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Title

Collar occupancy: a new quantitative imaging tool for morphometric analysis of oligodendrocytes

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Graphical abstract

Collar occupancy for in vitro quantitative oligodendrocyte morphological analysis

Highlights

- Collar occupancy is an automated high-throughput quantitative image analysis method
- Collar occupancy allows morphometric ranking during oligodendrocyte differentiation
- More differentiated oligodendrocytes have greater percentage of collar occupancy
- Kank2 is a new regulator of oligodendrocyte differentiation
- Dusp19 is a new regulator of oligodendrocyte myelination

Abstract

Background - Oligodendrocytes (OL) are the myelinating cells of the central nervous system. OL differentiation from oligodendrocyte progenitor cells (OPC) is accompanied by characteristic stereotypical morphological changes. Quantitative imaging of those morphological alterations during OPC differentiation is commonly used for characterization of new molecules in cell differentiation and myelination and screening of new pro-myelinating drugs. Current available imaging analysis methods imply a non-automated morphology assessment, which is time-consuming and prone to user subjective evaluation.

New Method - Here, we describe an automated high-throughput quantitative image analysis method entitled *collar occupancy* that allows morphometric ranking of different stages of *in vitro* OL differentiation in a high-content analysis format. *Collar occupancy* is based on the determination of the percentage of area occupied by OPC/OL cytoplasmic protrusions within a defined region that contains the protrusion network, the *collar*.

Results - We observed that more differentiated cells have higher *collar occupancy* and, therefore, this parameter correlates with the degree of OL differentiation.

Comparison with Existing Method(s) - In comparison with the method of manual categorization, we found the *collar occupancy* to be more robust and unbiased. Moreover, when coupled with myelin basic protein (MBP) staining to quantify the percentage of myelinating cells, we were able to evaluate the role of new molecules in OL differentiation and myelination, such as Dusp19 and Kank2.

Conclusions - Altogether, we have successfully developed an automated and quantitative method to morphologically characterize OL differentiation *in vitro* that can be used in multiple studies of OL biology.

Keywords

Oligodendrocyte, *collar occupancy*, morphometric analysis, imaging, automated, high-throughput, Kank2, Dusp19.

Introduction

In the central nervous system (CNS), oligodendrocytes (OL) are unique cells in their ability to produce myelin that potentiates saltatory nerve conduction and promotes axonal integrity and protection (Nave and Werner, 2014). Myelin homeostasis has been demonstrated to be essential in processes such as neural plasticity (Birey et al., 2015) and motor learning (McKenzie et al., 2014) and its disturbance is commonly associated to multiple sclerosis (MS), but also to other pathologies such as leukodystrophies, contusion type spinal cord injury, mental illness, and age-related cognitive decline (Haroutunian et al., 2014; Hinman and Abraham, 2007; Huang and Franklin, 2012; Papastefanaki and Matsas, 2015; Poggi et al., 2016; Pouwels et al., 2014). OL derive from oligodendrocyte progenitor cells (OPC), which hold the capacity to proliferate, migrate, and differentiate into myelinating OL. OPC comprise the progenitors from the subventricular zone (SVZ) (Menn et al., 2006) and the NG2 and PDGF α R positive OPC (or NG2 glia), which are homogeneously distributed within the CNS both in grey and white matter (Richardson et al., 2011; Rivers et al., 2008). Adult OPC are constantly proliferating in the CNS to maintain their homeostatic cell density (Hughes et al., 2013; Richardson et al., 2011), thus providing a substantial source of new OL and, thus, a potential reservoir for remyelination in case of injury or disease (Franklin and Goldman, 2015).

From the morphological point of view, OL differentiation is a highly dynamic process. Bipolar OPC differentiate first into immature OL by extending multiple cytoplasmic protrusions and, in the mature stage form complex protrusive networks that culminate in extensive myelin-like membrane sheets (Domingues et al., 2017). These morphological changes closely resemble those observed during *in vivo* developmental myelination (Kachar et al., 1986; Knapp et al., 1987) and, as a result, *in vitro* OL differentiation assays have been extensively used by many groups and in many different experimental contexts to address fundamental questions of OL biology (Colognato et al., 2007; Huang et al., 2016; Najm et al., 2015; Thurnherr et al., 2006). When quantification is necessary, protrusion complexity is usually determined by manual categorization of the different stages of differentiation according to their morphological complexity (Colognato et al., 2007; Olsen and Ffrench-Constant, 2005; Thurnherr et al., 2006). Also, the semi-automated Sholl analysis method is sometimes used to measure the extent of morphological changes occurring during OL differentiation (Gensel et al., 2010). Nevertheless, both methods are dependent on the user subjective evaluation and time-consuming and,

therefore, do not allow the analysis of a large number of cells within a useful timeframe. In this study we aimed at developing a new imaging method for analysis of OL morphology during differentiation. As such, we were able to develop a high-throughput, automated and quantitative method enabling robust and unbiased analysis that we named *collar occupancy*. Collar occupancy was validated by comparing with the existing manual categorization, and further used to characterize two molecules, Kank2 and Dusp19, whose role in OL differentiation and myelination had not yet been elucidated.

Kank2, also known as MXRA3, Ankrd25 and SIP, belongs to a family of 4 isoforms that include Kank1, Kank2, Kank3 and Kank4 and were initially suggested to be involved in actin stress fiber formation in NIH3T3 cells (Kakinuma et al., 2009). Kank2 was first identified as a protein co-expressed with known cell adhesion and matrix remodelling genes (Walker et al., 1999). Later, Kank2 was described to be a regulator of cell growth (Harada et al., 2005) and of availability of steroid receptor co-activators in the cytoplasm (Zhang et al., 2007). Kank2 has consistently been described enriched in cellular protrusions of different cells types such as the pseudopodia of NIH3T3 cells, astrocytic protrusions, neurites of neuroblastoma and foot pseudopodia of podocytes (Feltrin et al., 2012; Gee et al., 2015; Mili et al., 2008; Thomsen and Lade Nielsen, 2011). More recently, Kank2 was described to be involved in integrin-mediated mechanotransduction in cellular adhesion-sites by decreasing force transmission through interaction with the actin cytoskeleton (Sun et al., 2016). Such strong interaction of Kank2 with the cytoskeleton led us to hypothesize a potential role of Kank2 in OL biology.

