MethodsX 13 (2024) 102781

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/methodsx

Analysis of the spatial and morphological characteristics of oligodendrocytes from images of *in vitro* culture ☆

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ARTICLE INFO

Method name:
Spatial and morphological image analysis for *in vitro* oligodendrocytes

Keywords:
Oligodendrocyte
Oligodendrocyte progenitor cell
Spatial randomness
Sholl analysis
Morphological complexity

ABSTRACT

Oligodendrocytes (OLs) are glial cells responsible for the formation of myelin sheaths in the central nervous system. The characteristic features of the oligodendrocyte lineage, ranging from proliferative and migratory oligodendrocyte progenitor cells (OPC) to myelinating mature OLs, can be observed *in vitro* cultures of OL lineage cells. Here, we introduce a method for analyzing the spatial distribution of OPCs, which reflects their capacity for proliferation and migration, and the morphological complexity of mature OLs, which reflects their capacity for myelin formation, from immunostaining images of *in vitro* OL cultures. Through the methods described, we have demonstrated the tendency for OPCs to cluster in an environment with epidermal growth factor (EGF), and the differing morphological complexity of mature OLs according to culture medium and duration of differentiation.

- The proliferative and migratory characteristics of OPCs can be evaluated by analyzing their spatial distribution.
- The myelin-forming capacity of mature OLs can be measured by analyzing their morphological complexity.
- Image-based analyses may be a substitute for more convoluted experiments to assess OL function.

Abbreviations: CNS, Central nervous system; CSR, Complete spatial randomness; DAPI, 4′,6-diamidino-2-phenylindole; DMEM/F12, Dulbecco's modified eagle's medium / Ham's F12; EGF, Epidermal growth factor; FGF, Fibroblast growth factor; MBP, Myelin basic protein; MOG, Myelin-oligodendrocyte glycoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBM, Neurobasal medium; NSC, Neural stem cell; OL, Oligodendrocyte; OPC, Oligodendrocyte progenitor cell; PDGF, Platelet-derived growth factor; PDGFRα, Platetlet-derived Growth Factor Receptor Alpha; ROI, Region of Interest.

☆ **Related research article**

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https://doi.org/10.1016/j.mex.2024.102781
Received 11 January 2024; Accepted 27 May 2024
Available online 8 June 2024

Specifications table

Subject area:	Neuroscience
More specific subject area:	Oligodendrocyte morphology, Oligodendrocyte progenitor cell spatial distribution, Image analysis
Name of your method:	Spatial and morphological image analysis for *in vitro* oligodendrocytes
Name and reference of original method:	Baddeley, A. & Turner, R. spatstat: An R Package for Analyzing Spatial Point Patterns. Journal of Statistical Software 12, 1 - 42, doi:10.18637/jss.v012.i06 (2005). Ferreira, T. A. et al. Neuronal morphometry directly from bitmap images. Nat Methods 11, 982–984, doi:10.1038/nmeth.3125 (2014).
Resource availability:	Free open source software: Fiji/ImageJ [1], R/Rstudio, version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) https://imagej.net/software/fiji/downloads https://posit.co/download/rstudio-desktop/

Background

Oligodendrocytes (OLs) are glial cells in the central nervous system (CNS) responsible for the formation of myelin sheaths wrapping neurons, which enable saltatory conduction and provide metabolic support [2]. OL lineage cells are involved in various physiological CNS functions, and the compromise of such functions has been implicated in many CNS disorders, including ischemic brain diseases, multiple sclerosis, neuromyelitis optica spectrum disorders, neurodegenerative diseases, and traumatic brain injury [3-5]. The OL lineage spans from proliferating migratory oligodendrocyte progenitor/precursor cells (OPC) to myelin-generating mature OLs that ensheath axons. The distinct stages of the OL lineage can be identified by assessing the expression of OL stage-specific markers, such as platelet-derived growth factor alpha (PDGFRα) or neuroglia 2 (NG2) for OPCs and myelin basic protein (MBP) or myelin-oligodendrocyte glycoprotein (MOG) for mature OLs, and their stage-dependent functions can be measured by various *in vitro* and *in vivo* assays. OPC proliferation can be measured by proliferation markers such as BrdU or EdU, Ki67, or 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT)-based proliferation assays, and OPC migration can be measured using Transwell migration assays [6,7]. Myelin generation can be quantified using immunoblotting for myelin-associated proteins, such as MBP, and electron microscopy can be used to measure myelination *in vivo* [8]. Although these assays are valid methods for assessing OL function, they may be costly and require specific reagents. Quantitative assays, such as western blotting, transwell migration, and MTT assays, inevitably make qualitative analyses impossible. If possible, extracting data from images of OL markers may enable quantitative analysis while simultaneously visualizing cells through their markers without the need for other requirements. Here, we introduce a method for assessing the spatial distribution of OPCs and the morphological complexity of mature OLs based on immunostaining images of *in vitro* primary-cultured OL lineage cells. The proposed method, which uses commonly used software, can help detect information from images of primary-cultured OLs that may reflect their stage-specific characteristics, such as the proliferative and migratory capacity of OPCs or the generation of myelin by mature OLs.

