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

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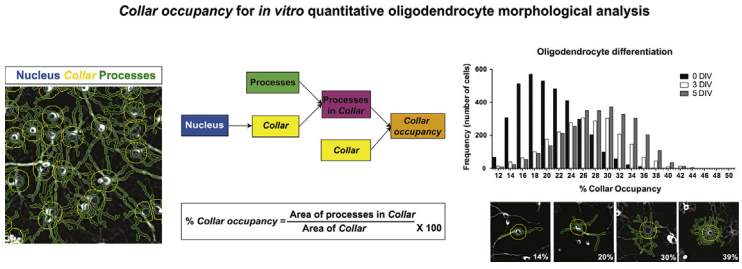
HIGHLIGHTS

- 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.
- Dups19 is a new regulator of oligodendrocyte myelination.

GRAPHICAL ABSTRACT



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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 methods: In comparison with the method of manual categorization, we found the *collar occupancy* to be more robust and unbiased.

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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.

1. 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 PDGFαR 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., 2018). 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 method, and further used to characterize two new 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 hypothesized that Dusp19 could be a regulator of such pathways.

2. Materials and methods

2.1. 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/I3S 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/I3S 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.

2.2. 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 10 ng/ml of human PDGFaa and 10 ng/ml FGF2 (Peprotech) were added. To induce OL differentiation, Sato medium was supplemented with 0.5% FBS