Dusp19, dual specificity protein phosphatase 19, is an atypical Dusp that dephosphorylates both tyrosine and serine/threonine residues and is widely distributed in mouse tissues. It is involved in the regulation of MKK/JNK and MAPK signalling pathways (Patterson et al., 2009; Wang et al., 2016; Zama et al., 2002a, b) and mitotic cell exit (St-Denis et al., 2016). Due to the importance of the MAPK MKK/JNK signalling pathways in regulating myelin expression (Chew et al., 2010), we hypothesised that Dusp19 could be a regulator of such pathways.

Materials and Methods

Animals. Wistar Hahn rats were used for mixed glial cell primary cultures. All animal experiments were performed with the approval of and in strict accordance with the IBMC/IS Animal Ethics Committee, the Portuguese Veterinary Office and the European Union animal welfare laws, guidelines and policies. The Portuguese law “Decreto-Lei 113/2013” regulates research with animals in IBMC/IS and is the national transposition of the European Directive 2010/63/EU. This legislation sets detailed regulations for how animals are to be housed and

handled as well as for the licensing of projects. Accordingly, all efforts were made to minimize animal suffering and reduce the number of required animals.

Cell culture media and reagents. Sato medium for OPC and OL culture is a high glucose DMEM based-medium (Gibco) supplemented with 5 µg/ml human insulin (Sigma), 100 µg/ml human apo-transferrin (Sigma), 100 µg/ml BSA (NZY Tech), 16 µg/ml putrescine (Sigma), 60 ng/ml progesterone (Sigma), 40 ng/ml sodium selenite (Sigma) and 30 ng/ml triiodo-L-thyronine (Sigma). In Sato proliferating medium 10ng/ml of human PDGF α and 10ng/ml FGF2 (Peprotech) were added. To induce OL differentiation, Sato medium was supplemented with 0.5% FBS (Sigma). Microtubules protecting (MP) buffer: 65mM PIPES (Millipore), 25mM HEPES (Sigma), 10mM EGTA (Sigma), 3mM MgCl₂ (Sigma), pH 6.9.

Mixed glial cell cultures. Cultures were prepared according to (Chen et al., 2007; McCarthy and de Vellis, 1980) with adaptations. Briefly, brain cortices of P1-P2 (1-2 post-natal days) Wistar Hahn rats were isolated in ice-cold Hank's Balanced Salt Solution (HBSS, Gibco) supplemented with 1% penicillin/streptomycin 10000U/mL. Cortices were dissected to remove the meninges and mechanically homogenized. Homogenized tissue was enzymatically treated with 0.1mg/mL DNase I (Sigma) and 0.0025% trypsin (Gibco) in HBSS for 15 minutes at 37°C. Reaction was stopped with serum-containing DMEM and cells were then washed, re-suspended in Glutamax high-glucose DMEM medium (Gibco) with 1% penicillin/streptomycin and 10% Fetal Bovine Serum (FBS, Sigma) and filtered through a 100µm nylon cell strainer (BD Falcon) to remove any cell debris. Finally, cells were plated on poly-D-lysine (PDL)-coated (Sigma) T75 flasks (Sarstedt) at a cell density of 2 brains per flask and further cultured at 37°C in 5% CO₂ for approximately 10 days. Medium was replaced every 2-3 days.

OPC culture and transduction with lentiviral shRNA. OPC were isolated from rat mixed glial cell cultures by mechanical dissociation (INFORS HT Minitron incubator) as previously described (McCarthy and de Vellis, 1980). Flasks were first shaken at 200 rpm, 37°C for 2 hours to remove the majority of microglia followed by 220 rpm, 37°C for 20h to detach OPC. OPC were further purified by a 2-3 hour-differential selective adhesion step with uncoated plastic Petri dishes. The unattached OPC were purified through a 40 µm cell strainer to remove cell debris. 4x10⁴ OPC were plated in HCl-treated 18mm glass coverslips previously coated with poly-D-lysine (10 µg/ml in boric acid buffer) and laminin-2 (10 µg/ml in phosphate-buffered saline). OPC cultures were obtained with a minimum of 90% purity. OPC cultures were then transduced with lentiviruses carrying a short hairpin RNA (shRNA) against the dsRED fluorescent protein, a control shRNA commonly used in mRNA depletion studies aiming at examining the role of a specific gene/protein in cell culture systems. These lentiviruses also expressed a green fluorescent protein (GFP) reporter. The dsRED shRNA sequence (5'-AGTTCCAGTACGGCTCCAA) was cloned into the lentiviral pSicoR vector

(Addgene) using HpaI-XhoI cleavage sites (Cotter et al., 2010). The lentiviruses were produced and quantified based on the protocol described by Tiscornia *et al.* (Tiscornia et al., 2006). OPC were transduced with shRNA-loaded virus using a multiplicity of infection (MOI, ratio of virus particles to cells) of 1:1 and maintained in proliferating Sato medium for two days for synchronization. OPC differentiation was further induced by replacing PDGF α and FGF2 by 0.5% FBS. Cells were collected for analysis at days 0, 3 and 5 post *in vitro* differentiation (DIV).

