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2D *vs* 3D morphological analysis of dorsal root ganglia in health and painful neuropathy

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ABSTRACT

Dorsal root ganglia (DRGs) are clusters of sensory neurons that transmit the sensory information from the periphery to the central nervous system, and satellite glial cells (SGCs), their supporting trophic cells. Sensory neurons are pseudounipolar neurons with a heterogeneous neurochemistry reflecting their functional features. DRGs, not protected by the blood brain barrier, are vulnerable to stress and damage of different origin (*i.e.*, toxic, mechanical, metabolic, genetic) that can involve sensory neurons, SGCs or, considering their intimate intercommunication, both cell populations. DRG damage, primary or secondary to nerve damage, produces a sensory peripheral neuropathy, characterized by neurophysiological abnormalities, numbness, paraesthesia and dysesthesia, tingling and burning sensations and neuropathic pain. DRG stress can be morphologically detected by light and electron microscope analysis with alterations in cell size (swelling/atrophy) and in different subcellular compartments (*i.e.*, mitochondria, endoplasmic reticulum, and nucleus) of neurons and/or SGCs. In addition, neurochemical changes can be used to portray abnormalities of neurons and SGC. Conventional immunostaining, *i.e.*, immunohistochemical detection of specific molecules in tissue slices, can be employed to detect, localize and quantify particular markers of damage in neurons (*i.e.*, nuclear expression of ATF3) or SGCs (*i.e.*, increased expression of GFAP), markers of apoptosis (*i.e.*, caspases), markers of mitochondrial suffering and oxidative stress (*i.e.*, 8-OHdG), markers of tissue inflammation (*i.e.*, CD68 for macrophage infiltration) etc. However classical (2D) methods of immunostaining disrupt the overall organization of the DRG, thus resulting in the loss of some crucial information. Whole-mount (3D) methods have been recently developed to investigate DRG morphology and neurochemistry without tissue slicing, giving the opportunity to study the intimate relationship between SGCs and sensory neurons in health and disease. Here, we aim to compare classical (2D) *vs* whole-mount (3D) approaches to highlight "pros" and "cons" of the two methodologies when analysing neuropathy-induced alterations in DRGs.

Key words: Dorsal root ganglia; sensory neurons; satellite glial cells; painful peripheral neuropathy; whole mount immunolocalization.

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Supplementary Video 1. Representative 3D reconstruction of a whole-mount mouse DRG in which CGRP+ neurons (blue) and SGCs (GS+, red) are immunolabelled.

Supplementary Video 2. Representative 3D reconstruction of a whole-mount mouse DRG showing CGRP+ (blue) and IB4+ (yellow) sensory neurons.

Introduction

Dorsal root ganglia (DRGs) are clusters of sensory neurons and satellite glial cells (SGCs) in intimate relationships. They are particularly vulnerable to stress of different origin and intensity due to the lack of the blood brain barrier in the peripheral nervous system. Peripheral neuropathy is the most common disorder of the peripheral nervous system in adults with increasing prevalence and heterogeneous causes. Since no effective treatment is available for patients suffering from this debilitating condition, the identification of molecular pathways is mandatory to elucidate mechanisms of disease and identify novel targets for neuroprotective therapies. Despite the classical immunostaining is a valuable analysis to go insight the morphostructural features as well as neurochemistry of DRG damage, new innovative whole-mount analysis can be valid alternative approaches for studying the 3D morpho-functional abnormalities in sensory neurons, SGCs and in their intimate exchanges. In the following paragraphs, we will: i) recall the distinctive anatomical features of DRGs; ii) revise the common approaches for investigating DRG alterations in peripheral neuropathies; and iii) introduce recently developed whole-mount methodologies. Advantages and disadvantages of the different techniques will be discussed.

Anatomy of the dorsal root ganglia

Sensory neurons

Primary sensory neurons of the DRGs are the first-order neurons along the pathway conveying sensory information from the periphery to the central nervous system. They are pseudounipolar neurons with a single axon that bifurcates in a branch projecting to the periphery and a branch projecting to the spinal cord. Sensory neurons detect and transduce information from a variety of specialized receptors, such as nociceptors, mechanoreceptors, and proprioceptors.[1]

Several classifications of sensory neurons have been proposed over the years based on: the cell body size, the neurochemical phenotype, the functional physiological properties, the degree of myelination of peripheral branches and the pattern of central termination in the spinal dorsal horn.[2-4]

Based on the cell body diameter, sensory neurons can be subdivided in small-, medium- and large-sized neurons.