Method details

Images were acquired from primary cultures of rat oligodendrocytes using a method devised in the laboratory [7]. Imaging was performed using an LSM800 confocal microscope (Carl Zeiss, Inc.). Images were preprocessed using Fiji/ImageJ [1], and R/RStudio, version 4.2.0 (R Foundation for Statistical Computing, Vienna, Austria) was used for subsequent statistical analysis.

Poisson point process-based evaluation of the spatial distribution of OPCs

To acquire accurate information on the location of OPCs, the ICC image should include a nuclear counterstain such as 4′,6-diamidino-2-phenylindole (DAPI) or Hoechst 33342 (if the culture is of high purity, above 90 %, then nuclear counterstaining alone may suffice) or staining for a nuclear antigen such as Olig2, which is a transcription factor specific to all OL lineage cells. All image formats compatible with Fiji/ImageJ were considered acceptable. The process of obtaining the x–y coordinates of the cell nuclei in an image is explained below and is shown in Fig. 1.

1. Open the image in Fiji/ImageJ. The image should exclusively contain OPC nuclei.
2. Convert the image into binary format by running *Make Binary (Process–Binary–Make Binary)*
    2.1. Depending on the background noise level, the image may not be of sufficient quality for direct conversion into binary format. In this case, functions such as *Despeckle (Process–Noise–Despeckle)* or *Remove Outliers (Process–Noise–Remove Outliers)* may aid in noise removal.
3. Run *Fill Holes (Process–Binary–Fill Holes)* to eliminate dark spots within the nuclei.
4. Run *Median (Process–Filters–Median)* to remove residual background noise and smoothen the nuclei.
    4.1. *Median* requires the application of a radius (pixels). The optimal radius may depend on the size of the nuclei (pixels).
5. Run *Watershed (Process–Binary–Watershed)* to separate overlapping nuclei.
6. Run *Analyze Particles (Analyze–Analyze Particles)* to obtain x–y coordinates of the OPC nuclei.