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in microtubules protecting buffer for the preservation of the cytoskeleton integrity. Next, cells were permeabilized in 0.1% Triton X-100 in PBS for 10 minutes at room temperature and incubated in blocking solution (5% normal goat serum in PBS) for 1h at room temperature. Samples were immunolabeled with antibodies against α -tubulin (dilution 1:2000, T5168 Sigma), a cytoskeleton protein that enables a clear visualization of OPC and OL protrusions, and Olig2 (rabbit anti-Olig2 (pAb, dilution 1:500, AB9610 Chemicon International), a transcription factor mainly expressed by OPC/OL (Coprav et al., 2006), to ensure the identity of all cells analyzed, thereby excluding potential contaminating cells such as astrocytes and microglia from the analysis. Overnight incubation at 4°C with primary antibodies was followed by 1h incubation at room temperature with Alexa Fluor-568 or 647 conjugated secondary antibodies (dilution 1:1000, Molecular Probes). Finally, nuclei were counterstained with 0.25 μ g/ml DAPI (Invitrogen) for 10 minutes and mounted on glass slides using Fluoroshield mounting medium (Sigma).

High-throughput widefield fluorescence microscopy image acquisition and analysis. Cells images were acquired in the IN Cell Analyzer 2000 (GE Healthcare), a computer-controlled high-throughput widefield fluorescence microscope. Fluorescence images were obtained using a Nikon 20X/0.45 NA Plan Fluor objective, 2x2 binning and the 2.5D acquisition mode with a Z section of 3 μ m to allow focusing in all planes. Between 36 and 100 fields were obtained per sample, enabling the acquisition of thousands of cells. Exposure times were adjusted for each experiment, but maintained for all samples within experiments, to allow comparison of results. The image analysis protocol was developed using the IN Cell Investigator-Developer toolbox 1.9.2 (GE Healthcare), the image analysis software associated with the IN Cell Analyzer 2000 microscope. For comparison, the same experiments were analysed by manual categorization. A minimum of 300 cells per condition, per experiment, were classified on a scale of 1 to 4 where 1 represents bipolar/multipolar OPC stage and 4 the mature OL stage with highly protrusive arborisations. Manual analyses were performed by the same person, to reduce variability.

Image analysis with CellProfiler. The same microscopy images used for the development of the *Collar occupancy* image analysis protocol under the IN Cell Investigator-Developer toolbox

were used to create an analysis pipeline using the open-source software CellProfiler (Carpenter et al., 2006). The collar occupancy CellProfiler pipeline can be freely downloaded from <https://github.com/andrefilipemaia/Collar-occupancy>. Briefly, we first segmented the nuclei from the DAPI channel using an Otsu intensity threshold method. Next, we expanded the nucleus area in order to create a larger circular area, which after subtraction of the nuclear area becomes the “collar” region. Subsequently, we segmented the OL protrusions from the α -Tubulin channel using a RidlerCalvard intensity threshold method. We then deleted (masked) the protrusions outside the collar region in order to be able to quantify the area occupied by the protrusions inside the collar region. The analysis pipeline generates images that allow visual inspection of the segmentation and corresponding spreadsheets with the measurements. The data contained in these spreadsheets need to be combined in order to select the “well segmented cells” and calculate the % collar occupancy (through division of the area of the protrusions in collar by the area of the collar region).

Statistical Analysis. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). Data show mean plus/minus standard error of mean (mean $\pm$ s.e.m.). Medians were calculated in the analysis of frequency distributions. Statistical significance was performed using paired t-tests with Holm-Sidak method for multiple comparison or 2-way ANOVA with Tukey method for multiple comparisons. The criterion for statistical significance was $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

Results

With the ultimate goal of contributing to develop new therapies that promote CNS remyelination, *in vitro* models of OL differentiation have been not only crucial in identifying new molecules relevant in OL biology (Thurnherr et al., 2006), but are also ideal for screening potential pro-myelinating drugs for further studies in more complex *in vivo* systems (Deshmukh et al., 2013; Najm et al., 2015). In this regard, we aimed at developing an automated high-throughput quantitative image analysis method able to characterise morphologically the OL cell lineage in various experimental settings at different stages of differentiation. We first tested this image-analysis method in OPC transduced with an irrelevant shRNA and a GFP reporter that were differentiated *in vitro* into OL for 5 days, and then applied it to examine the role of two yet uncharacterized molecules in OL differentiation and myelination.

Definition of collar occupancy

In this study, we used primary cultures of rat OL, which were analysed at different timepoints of cell differentiation: day 0 (enriched in OPC), day 3 (mostly immature OL) and day 5 (more mature OL) of *in vitro* (DIV) differentiation. Cells were immunostained with Olig2 to ensure

the exclusive analysis of cells of OL lineage, DAPI counterstain to analyse cells with well-defined nucleus, and α -tubulin to identify the total cell protrusions network and enable a complete morphometric analysis. GFP fluorescence from the lentiviral shRNA guaranteed the analysis of only transduced cells (**Figure 1A**).

The automated morphometric method implies sequential steps: 1) image acquisition; 2) cell segmentation and, 3) concomitant quantification. Certain terminologies were used to identify different image processing steps, which are explained in **Table 1**.

Cell image acquisition and identification of objects of interest for segmentation. Images were acquired using a computer-controlled high-throughput widefield fluorescence microscope in “2.5D mode,” consisting in acquisition beginning above and ending below the focus point (total Z section of 3 μ m) and further projected into a single plane. This outputted a deconvoluted image with 2x2 binning, which was further analysed in the microscope image analysis software. Next, specific subcellular compartments were identified as objects of interest in the images for segmentation - nuclei, cell bodies and protrusions - that by combination generated new objects of interest - the collar and the collar occupancy, explained in detail below (**Figures 1B, C, D and E**).