Small-sized DRG neurons (10-30 µm in diameter) give rise to small diameter unmyelinated C fibers that display a slow conduction velocity and mostly convey noxious and/or thermal stimuli to the CNS.[5] Based on the phenotype, small neurons can be further split into peptidergic or non-peptidergic neurons depending on the expression of transmitter neuropeptides.[3]Peptidergic nociceptors express one or more neuropeptides, such as the calcitonin gene-related peptide (CGRP),[6-10] substance P (SP),[9-11] somatostatin (SST).[9,12] Further subdivisions of peptidergic neurons have been proposed based on the neurotrophic content (*i.e.*, BDNF and GDNF).[13] Non-peptidergic nociceptors typically bind the isolectin B4 from *Griffonia simplicifolia* (IB4) and are immunoreactive to the LA4 antibody.[14] These neurons also express GDNF receptors RET, GFRα1, GFRα2[9,15,16] and the purinergic receptor P2X3.[17]

Medium-sized DRG neurons (30-50 µm in diameter) are mainly nociceptors and give rise to fine myelinated Aδ-type primary afferent fibers displaying faster conduction velocity as compared to C fibers.[1] These neurons may express the neuropeptide CGRP,[6,18] but do not contain binding sites for IB4. Large-sized DRG neurons (>50 µm in diameter) give rise to heavily myelinated Aβ-type primary afferent fibers displaying fast conduction velocities. Large neurons typically encode touch or proprioception and express the neurofilament NF200.[19]

The continuous discovery of novel neurochemical and functional markers,[4] as well as the application of new molecular techniques and single-cell proteomics to DRG neurons[20,21] have identified additional neuronal phenotypes, including C-fiber small neurons that do not express peptides and do not bind IB4, but express the vesicular glutamate transporter type 3 (VGLUT3), the tyrosine hydroxylase (TH),[22-24] the chemokine-like protein TAFA4[25,26] and the Gαi-interacting protein GINIP.[23,27,28]

Functionally, the different neuronal phenotypes have been associated with specific sensory modalities. Indeed, the thermal receptor TRPV1 is mostly found in peptidergic neurons,[29] while mechanotransducers such as PIEZO1[30] and TACAN[31] are chiefly found in non-peptidergic neurons. On the other hand, recent evidence obtained by *in vivo* calcium imaging have demonstrated that most small sensory neurons are polymodal.[32] In addition, important species differences could be observed in phenotypic marker expression. For instance, the vanilloid receptor TRPV1 is expressed in both peptidergic and non-peptidergic sensory neurons in rat,[33-37] and a small population of non-peptidergic neurons expressing TRPV1 has also been reported in mice.[23,37-39] In a similar manner, GINIP is exclusively expressed in non-peptidergic neurons in mouse,[23,39,40] while it has been also described in peptidergic neurons in rat.[28] Even in human DRGs, the neurochemical boundaries between peptidergic and non-peptidergic populations of sensory neurons are not as sharp as originally described in rodents.[41] Thus, neuronal phenotypes may be important, but not sufficient, for defining the role of DRG neuronal populations in sensory encoding. Indeed, several lines of evidence strongly indicate that a crucial role in the definition of specific sensory modalities is played by the central arrangement of primary afferent projections in the spinal dorsal horn.[42,43]

Satellite glial cells

Satellite glial cells (SGCs) of the DRGs are a group of perineuronal cells that envelop cell bodies of sensory neurons providing metabolic support and influencing neuronal function.[44-47] SGCs were described as cells with a relatively large nucleus and perineuronal processes.[46] Then, subsequent investigations led to identify SGCs as laminar cells with a flattened glial sheath surrounding sensory neurons.[44] Specifically, several SGCs surround a single neuron forming a morphological and functional unit,[44,48] which is separated from adjacent SGCs/neurons units by connective tissue.[44,46,47] The number of SGCs rises with the increase of the neuron's volume.[46,49] In some species such as lizard, rat and rabbit, it has been described that a common glia sheet could wrap different neurons forming a "cluster", mostly in young animals.[47,50] In these units, glial and neuronal membranes are in close apposition, with a distance of 15-20 nm.[46] The glial layer could also be discontinuous leading to a direct contact between membranes of two adjacent neurons.[47,51]