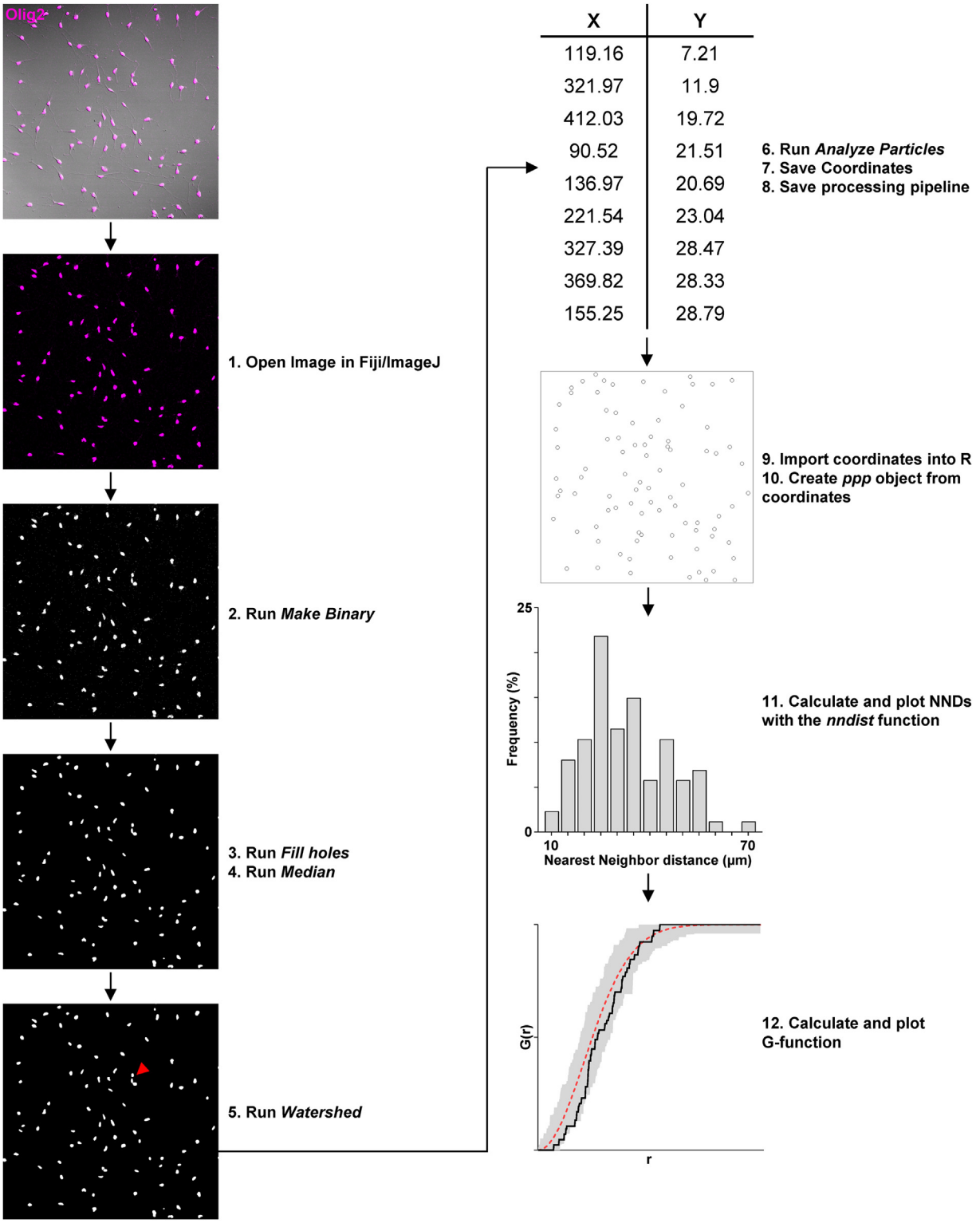

Fig. 1. Analysis pipeline to evaluate the spatial distribution of OPCs. Analysis pipeline to first process images of OPC nuclei in Fiji/ImageJ, obtain xy coordinates of OPCs, and calculate NNDs and the G-function in R to evaluate spatial distribution. Red arrowhead = overlapping nuclei separated by the Watershed function in Fiji/ImageJ. NND = nearest neighbor distance. OPC = oligodendrocyte progenitor cell.

6.1. The "Centroid" parameter in *Set Measurements (Analyze–Set Measurements)* should be checked to obtain coordinates, and if not already, the scale of the image should be set through *Set Scale (Analyze–Set Scale)* to acquire the coordinates in actual distances instead of pixels.

7. Save the resulting table of x–y coordinates as a .csv file
8. The processing pipeline can be recorded as macro code using the *Record (Plugins–Macro–Record)* function and applied to multiple images through *Batch–Macro (Process–Batch–Macro)*.
   8.1. An argument must be included to have the resulting .csv table of x–y coordinates include the name of the image file when saving. The following code can be used to achieve this name = getTitle();
   saveAs("Results",name+".csv")
   Through the above steps, tables of the x–y coordinates of the OPCs in an image can be obtained. To measure NNDs and evaluate the degree of spatial randomness, the *spatstat* package in R, developed for spatial statistics, can be used [9,10]. Point patterns can be generated from the x–y coordinates and tested to observe whether the distribution of OPCs falls under complete spatial randomness (CSR). The detailed steps are listed below and in Fig. 1.
9. Import the .csv results table as a dataframe (named "df" in the example code) into R with the *read_csv* function. The x coordinates will be under the column "X" and the y coordinates under the column "Y."
   9.1. *read_csv* can be activated by installing the library *tidyverse*.
10. 10. Convert the dataframe into a 2D point pattern through the *ppp* function in *spatstat*.
   10.1. Arguments specifying the columns with x and y coordinates, and the size of the window (referring to the size of the image) are needed.
   10.2 The successful conversion to a *ppp* class object can be validated by plotting the object with plot() and comparing the plotted *ppp* object with the source image.
11. Calculate NNDs through the *nndist* function in *spatstat*.
12. Test for CSR with the G-function. Generate a data frame (named "Gfunction" in the example code) by running *envelope(, Gest)*, and plot the dataframe by running *plot()*.
   12.1 The G-function is the cumulative frequency distribution of NNDs. The gray area of the G-function plot demarcates the boundary of CSR, and the red dotted line represents the theoretical Poisson curve. 12.2 The built-in *plot()* function in R does not visualize the entire data frame. To visualize the entire G-function curve, we exported the data frame to a data table.
   Thus, the spatial distribution of OPCs can be assessed and compared using the following method.