Segmentation of nuclei and protrusions and definition of collar occupancy. Cell segmentation is a crucial step for a proper quantitative image analysis. *In vitro* cultures of primary OL require a certain degree of cell confluence in order to differentiate properly and, in these conditions, it is extremely challenging to associate the complete cell protrusion network to its corresponding nucleus, where many cells are in close proximity. As such, we aimed to develop a strategy for cell segmentation that could overcome this issue while, at the same time the corresponding measurements could correlate with different stages of OL differentiation. First, a series of informatics tools were used for cell segmentation with the following purposes: (1) preparation of fluorescence images for segmentation, by reducing background noise and accentuating foreground objects; (2) segmentation of foreground objects, by creating masks; and, finally, (3) combination of masks to create new objects of interest to be measured. These consisted in a first optional pre-processing step to accentuate foreground objects, followed by segmentation based on shape (object segmentation) or on fluorescence intensity (intensity segmentation). Acceptance criteria such as object area, circularity (form factor of 1 equals a perfect circle) or minimum/maximum fluorescence intensity (density levels) were further included to clear the output image of artefacts (data not shown). Segmentation of OPC/OL nuclei and protrusions was done in a series of sequential steps. Nuclei segmentation was done with DAPI staining (**Figure 1B**). We observed that in regions of high cell confluence, segmentation errors could easily occur as adjacent nuclei could be segmented as a single object. To avoid this, we created additional steps such as *seed*, *erosion* and *clump breaking*, which resulted from nuclear

segmentation and enabled the separation of adjacent nuclei. After proper segmentation of the nuclear area, the *dilation* step consisted in an expansion of the nuclear area in order to overlap with the cell body and, later, be used to intersect with the protrusion network segmentation. The *protrusion network* was segmented using the α -tubulin staining (**Figure 1C**). To identify the individual protrusions that belonged to each cell, we first added the segmentation of the total protrusion network to the segmentation of the nucleus and cell body and then excluded the protrusions that do not touch the cell body to get the *cell network*. From this, we subtracted the nuclear segmentation to obtain exclusively the cytoplasm and protrusions. Next, the nuclear area was dilated again to create a new object of interest, which we designated *collar* and consists in a region that encircles the nucleus, the cell body and part of the protrusion network. Finally, the *collar* and the protrusions were combined to obtain only the fraction of protrusions that exist within the *collar*. Altogether, we segmented the nuclei and cell protrusions of OPC/OL to create new objects of interest - the *collar* and the *protrusions inside collar* - and, thus, measure the *collar occupancy* (**Figures 1D, E**). This new measurement parameter consists in calculating the percentage of *collar* area occupied by protrusions as described in the following equation:

$$\% \text{ Collar occupancy} = \frac{\text{Area of Protrusions in Collar}}{\text{Area of the Collar}} \times 100 \quad \text{Equation 1}$$

As OPC differentiate, they extend protrusions that become increasingly branched and interconnected (Song et al., 2001). Therefore, we hypothesized that the degree of OL differentiation could be correlated with the protrusion *occupancy* within the *collar* region by determining the percentage of area within the *collar* occupied by protrusions (**Equation 1**), expecting that more differentiated cells will have greater *collar occupancy*, our final measurement.

Classifiers and measurements. Cells were classified as Olig2 positive or negative and GFP positive or negative based on a threshold of the mean pixel intensity (density levels) within the nuclear segmentation mask. Only transduced OPC/OL – Olig2 positive and GFP positive – were considered for further analysis. In parallel, we also applied this protocol to determine the percentage of cells immunostained for myelin basic protein (MBP), a marker of OL myelination, after 5 days of *in vitro* differentiation, by classifying as MBP-positive or –negative (**Figure 2A**). In order to thoroughly analyse the datasets, several measurements were incorporated into the analysis protocol. These included the cells centroid (X and Y) positions to allow cell identification, the mean pixel intensity of fluorescence within the nuclear area (to classify cells as Olig2/GFP/MBP-positive or -negative), the areas of the nucleus and *collar* (to

eliminate cells with segmentation errors), the protrusions total length and area occupied per cell (to exclude protrusion segmentation errors) and within the collar and, finally, the *collar occupancy* (the percentage of *collar* area occupied by protrusions) (**Figures 2B, C**).

Altogether, we were able to define *collar occupancy* as a quantitative tool to measure the degree of cell differentiation, specifically in transduced cells of OL lineage and based on the segmentation of cell nucleus and protrusions.

Data Analysis Procedure

High throughput microscopy produces large datasets. During analysis, one of the biggest challenges is their visualization, inspection and quality control, being crucial for data quality assessment and ensure that biologically meaningful quantitative data is extracted.

Selection of well-segmented cells. Accurate cell segmentation was assured by a quality control step. By visual inspection of random individual cells, we verified that some cells were not properly segmented and that this was related to the areas of the collar and nucleus. We analysed the frequencies and distributions of the areas of *collar* and nucleus and verified that a specific range of nuclei and *collar* areas corresponded to well-segmented cells (**Figures 3A, B**). In the *collar* area frequency distribution we identified (1) a population of cells with less than 400 μm^2 *collar* areas that corresponded to *collar* segmentation errors and occurred specifically in very high confluence regions (cells in this area had very small *collars* that did not allow identification of cell protrusions within the *collar*); (2) a population of cells with *collar* areas between 400 to 600 μm^2 that corresponded to well-segmented cells; (3) and a population with *collar* areas above 600 μm^2 that corresponded to a “collar of 2 cells” (cells for which the first step of nuclear and *seed* segmentation failed to properly separate adjacent nuclei) (**Figure 3A, left**). Moreover, when plotting a scatter of the areas of the nucleus as a function of the *collar*, we also found that cells with less than 55 μm^2 nuclear area also had segmentation errors or were likely dead cells, whereas cells above 150 μm^2 were “bi-nucleated” (**Figure 3B, left**). Therefore, only the cells with *collar* area between 400 to 600 μm^2 and with nuclear area between 55 to 149 μm^2 were considered for further analysis (**Figures 3A, right and 3B, right**). As expected, the majority of well-segmented cells had *collar* areas directly proportional to the nuclear area, falling on a straight line. We observed that cells that are scattered below this line had minor segmentation errors due to constraints caused by neighbouring cells (**Figure 3B, right, examples**). However, these did not introduce a bias in the analysis because their *collar occupancy* values have a similar frequency distribution in the population and, therefore, were not excluded from the analysis (data not shown). Finally, it was observed that cells with more than 1200 μm^2 of total protrusion length and 1700 μm^2 of total protrusion area corresponded to