SGCs around DRG neurons are connected each other by gap junctions consisting of protein connexins[46] and the most abundant so far described in SGCs are Cx43 and Cx32.[52,53] Moreover, SGCs show dye coupling as observed after injection with Lucifer yellow.[54] These features clearly indicate that SGCs carry out several important functions for DRG homeostasis, providing trophic and mechanical support for sensory neurons, regulating ion concentration, diffusion and trafficking of molecules between neurons.[48]

SGCs can be identified by the expression of several glial markers such as glutamine synthetase,[51,55] GFAP,[44,56,57] S-100,[58,59] vimentin,[58] Cx42,[53] Kir4.1 potassium channels[60] and glutamate

transporters.[45,61] In particular, Nascimento and colleagues[58] have postulated the existence of a heterogeneous population of SGCs in DRGs after the observation that the expression pattern of S-100 and vimentin in SGCs is related to the neuron size they enwrapped. The heterogeneity of SGCs is consistent with our recent observation that different neuron-glia arrangements can be found in DRG for specific neuronal phenotypes.[51]

Due to the peculiar neuron-glia arrangement, an increasing number of studies have investigated the potential impact of transglial communication in shaping sensory transmission. The seminal work of Devor and Wall[62] demonstrated electrical coupling between DRG neurons arranged in small clusters. Subsequently, Rozansky and colleagues[63,64] showed that the spread of excitation from stimulated sensory neurons to adjacent neurons is mediated by the interposed SGCs. The authors defined this type of neuron-glia-neuron communication as "sandwich synapse". Following a train of stimulation, sensory neurons signal to the associated SGCs via purinergic signaling (*i.e.*, through P2Y2 receptors).[64] In turn, activated SGCs depolarize adjacent neurons *via* glutamate release and NMDA receptor activation[64] or nitric oxide.[65] The electrical coupling between SGCs *via* gap junctions may additionally amplify the signal allowing the recruitment of several adjacent neurons.

Conventional approaches for morpho-functional analysis of DRGs during peripheral neuropathy

The damage of DRG determines the establishment of a sensory neuropathy, which can be due to a primary damage of sensory neurons and/or SGCs (for example after platinum-derived chemotherapy) or to a damage secondary to peripheral nerve injury (for example after peripheral nerve trauma, as compression or transection). Patients with sensory neuropathy may have neurophysiological abnormalities in peripheral nerves, numbness, paraesthesia and dysesthesia, proprioceptive ataxia, tingling and burning sensations, widespread areflexia, neuropathic pain. Several are the causes of peripheral neuropathy, as autoimmune disease (*e.g.*, Guillain-Barre syndrome), diabetes, infections, inherited disorders (*e.g.*, Charcot Marie Tooth), solid and blood tumors, alcoholism, exposure to toxic substances (drugs as chemotherapy, poisons, as heavy metals and industrial chemicals), traumas or pressure of the peripheral nerves and vitamin deficiencies (B-1, B-6 and B-12, E). The features of DRG abnormalities can be determined by the nature of the insult and can involve sensory neurons, SGCs or, considering their intercommunication, both cell populations. Traditional immunohistochemical/histological approaches on DRG sections at both optical and ultrastructural level have been extensively used to address both morphological and molecular changes in pathological conditions. In the following paragraphs, we will review the main applications of these approaches in painful peripheral neuropathy.

Alterations in DRG sensory neurons

Depending on the cause and exposure extension, different cellular compartments can be damaged in sensory neurons. Electron microscopy observations, for example, revealed mitochondria swelling, dilatation in the cisternae of rough endoplasmic reticulum and Golgi complex, microtubule and neurofilament disarranging after chemical exposure. Moreover, neuronal enlargement, rounding of the cytoplasmic membrane, eccentric displacement of the nucleus and nucleolus, loss of Nissl substance can be evident.[66,67]

Neuronal suffering can lead to neuronal death through different pathways that can be biochemically and/or morphologically determined (autophagy, necrosis, apoptosis). Morphological features of necrotic sensory neurons involve cell swelling and degeneration of cytoplasmic organelles under the action of cellular lytic enzymes (lipases, proteases, endonucleases) that produce alterations in nuclear and cellular membranes, resulting in cellular disgregation and recruitment of inflammatory cells. Depletion of ATP can influence a progressive decrease of cellular processes and organelles' failure.[68]