Sholl analysis of mature OLs to assess the morphological complexity of mature OLs

To quantify and compare the degree of morphological complexity, we used Sholl analysis, a method originally devised to analyze neuronal morphology [10]. The key principle of Sholl analysis is to measure the number of intersections an imaged cell makes with concentric spherical shells of increasing diameter. Sholl analysis of images of cultured mature OLs stained for MBP can be performed using Fiji/ImageJ with the *Neuroanatomy* plugin, and the process is described below and in Fig. 2.

1. Open the image with MBP staining in Fiji/ImageJ.
   1.1. Set the scale of the image with *Set Scale*.
2. Run *Smooth (Process–Smooth)* to smoothen the image.
3. Convert the image into binary format using *Make Binary*.
4. Draw a region of interest (ROI) around the cell to be analyzed using the freehand selection tool.
5. Run *Clear Outside (Edit–Clear Outside)* to clear processes from cells other than the one to be analyzed.
6. Mark an ROI at the center of the cell soma using the point/multi-point tool.
7. Execute Sholl analysis using *Sholl Analysis (From Image) (Plugins–Neuroanatomy–Sholl–Sholl Analysis (From Image))*. A prompt should appear when setting the parameters for the analysis.
   7.1. The *Neuroanatomy* plugin should be installed into Fiji/ImageJ to use the Sholl Analysis feature.
   7.2. The ROI at the center of the cell soma was automatically set to that at the center. The step size (increment of shells) and end radius (radius of the last shell) were set before the analysis was performed.
   7.3. The end radius should be set as the length of the longest line that can fit within the image frame (for a square image, the length of the diagonal line linking the corners) to prevent processes from being omitted from the analysis.
8. The "Analyze Image" button in the Sholl analysis prompt generates an overlay of the analysis and a table with the radius of each shell and the corresponding number of intersections.
   Thus, the morphological complexity of cultured mature OLs can be evaluated using Sholl analysis of MBP-stained images.

Method validation

Spatial distribution of OPCs in in vitro *culture*

OPCs arise from neural stem cells (NSCs), which are known to form aggregated neurospheres *in vitro*; OPCs can form *in vitro* "oligospheres" as well [11]. During the development of a method for primary OL culture from neonatal rodent brains, we discovered