protrusion segmentation errors as they included the protrusions of neighbouring cells and, therefore, they were also excluded from the analysis (data not shown). Altogether, this automated protocol selected for analysis only well-segmented cells that were Olig2-positive, GFP-positive, with a *collar* area between 400 and 600 μm^2 and a nuclear area between 55 and 150 μm^2 . Accordingly, these exclusion/selection steps ensured that, first, we were looking only at shRNA-transduced oligodendrocytes; second, that there was similarity between *collar* sizes across experiments and conditions; and, finally, that cells that were not well segmented were eliminated.

Validation of collar occupancy protocol by comparison with manual categorization during OL differentiation. Next, we quantified the percentage of *collar occupancy* for each cell to determine the degree of OL differentiation as described in **Equation 1** and its frequency distribution was analysed at different timepoints of OL differentiation: 0, 3 and 5 days of *in vitro* differentiation (DIV). We verified a clear and positive correlation between the *collar occupancy* and the different OL differentiation stages (**Figure 4A**). At day 0 DIV most cells are in OPC stage, presenting a bipolar morphology. As expected, cells had the lowest values of *collar occupancy*, the majority between 14 and 24% (medians of 3 experiments = 18.0, 20.2 and 20.3). At day 3 DIV, most cells are in the immature stage and had intermediate values of *collar occupancy*, ranging between 20 and 30% (medians of 3 experiments = 23.1, 25.4 and 26.7). Finally, at day 5 DIV, a significant proportion of cells had more complex protrusions arborized structures that corresponded to higher values of *collar occupancy*, between 26 and 36% (medians of 3 experiments = 25.8, 28.4 and 28.7). Cells with less than 12% of *collar occupancy* were likely to correspond to segmentation errors and therefore were eliminated.

In order to make the collar occupancy method available to all researchers, we created an analysis pipeline with CellProfiler (**Supplementary Figure 1**), a freely available modular image analysis software (<http://cellprofiler.org>) (Carpenter et al., 2006). As expected, we observed exactly the same trend in terms of % collar occupancy during OL differentiation using either software, the IN Cell Developer (**Figure 4A**) and CellProfiler (**Supplementary Figure 1**). This clearly demonstrates the universality of the collar occupancy method across different image analysis platforms. The *collar occupancy* IN Cell Developer protocol (.xep file) and CellProfiler pipeline (.cppipe file) can be freely downloaded from <https://github.com/andrefilipemaia/Collar-occupancy>.

Altogether, we were able to develop an automated protocol with a series of sequential steps to quantify only well-segmented cells of the OL lineage. Being aware of the complex architectural nature of these cells, we were able to identify the *collar occupancy* as a trustworthy tool to quantitatively measure the degree of OL differentiation, as it was possible to observe an unquestionable increase of *collar occupancy* during OL differentiation that correlates with the

increase in OL morphological complexity. In this sense, we established 4 stages of *collar occupancy* to categorize the morphological complexity during OL differentiation in order to compare with the current method of manual categorization. Cells in stage 1 had *collar occupancy* between 12-18%, cells in stage 2 had *collar occupancy* between 19-25%, cells in stage 3 had *collar occupancy* between 26-32% and, finally, cells in stage 4 had *collar occupancy* higher than 32% (**Figures 4B, C**).

Finally, we validated this automated analysis by quantifying the percentage of Olig2- and GFP-positive cells in each of the 4 established categorization stages during OL differentiation (**Figure 5A**) and compared the results with a manual categorization of the same experimental data, as we have done before (Thurnherr et al., 2006). Here, we also established 4 categories, representative of different stages of differentiation (**Figure 5B**). Using the Cell Counter plugin from the open-source Image J software, a minimum of 300 cells per condition and per experiment were classified as belonging to one of four categories. Cells were visually inspected for their expression of Olig2 and GFP. Only cells that were undoubtedly classified as belonging to a single category were considered (intermediate morphologies, technical artefacts and areas of extremely high confluence were excluded from analysis). Manual analysis was performed by the same person to minimize variability. We observed that both analyses generated similar results and presented an almost identical OL differentiation pattern, consistent with a decrease in the percentage of cells in the first stages of differentiation (stages 1 and 2) and a concomitant increase of cells in the late stages of differentiation (stages 3 and 4) over the 5 days of OL differentiation in culture. Of note, with the automated quantification we analysed 4.2 times more cells when compared with the manual quantification (1546 ± 369 vs. 368 ± 78 , mean $\pm$ s.e.m. of all three timepoints from 3 independent experiments). This led to a more robust analysis. Globally, these data clearly show that the automated method here described was fully competent in assessing OL differentiation in culture, as it was able to quantify a decrease in the percentage of undifferentiated OPC and an inversely proportional increase of mature OL along differentiation.

