o Panserin 401, PAN Biotech
- o HEPES (23.4 mM), Sigma
- o Glucose (0.15% w/v), Sigma
- o Insulin (8.7 µg/ml), Sigma
- o N2 Supplement (0.3x), Life Technologies/Thermo Fisher
- o Penicillin (30 U/ml), Sigma
- o Acetone, Sigma
- o Methanol, Sigma
- o Goat Serum (5% v/v), Sigma
- o Bovine Serum Albumin (BSA, 1% w/v), Roth
- o Triton X-100 (0.1% v/v), Sigma
- o Beta Tubulin III (Anti-Tuj1 antibody, 1:2000), Bio Legend (801202)
- o Anti-mouse FITC antibody (1:200), Jackson Immuno Research (115-095-146)
- o DAPI (4',6-Diamidino-2-phenylindole, 1:1000), Invitrogen/Thermo Fisher

Equipment:

- o Stereomicroscope
- o Chamber slides (eight well, glass bottom), Ibidi
- o Laminar Flow: Clean Bench
- o Incubator (37°C, 5% CO₂)
- o Axio Imager.M2 microscope, AxioCam MRm, Zeiss
- o Scissors and Forceps

Software:

- o ImageJ Fiji (2.14.0/1.54f plugins: Neuroanatomy, NeuronJ)
- o IBM SPSS Statistics (29.0.2.0 (20))
- o Adobe Photoshop 2024 (25.11)
- o Zeiss AxioVision (4.8.1)

Animal preparation

- 1 **Collect Temporal Bones from postnatal rats**
 - Use postnatal Sprague Dawley rats aged 3-5 days, both male and female.
 - For each experiment, use 5 rats.
 - Perform decapitation to euthanize the animals, following ethical guidelines for treatment.
 - Carefully collect Temporal Bones from postnatal rats with scissors and place the collected temporal bones immediately in ice-cold HBSS to preserve the tissues.
- 2 **Isolate the Spiral ganglion:**
 - In the Clean Bench, under the stereomicroscope, carefully separate the cochlear capsules from the temporal bones using Forceps
 - Isolate the spiral ganglia from the modiolus.
 - Place the isolated spiral ganglia in DPBS to preserve them until further processing.
 - Cut the spiral ganglia into small explants, approximately 500 μm in size.
 - Categorize the explants according to their position in the cochlea into three groups: Basal, Medial, Apical

Cell Culture: spiral ganglion explant

- 3 **Preparation of Chamber Slides:**
 - Use eight-well chamber slides with glass bottoms
 - Coat the slides with Laminin (10 $\mu\text{g}/\text{ml}$) and Ornithine (0.01% (w/v)).
- 4 **Prepare the culture medium consisting of:**
 - Panserin 401
 - 23.4 mM HEPES buffer
 - 0.15% (w/v) Glucose
 - 8.7 $\mu\text{g}/\text{ml}$ Insulin
 - 0.3x N2 Supplement
 - 30 U/ml Penicillin
 - Add growth factors to the medium in varying concentrations
- 5 **Culture Conditions**
 - Place the explants in an incubator at 37°C with 5% CO_2 .
 - Culture the explants for 72 hours.
- 6 **Fixation of Explants**
 - After 72 hours, fix the explants using an acetone-methanol solution (1:1).

Immunohistochemistry

7 **Blocking**

- Block the slides using Dulbecco's Phosphate Buffered Saline (DPBS, pH 7.3) with the following components:
 - 5% (v/v) Goat Serum (Sigma)
 - 1% Bovine Serum Albumin (BSA; Roth)
 - 0.1% (v/v) Triton X (Sigma)

8 **Primary Antibody Staining**

- Stain the samples overnight using the neuronal marker beta Tubulin III (Anti-Tuj1 antibody, 1:2000 in 1xDPBS, 5% goat serum, 1%BSA, 0,1% Triton), at 4°C.

9 **Secondary Antibody Staining**

- Apply the anti-mouse FITC antibody (1:200 in DPBS with 0.1% Triton X), 1 hour at room temperature.

10 **DAPI Staining**

- Perform DAPI staining to visualize nuclei (1:1000 in DPBS), 20 minutes at room temperature.

11 **Microscopy**

- Use an Axio Imager.M2 microscope and AxioCam MRm with 5x magnification to capture images.
- Capture images with a constant exposure time of 8 seconds.
- If necessary, align the microscopic fields of each explant using Adobe Photoshop (Version 25.11).

Sholl Image analysis with ImageJ Fiji (2.14.0/1.54f, Neuroanatomy)

- 12 **Open the Tuj1 Staining Image** This is the image of neurite outgrowth.
The commands can be entered by navigating through the dropdown menus or directly (Plugins > New Macro, insert the line, click Run)

13 **Set Scale to Pixels:**

- set the scale in pixels. (Analyze > Set Scale)

```
run("Set Scale...");
```

14 **Resize the Image Canvas**

- Increase the image canvas size to ensure that the concentric circles do not touch the image borders to set the canvas size (e.g., 2500×2500 pixels).

```
run("Canvas Size...");
```

15 Remove the explant from the neurite outgrowth image

- Open the DAPI-stained image to define the size of the explant.
- Use the Wand Tool to outline the explant by adjusting the tolerance settings.
- copy Explant Outline, paste it onto the neurite outgrowth image, and remove the DAPI stain so that only the outline remains by pressing strg + z.
- Enlarge and Measure Explant Size by 5 pixels to ensure it covers the entire explant.
- Measure and cut out the explant area from the neurite outgrowth image.

```
run("Copy");
```

```
run("Paste");
```

```
run("Enlarge...", "enlarge=5");
```

```
run("Measure");
```

```
run("Cut");
```