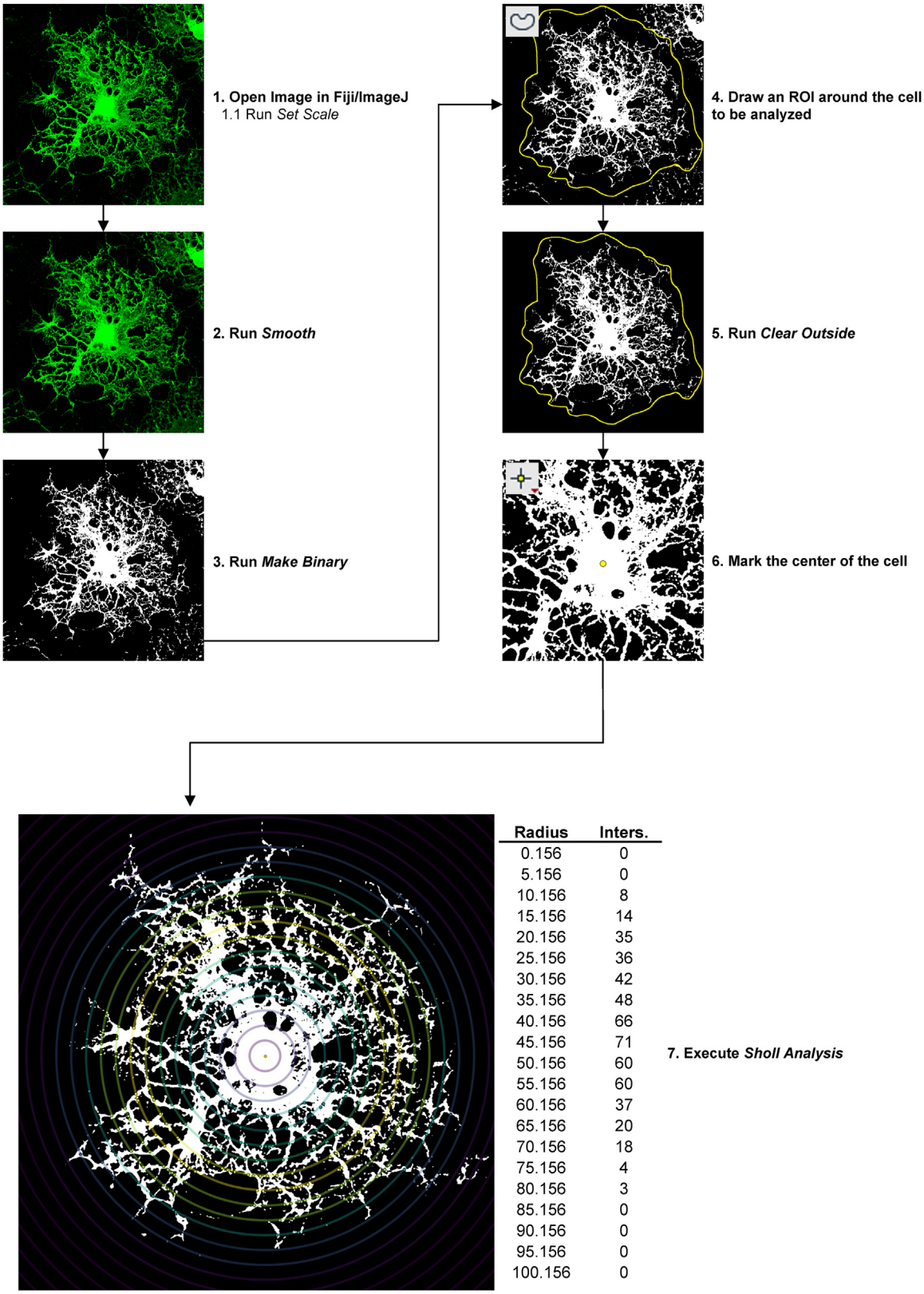

Fig. 2. Analysis pipeline for Sholl analysis to measure the morphological complexity of mature oligodendrocytes *in vitro*. Analysis pipeline to perform Sholl analysis, to quantify the morphological complexity of mature oligodendrocytes from immunocytochemistry images of myelin-associated proteins, using the Neuroanatomy plugin in Fiji/ImageJ. ROI = Region of interest.