Identification of Kank2 and Dusp19 as new regulators of OL differentiation and myelination, respectively. In order to show that this method was effective in identifying new molecules involved in OL differentiation and myelination, we selected two yet uncharacterized molecules in OL biology and addressed their role in differentiation and myelination. We first analysed the expression of Kank2 and Dusp19 during the different stages of *in vitro* OL differentiation. We observed that both Kank2 and Dusp19 are expressed during OL differentiation, at the protein and mRNA levels (**Figures 6A, B**). *In vivo*, we also identified the expression of Kank2 and Dusp19 in CNS white matter regions such as cerebellum, brain stem and optic nerve (data not shown). Next, to address the functional role of Kank2 and Dusp19 in OL differentiation and

myelination, specific shRNAs were designed and cloned into a lentiviral plasmid to deplete mRNA expression. The knockdown efficiency of each of these shRNAs was evaluated by quantitative real time PCR at 0 DIV. It was found that both shRNAs for Kank2 and Dusp19 had a significant knockdown efficiency (**Figure 7A**). In the case of Kank2 shRNA, we verified that there was no compensation by the other isoforms Kank1, Kank3 and Kank4 (data not shown). In order to assess the effect of Kank2 and Dusp19 knockdown in OL protrusion extension and branching, we compared the methods of collar occupancy and manual categorization at 3 DIV. We observed similar results with both methods, where Kank2-depleted OL showed an arrest in less differentiated stages when compared with the control OL (**Figures 7B, C**). However, Dusp19 depletion showed no effect in OL morphological differentiation (**Figures 7B, C, D**). One of the parameters that are measured in the *collar occupancy* protocol is the OL total protrusion length and consists in the sum of the length of all protrusions for a given cell (**Figure 2B**). We observed that Kank2, but not Dusp19 mutant cells had a decrease in the OL total protrusion length (**Figure 7D, E**). Finally, myelin production was also assessed at 5 DIV in Kank2 and Dusp19 mutant and control cells. We quantified the percentage of MBP positive cells in transduced cells of OL lineage using the automated protocol. Interestingly, we observed that depletion of Dusp19, but not Kank2 reduced significantly the production of MBP (**Figure 8**). Altogether, these results suggest an important role of Kank2 in OL morphological differentiation, while Dusp19 seems to regulate OL myelination.

Discussion

OL differentiate *in vitro* by extending and ramifying multiple protrusive structures (Bauer et al., 2009; Pfeiffer et al., 1993; Song et al., 2001). These cells are morphologically very complex and, in comparison with astrocytic protrusions or neurites in neurons, OL have thin protrusions and, therefore, cell segmentation is very challenging for quantitative imaging analysis of their morphology. Moreover, these cultures require a reasonable degree of confluence for proper differentiation, making difficult a complete association of the OL protrusion network with the corresponding nucleus. Of note, other quantitative methods assessing OL differentiation, such as the Sholl analysis, can only quantify a small cell number in a feasible manner and require low cell density (Barateiro et al., 2015; Colognato et al., 2007; Gensel et al., 2010; Thurnherr et al., 2006), which may compromise the results. Therefore, in this study we developed a quantitative, automated and high-throughput method able to correlate with the degree of *in vitro* OL differentiation and independent of confluence of cell cultures. We called it *collar occupancy*, as it is the ratio of area occupied by OPC/OL protrusions within the *collar* area, a region that is an extension of the nuclear area and comprises also the cell body and part of the protrusions network.

Using a widefield high-throughput microscope, we were able to exclusively analyse thousands of transduced cells of OL lineage - Olig2 and GFP-positive cells - and estimate their degree of differentiation. We found that the *collar occupancy* correlates positively with the degree of OL differentiation. Moreover, when compared with the manual categorization, we found the method to be more robust, consistent and faster by analysing many more cells in a much shorter period of time, easier to perform and not dependent on the user subjective evaluation (bias). In opposition, manual methods such as categorization rely on the user ability to recognize and distinguish different stages of morphological development. This is usually prone to errors due to exhaustion, but also the analysis criteria may change over time. Furthermore, categorization in a limited number of stages is an oversimplification of reality while different cell morphologies exist in a continuum spectrum. With the *collar occupancy* method, we overcome this issue by calculating a specific % of the *collar occupancy*, whose frequency can be plotted in a more continuous spectrum than limited number of categorizations and the classification criteria are equally applied to every cell, thus eliminating such biases. Though here we have compiled our results of % collar occupancy into 4 categories in order to compare with the manual categorization results, the future use of the percentage values of *collar occupancy* of experimental and control conditions will surely enable the identification of more subtle changes when compared with the categorization methodology.

Additionally, while the manual method requires 100% of user supervision, the automated one reduces this to two steps: 1) before analysis, the user must define the segmentation criteria (adapted to the cells being analysed) such as fluorescence intensity threshold, kernel size for erosion, and size of collar expansion; 2) after analysis, the user must define the classification criteria for well-segmented cells vs. segmentation errors. The automated protocol can be applied to a whole dataset overnight/over the weekend without supervision. The post processing analysis can then be easily performed in spread sheet-type files, e.g. Excel, by simply ordering cells based on nuclear size, total process length and *collar occupancy*, to eliminate cells outside the classification criteria intervals.

The *collar occupancy* method is able to solve some limitations usually encountered in manual morphometric analysis. Sholl analysis is very time consuming, limited to few tens of cells to be analysed and difficult to perform in high cell confluence due to superimposition of cellular protrusions from neighbour cells. In alternative, here in the *collar occupancy* method the *collar* contains an area with sufficient cell protrusions correlative of the OL differentiation status while, at the same time, not touching neighbour *collars*. Moreover, with the application of some thresholds in *collar* and nuclear areas, we were able to, in an automated manner, exclude cells with segmentation errors, guaranteeing accurate analysis of only well-segmented OL in culture and, importantly, with no limit number of cells to be analysed. Of note, one should be careful

and verify which criteria are used to eliminate cells with segmentation errors, as this may be dependent on the experimental study. Also, the classification criteria intervals shown here are merely demonstrative and specific to these experimental conditions.