16 Noise Reduction:

- Apply a despeckle filter to reduce noise

```
run("Despeckle");
```

17 Convert to Binary

- Convert the image into a binary format by setting a manual threshold for brightness. The threshold is set between 15 and 255, making the background black and neurites white

```
run("Threshold...");
```

```
setOption("BlackBackground", false);
```

18 **Mark the Explant Center**

- Using measurement data from the cut-out, place a point at the center of the explant with the point tool to set the center (data from the explant measurement (Step 15))

```
setTool("point");
```

```
makePoint(x, y);
```

19 **Run Sholl Analysis Plugin**

- Open the Sholl Analysis plugin from ImageJ Fiji Plugins > Neuroanatomy> Sholl > Analysis from Image and set parameters for the analysis:
- Start Radius: Defines the distance from the center where the first circle will be drawn (should cover the incipient neuritis of the explant).
- Step Size: Determines the distance between consecutive circles (e.g., 10 pixels).
- End Radius: (should cover the tip of the longest neurite of the explant)

20 **Remove Artifacts:**

- If necessary, manually remove staining artifacts by using the paintbrush tool and the background color, then re-run the Sholl analysis.

```
setForegroundColor(255, 255, 255);
```

21 **Data Export:**

- Copy the data (distance from the center and the number of neurite intersections) and export it to Microsoft Excel for further analysis.

Gray Value analysis with ImageJ Fiji (2.14.0/1.54f)

22 **Open the Tuj1 Staining Image:** This is the image of neurite outgrowth. Transform into a 8-bit image type (Image, Type) and save the file.

23 **Set Scale to Pixels:**

- define the scale in terms of pixels.

```
run("Set Scale...");
```

24 **Measure Background Brightness:**

- Use the rectangle tool to measure brightness in four corners of the image. Average the values to calculate the mean background brightness using

```
setTool("rectangle");
```

```
run("Measure");
```

25 **Resize the Image Canvas:**

- Increase the image canvas size to ensure that the concentric circles do not touch the image borders to set the canvas size (e.g., 2500×2500 pixels)

```
run("Canvas Size...");
```

26 **Remove the explant from the neurite outgrowth image**

- Open the DAPI-stained image to define the size of the explant.
- Use the Wand Tool to outline the explant by adjusting the tolerance settings.
- Paste Explant Outline, paste it onto the neurite outgrowth image, and remove the DAPI stain so that only the outline remains.
- Enlarge and Measure Explant Size by 5 pixels to ensure it covers the entire explant.
- Measure the explant area and Brightness from the neurite outgrowth image.

```
run("Copy");
```

```
run("Paste");
```

```
run("Enlarge...", "enlarge=5");
```

```
run("Measure");
```

27 **Measure Brightness in Successive Rings:**

- Continue enlarging the outline by increments of 10 pixels to cover the surrounding neurites, measuring brightness after each enlargement
- Measure, then repeat the enlargement until all neurites are completely covered.

```
run("Enlarge...", "enlarge=10");
```

```
run("Measure");
```

28 **Calculate Ring Brightness:**

- Export the data (area and cumulative brightness) to Microsoft Excel.
- Calculate the following parameters:
 - Ring Area: Calculate by subtracting the area of the previous ring from the current one.
 - Cumulative Brightness: Subtract the cumulative brightness of the previous area from the current one.
 - Mean Brightness per Ring: Divide the cumulative brightness by the corresponding ring area.
- Subtract the background brightness (Step 24)

Manuel Measurement with ImageJ Fiji (2.14.0/1.54f, NeuronJ 1.4.3)

29 **Open the Tuj1 Staining Image:** This is the image of neurite outgrowth. Transform into a 8-bit image type (Image > Type) and save the file.

30 **Neuronal Survival**

- Click on multiple point tool and mark the neurons in the explant.
- Count neurons with visible soma, nucleus, and neurites

31 **Open the NeuronJ Plugin** (Plugins > NeuronJ)

32 **Open the Tuj1 Staining Image:** This is the 8-bit image of neurite outgrowth. (Click on Load image/tracing)

33 **Primaries**



- Click on Add Tracing. The route of all primary neurites is traced by hand starting from the explant (single click to start)
- The tracing following the longest branch (double click to finish)
- Label Tracings as Primary (click on Label tracings)

34 **Branching**

- Further branches are marked and traced. Click on Add Tracing.

35 **Measure Tracings**

- Click on measure tracings for the following parameters: number, length, and brightness
- Measure Primary and Branchings separately
- Copy data to Microsoft Excel

Repeated Measurement ANOVA with IBM SPSS Statistics (29.0.2.0 (20))

36

Instructions for Importing and Arranging Data in SPSS from Excel

- Rearrange the dataset in Excel: each explant is represented by a row and each measurement point (distance) by a column.
- In SPSS, go to File > Import Data > Excel and open the Excel file

37 **Measurement Setup:**

- Go to Analyze > General Linear Model > Repeated Measures.
- Name the Within-Subject Factor: Distance
- Set Number of Levels to the Amount of measurement points
- Click Add and then Define.
- Select Measurement Points: Choose the measurement points from the list for your within-subject variable.
- Define Between-Subjects Factor: Select the Grouping parameter (e.g., growth factor concentration) as the Between-Subjects Factor.
- Plots: Click on Plots to create a graph. Choose the Grouping factor (e.g., growth factor concentration) and select it to display as separate lines.
- Post Hoc Comparisons: Click on Post Hoc to compare the groups.