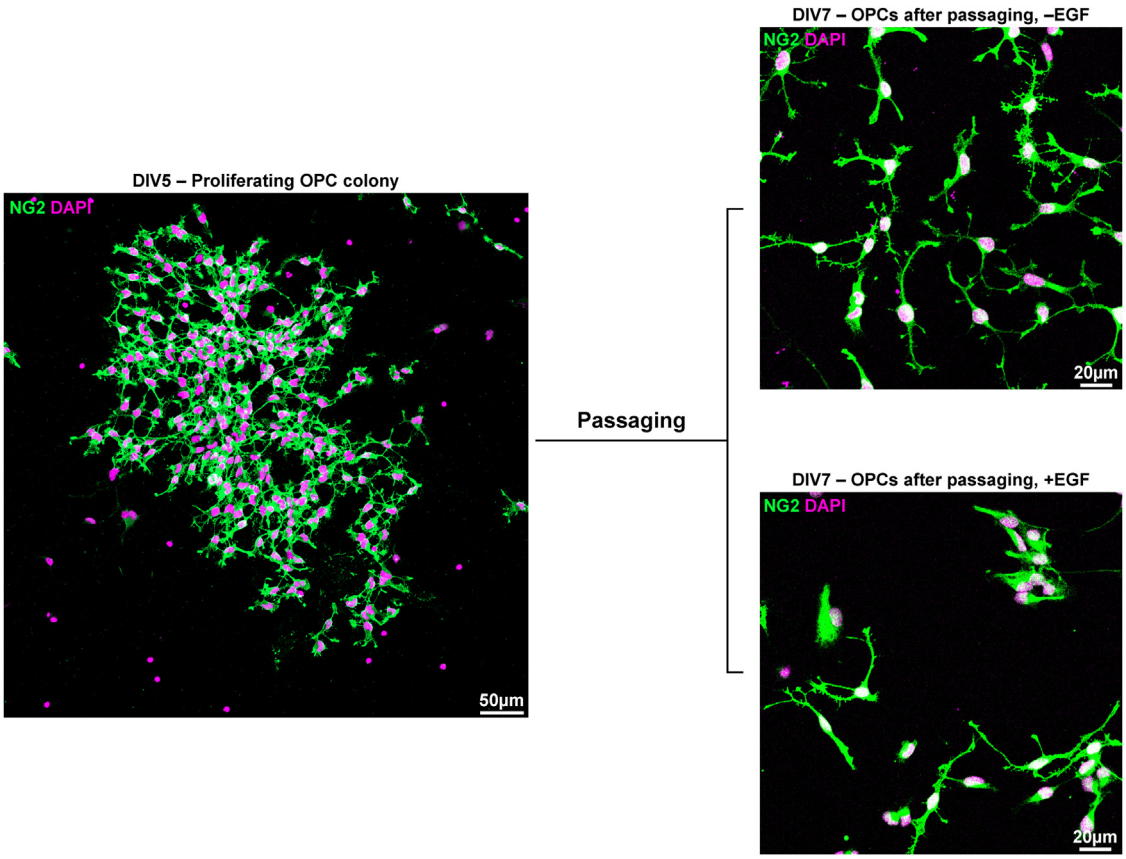

Fig. 3. Spatial distribution of OPCs in *in vitro* culture. The spatial distribution and tendency to cluster in OPCs *in vitro*, according to time and the presence of EGF in culture media. DIV = days *in vitro*. EGF = epidermal growth factor. OPC = oligodendrocyte progenitor cell.

that OPCs form aggregated colonies when initially isolated from the brain and expanded in an OPC proliferation medium containing platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF). After the cells were passaged and homogenously seeded, the tendency to form aggregates diminished; however, when epidermal growth factor (EGF) was added to the environment, the OPCs retained the tendency to cluster together (Fig. 3). We applied the spatial distribution analysis to compare the distribution between OPCs cultured (after passage) with or without EGF in the culture medium. OPCs cultured in a medium containing EGF showed a significantly closer NND between cells, and a significant left shift of the G-function graph beyond the boundaries of CSR indicated a clustered pattern of distribution. OPCs cultured without EGF exhibited a distribution within the bounds of CSR (Fig. 4).

Morphological complexity of mature OLs according to culture condition

While the primary function of mature OLs is to ensheath adjacent axons, in *in vitro* two-dimensional culture environments, where OLs are cultured on flat surfaces, they acquire a web-like morphology that can be visualized using immunostaining for myelin-associated proteins such as MBP. We observed that during *in vitro* differentiation of OLs using media containing the thyroid hormone T3, the degree of morphological complexity differed according to the base medium used and the duration of differentiation (Fig. 5). We compared 4 groups of mature OLs cultured in different conditions; the base medium used was either Dulbecco's modified eagle's medium / Ham's F12 (DMEM/F12) or Neurobasal medium (NBM), and the duration of differentiation was either 2 or 4 days. We observed that on Day 4, mature OLs cultured in NBM showed more elaborate, complex processes that could be discerned in ICC images for MBP, and quantified by the OL morphological complexity analysis (Fig. 5).

Limitations

For the OPC spatial distribution analysis, images with too much noise may not be able to be thresholded by Make Binary into a suitable image for analysis. Also, the Watershed function in Fiji/ImageJ may not be 100 % accurate, which may introduce minor inaccuracies.