Additionally, we validated this protocol in an experimental setup of transduced OPC with a GFP reporter to contextualize in a possible gene knockdown/overexpression study. Nevertheless, this protocol will work similarly well with non-transduced cells if necessary. This automated protocol also demonstrated the possibility to include a marker of OL myelination by the quantification of the percentage of MBP-positive cells to enable a more complete assessment of the role of specific proteins or drugs in OL differentiation and myelination. Recently, Najm and colleagues described a high-throughput screening platform to identify new remyelinating therapeutics using mouse epiblast stem cell (EpiSC)-derived OPCs (Najm et al., 2015). Here, they quantified OL differentiation by high content imaging of MBP expression through the measurement of length and intensity of MBP labelled oligodendrocyte protrusions. Similarly, a previous study developed a high content imaging assay based on the induction of MBP expression in primary rat optic nerve-derived OPC to identify new pro-myelinating drugs (Deshmukh et al., 2013). However, these screening studies were focused on the ultimate outcome of myelin production by OL and not based on a morphometric assessment. Additionally, they did not identify individualized cells as data was generated on a per well basis.

To the best of our knowledge, the *collar occupancy* method we describe here is the first morphometric method for analysing OL morphological complexity during *in vitro* differentiation in an automated and high-throughput format. Importantly, for those researchers with no access to high-throughput microscopes, this new concept of *collar occupancy* can be easily adapted to image acquisition in normal widefield microscopes and further analysis in an open-source image processing programs such as CellProfiler and Image J/Fiji. We generated a pipeline in CellProfiler software using the same steps used in IN Cell Developer software and demonstrated that very similar results were obtained. These results also show that the range of values obtained for collar areas and thus for % collar occupancy in both platforms may vary with the software used but the trend observed is the same. Depending on the culture conditions, the collar diameter can be adjusted, as long as a superimposition with that of neighbour cells does not occur. This gives flexibility to this method and, therefore, the values shown in our data are merely demonstrative. Moreover, we consider that the concept of *collar occupancy*, besides an innovative way to study OL differentiation, may have the potential to be used to study other cells producing protrusive structures in the nervous system, such as neurons, astrocytes, microglia and Schwann cells.

Finally, we showed that the *collar occupancy* is a valuable method for screenings aiming at identifying novel OPC/OL differentiation-regulating molecules and/or pro-(re)myelinating

drugs. In this particular study, we were able to identify two new regulators of OL differentiation and myelination. Using the *collar occupancy* method, we observed that Kank2 plays a role in OL differentiation as its depletion causes an arrest in less differentiated cells. In Kank2-depleted OL, we observed an increased cell number with lower percentage of *collar occupancy* and total protrusion length. However, Kank2 depletion did not affect the percentage of cells producing MBP. Literature evidences a role of Kank proteins in cytoskeleton by interaction with actin, talin and Rho GTPases (Gee et al., 2015; Kakinuma et al., 2008; Kakinuma et al., 2009; Roy et al., 2009; Sun et al., 2016). We, and others, have shown previously the importance of these cytoskeletal molecules in OL morphological differentiation (Nawaz et al., 2015; Olsen and Ffrench-Constant, 2005; Thurnherr et al., 2006). Therefore, the elucidation of the signalling mechanisms by which Kank2 regulates OL differentiation should be addressed in the future. With an opposite profile and as initially hypothesized, we found that Dusp19 does not play a significant role in OL morphological differentiation but rather is important for myelination. Future studies should address through which signalling mechanisms Dusp19 is regulating myelin gene expression. MKK7/JNK/c-Jun are possible pathways (Chew et al., 2010; Wang et al., 2016; Zama et al., 2002a).

Altogether, *collar occupancy* is a quantitative automated high-throughput image analysis method to analyse OL morphological complexity during *in vitro* differentiation that can be applied to more fundamental studies of OL biology, aiming at identifying new molecules involved in cell differentiation, or applied studies aiming at screening drugs for potential remyelinating therapies.

Competing interests

The authors declare that they have no competing interests

Availability of data and materials

The collar occupancy IN Cell Developer protocol (.xeap file) and CellProfiler pipeline (.cppipe file) can be freely downloaded from <https://github.com/andrefilipemaia/Collar-occupancy>.

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Authors' contributions

JBR and HSD conceptualized the project. HSD, JBR and FB wrote the manuscript. HSD, FB and AC performed the cell culture experiments. FB did the immunostainings. FB and AFM acquired the images. FB, AFM and VM developed the collar occupancy protocol. AFM developed the collar occupancy protocol in CellProfiler open-source platform. FB, HSD, AC and IMP analysed the data.

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ACCEPTED MANUSCRIPT

FIGURES LEGENDS

Figure 1 – Development of an automated high-throughput method for quantitative image analysis of OL under differentiation – from cell segmentation to definition of *collar occupancy*. **A.** Example of cells used for analysis. Images show *in vitro* differentiated OL transduced with GFP-dsRED shRNA (green) at 3 days IVD, immunostained with Olig2 (white) and α -tubulin (red) antibodies and counterstained with DAPI (blue). Scale bar 30 μ m. **B.** Steps of OPC/OL nuclear segmentation: scheme and representative images. Fluorescence image of DAPI staining is segmented and eroded to create a “seed” target. Then, nuclei are segmented and “declumped” using the seed to separate adjacent cells. Nuclear segmentation is slightly dilated to overlap with the cell body. Finally, nuclei are expanded by a radius of 10 pixels to create the *collar*. Scale bar 50 μ m. **C.** Steps of OPC/OL protrusions segmentation: scheme and representative images. α -Tubulin staining is segmented to identify the protrusion network and then added to the cell body segmentation to create a “final network”. Next, nuclei are subtracted from the “final network” to create the “protrusions” mask. Protrusions and *Collar* segmentation are combined to create the “protrusions in *collar*” mask. This will be used to determine *collar occupancy*. Scale bar 50 μ m. **D.** Output image of the automated protocol. Nuclei are outlined in blue, *collars* in yellow and protrusions in green. **E.** Scheme of linking the individual objects of interest to create the final mask that permits the calculation of the *collar occupancy*.

Figure 2 – Classifiers and measurements used to analyse samples. A. Cells were classified as Olig2-positive or negative, GFP-positive or negative, and MBP-positive or negative based on arbitrary thresholds of fluorescence intensity. **B.** Scheme of all measurements included in the protocol to allow sample analysis. **C.** Example of data output results obtained for 10 cells.

Figure 3 – Selection of well segmented cells. **A.** Left – Frequency distribution *collar* area of Olig2- and GFP-positive cells at OL 3 days of IVD, evidencing the existence of 3 situations: segmentation errors (lower *collar* areas), well segmented cells (intermediate *collar* areas) and binuclear cells (higher *collar* areas). Right – inset of the first graph, showing in greater detail the frequency distribution of well segmented cells according to the area of their *collars*, between 400 and 600 μm^2 . Histograms show the frequency distribution of one representative experiment and timepoint (3 days IVD). **B.** Left – Scatterplot of area of the *collar* versus area of the nucleus of Olig2- and GFP-positive cells. Right – inset of the first graph, after selecting cells within the peak region of the histogram in **A.** (*collar* area between 400 and 600 μm^2) and with appropriate nuclear area (between 55 and 150 μm^2). Cells with *collars* directly proportional to their nuclei fall on a straight line. The other cells are evenly distributed and correspond to situations where the *collar* is slightly constrained due to neighbouring cells. *Scatter* graphs are representative of one experiment and timepoint (3 days IVD). On the images insets, nuclei are outlined in blue, *collars* in yellow and protrusions in green.

Figure 4 – Validation of *Collar occupancy* protocol: categorization and quantification of OL differentiation stages. **A.** Frequency distribution of the % of *collar occupancy* at different timepoints of OL differentiation. Histogram shows the frequency distribution of one representative experiment out of 3 independent experiments. **B.** Representative images of cells with different percentages of *collar* occupancies. Identification of 4 stages of differentiation according to different ranges of *collar occupancy* percentages: stage 1 - 12-18% *collar occupancy*; stage 2 - 19-25% *collar occupancy*; stage 3 – 26-32% *collar occupancy*; and stage 4 - more than 32% *collar occupancy*. **C.** Flow chart of the data analysis protocol.

Figure 5 – Validation of Collar occupancy method. Quantification of the percentage of Olig2- and GFP-positive cells in each stage of OL differentiation - stages 1 to 4, according to their morphological complexity at 0, 3 and 5 DIV differentiation, using the *collar occupancy* (A) and manual categorization (B) methods. Results are displayed as mean+s.e.m. of 3 independent experiments.

Figure 6 – Kank2 and Dusp19 are expressed during OL differentiation.
Immunocytochemistry (A) and mRNA expression of Kank2 and Dusp19 in OL at days 0, 3 and 5 DIV differentiation (B).

Figure 7 – Knockdown of Kank2 but not Dusp19 inhibits morphological OL differentiation. Knockdown efficiency of Kank2 and Dusp19 lentiviral shRNAs (A). Quantification of the percentage of Olig2- and GFP-positive cells in each stage of OL differentiation - stages 1 to 4, according to their morphological complexity at 3 DIV using the *collar occupancy* (B) and manual categorization (C) methods in control OL and Kank2 and Dusp19 mutants. Results are displayed as mean+s.e.m. of 6 independent experiments. Quantification of the total protrusion length parameter obtained from the collar occupancy method in control and Kank2 and Dusp19 mutant OL (D). Representative images of control and Kank2 and Dusp19 mutant OL immunostained with Olig2 (blue) and alpha-tubulin (red). GFP (green) fluorescence derives from the shRNA- associated reporter (E).

Figure 8 – Knockdown of Dusp19 but not Kank2 inhibits OL myelination. Quantification of the percentage of MBP-positive cells in control and Kank2 and Dusp19 mutant OL at 5DIV using an automated protocol (A). Representative images of control and Kank2 and Dusp19 mutant OL immunostained with Olig2 (blue) and MBP (red). GFP (green) fluorescence derives from the shRNA- associated reporter (B).

Table 1 – Description of the terminologies used in the *collar occupancy method*.

Terminologies	Description
2.5 D mode	Intermediate acquisition mode between 2D and 3D, beginning above and ending below the focus point and further projected into a single plane.
Object of interest	Subcellular region within the cell, e.g. nuclei, cell bodies, protrusion network, collar, etc.
Pre-processing	Image-processing algorithms applied to an image prior to segmentation, in order to make a given target-set easier to detect.
Denoising	Weighted smoothing operation that depends on the intensity difference between neighboring pixels. A higher degree of smoothing is applied to areas of the image where the gradient is small than in areas where the gradient is steep.
Segmentation	Process of separating and isolating targets from other image features and background.
Object segmentation	Segmentation based on shape. Uses kernels, sets of at least 9 pixels (3x3), in which the center pixel is replaced by the value computed using the eight pixels that surround it.
Intensity segmentation	Segmentation based on fluorescence intensity. Segments pixels within a defined range of fluorescence intensities, set by the user.
Post-processing	Image-processing algorithms that can be applied to an image after segmentation, in order to refine a target-set prior to applying measurements
Erosion	Tool that shrinks a region's outer boundary while enlarging any holes within it.
Dilation	Tool that enlarges a region's outer boundary while reducing holes within it.
Clump breaking	Tool that calculates approximate boundaries between neighboring cells at the equidistance from a central point such

	as the nucleus.
Combination of minimum signal	Tool that compares pixel values in image 1 with image 2. Lower value pixels in image 2 are used to replace higher pixel values in image 1.
Seed	Small region at the center of a nucleus, used as a reference point for subsequent image processing steps
Collar	Region that contains the nucleus, the cell body and part of the protrusion network. Can be thought of as the "area of influence" of a cell.
Protrusion network	Collection of all cellular protrusions in an image.
Cell network	Collection of all cellular protrusions and cell bodies in an image.
Protrusions inside collar	Part of a cell's network within the collar.
Collar occupancy	Percentage of collar area occupied by cellular protrusions.