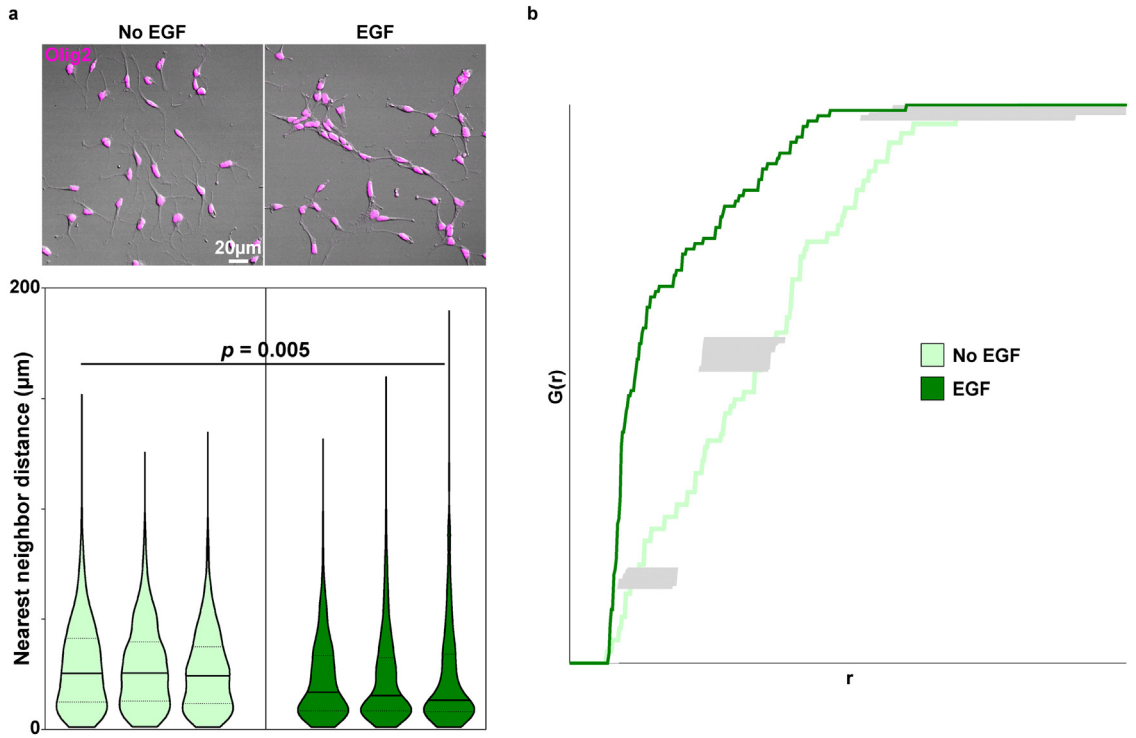

Fig. 4. Comparison of spatial distribution between OPCs cultured with or without EGF. a Representative images of OPCs cultured with or without EGF, stained for Olig2, and violin plot of NNDs. $N = 3$ biologically independent samples, 2 groups per sample, 16 images per group, approximately 200 DAPI+ cells per image. Nested *t*-test, two-tailed. Data is presented as median values ± quartiles. b Graph of spatial G-function of OPCs cultured with or without EGF. Grey areas demarcated the boundaries of complete spatial randomness. EGF = epidermal growth factor. NND = nearest neighbor distance. OPC = oligodendrocyte progenitor cell.

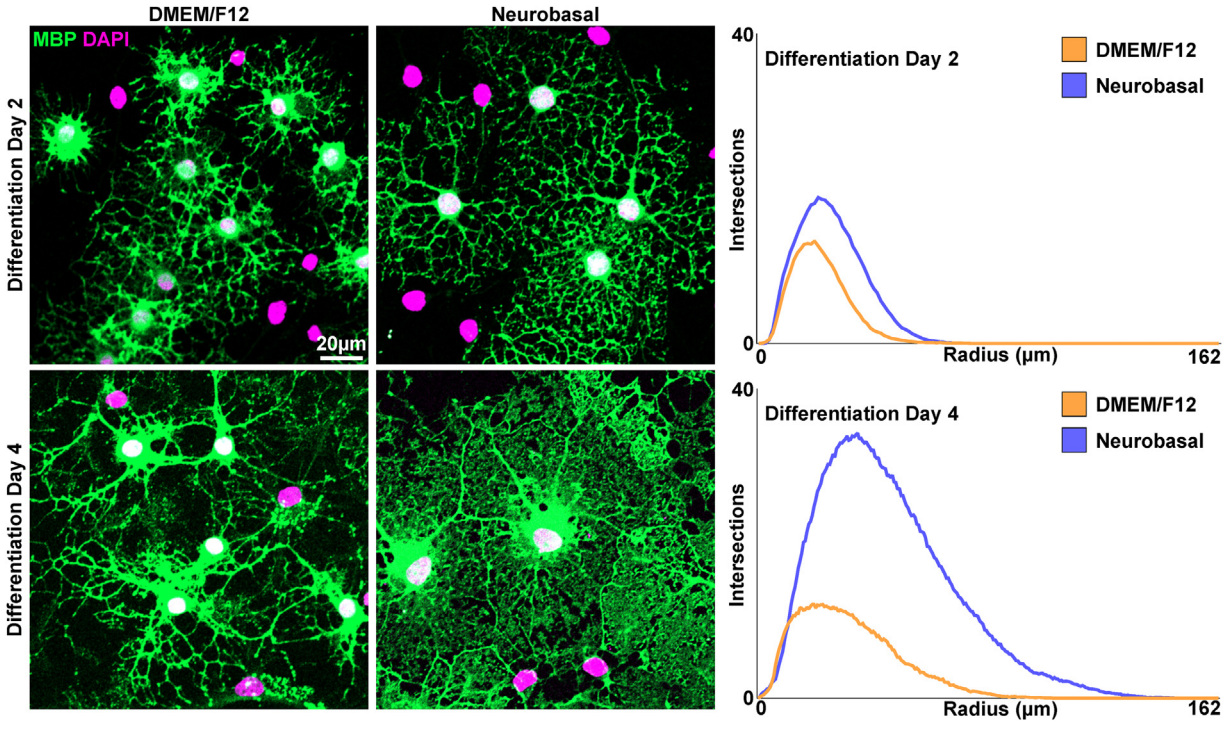

Fig. 5. Quantification of the morphological complexity of *in vitro* mature oligodendrocytes using Sholl analysis. Representative images of *in vitro* mature oligodendrocytes in designated culture conditions, stained for MBP, and results of Sholl analysis. DMEM/F12 = Dulbecco's modified eagle's medium / Ham's F12. MBP = Myelin basic protein.

For the OL morphological complexity analysis, Sholl analysis requires only the cell of interest to be included in the image, which necessitates the inclusion of a manual step to draw an ROI around the cell and clear the outside of the ROI. This makes it difficult to automate the analysis pipeline.

Discussion

The OL lineage comprises diverse stages, from OPCs to mature OLs, and intermediate stages such as pre-oligodendrocytes and immature OLs. The diversity of stages and the fact that those stages are subject to change tend to make OL lineage cells challenging to analyze, as the stage and status of the cell must be monitored at the same time the analysis is ongoing to ensure that an unexpected change has not occurred during or as a result of the analysis. Therefore, extracting quantifiable data from images of OL stage markers without using additional reagents or measures that may impact the OL stage or physiology can be a valuable tool to assess OL function. In this article, we describe two methods to extract data from *in vitro* ICC images: one is to quantify the spatial distribution of OPCs in culture, which may reflect their state of stemness and proximity to the NSCs from which they originate, and the other is to quantify the morphological complexity of mature OLs, which may reflect the extent of maturation and capacity for myelin generation. Different methods for extracting data from images, along with the two methods discussed in this article, may help improve our understanding of OL-lineage cells and their functions, which have been shown to play an important role during development and in a wide range of CNS diseases.

Conclusion

In this study, we describe a method to extract information reflecting the characteristics of OL lineage cells at both the OPC and mature OL stages from immunostained images. These analyses may substitute for more convoluted analyses to assess the same functions and thus contribute to a better understanding of OLs and their role in the CNS.

Ethics statements

The article does not contain content relevant to this section.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Hanki Kim: Conceptualization, Methodology, Software, Writing – original draft. **Bum Jun Kim:** Methodology, Validation, Data curation. **Seungyon Koh:** Validation, Data curation. **Hyo Jin Cho:** Validation, Data curation. **Xuelian Jin:** Validation, Data curation. **Byung Gon Kim:** Writing – review & editing. **Jun Young Choi:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Data availability

The authors have provided the example images needed to replicate results in the Supplementary Materials section.

Acknowledgments

This work was supported by a grant of the M.D.-Ph.D./Medical Scientist Training Program through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (to H. K.). This work was also supported by National Research Foundation of Korea (NRF) grants funded by the Korean government (MSIT; Ministry of Science and ICT) (NRF2019R1A5A2026045 and NRF-2021R1F1A1061819), a grant from the Korean Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HR21C1003), and new faculty research fund of Ajou University School of Medicine (to J.Y.C.).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102781.

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