By contrast, apoptosis requires energy and protein synthesis to proceed and is regulated by several specific molecules that orchestrate the cascade of apoptotic events (*i.e.*, caspases). Morphological features of apoptotic sensory neurons include cytoplasmic shrink, irreversible fragmentation and condensation of chromatin (piknosis) with intact nucleus. Organelles and plasma membranes are unaltered, thus avoiding an inflammatory response. Neurotoxic platinum drugs as cisplatin and oxaliplatin, employed in chemotherapy regimens against several solid tumors (*i.e.*, testis, lung, ovarian, colon cancers), produce a "neuronopathy" with DRG sensory neurons damage[69] and triggering of apoptotic pathways (Figure 1).

In diabetic sensory neuropathy, peripheral microvascular injury associated with the cellular consequences of high glucose levels in the blood leads to the formation of advanced glycation end products in neurons and increased production of reactive oxygen species (ROS) that alter neuronal metabolism and function.[70-74] Morphological and quantitative evaluations on DRG of animals affected by experimental diabetic neuropathy showed somatic atrophy[75] and a disorganized neuronal structure with cellular shrinkage and nuclear and nucleolar blurring with the formation of vacuolar-like structure in the cytoplasm. A computer-assisted quantitative analysis of Nissl/stained neurons also showed an evi-

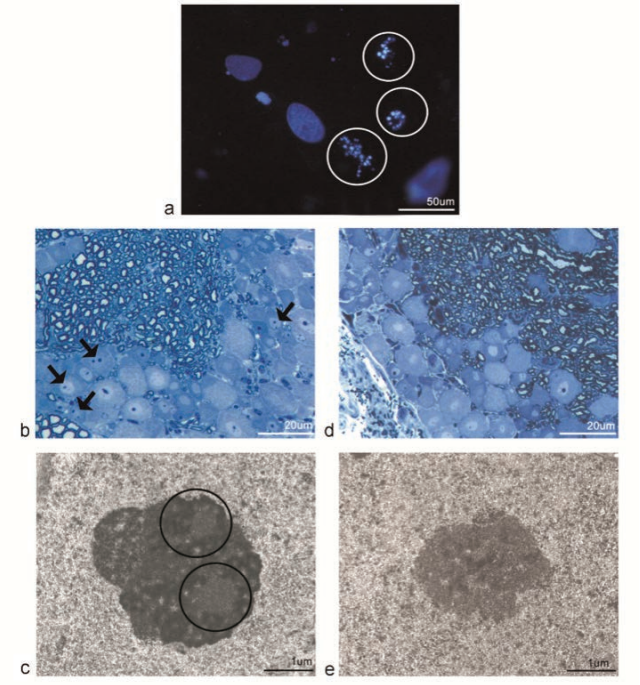

Figure 1. Examples of neuronal alterations detected by standard histological procedures on DRG sections after exposure to neurotoxic chemotherapy (cisplatin): nuclear condensation and fragmentation can be observed in DRG sensory neurons exposed to cisplatin *in vitro* (a, Hoechst staining). Cisplatin determines an increased incidence of multiple nucleoli *in vivo* (b) as well as alterations in nucleolar segregation of granular and fibrillar components (c) in DRG sensory neurons compared to non-treated control (d-e).

dent neuronal loss in the DRG of diabetic neuropathic rats.[76]

The morphofunctional changes of some subpopulations of sensory neurons can be relevant also during neuropathic pain. In the onset of neuropathic pain, peripheral nerve injury increases the excitability of sensory neurons in DRGs leading to spontaneous activation and discharge (hyperexcitability), then involving upper central circuits. Neurochemical properties can be used to identify those neurons who are involved in nociception and are altered due to the establishment of neuropathic pain by immunostaining. For instance, the capsaicin receptor TRPV1[77-79] can be activated and/or sensitized by mechanical stress, noxious heat, protons, and other endogenous compounds released following tissue injury. TRPV1 activation determines neuronal depolarization, which causes the release of sensory neuropeptides such as CGRP and SP from peripheral and central nerve terminals which in turns enhance the sensitization of nociceptor through the interaction with their effector cell receptors.[80] As previously stated, the presence of TRPV1 is restricted to small- and medium-sized neurons with reactivity in both peptidergic (CGRP+) and non-peptidergic (CGRP-) sensory neurons.[33,81] In DRG of neuropathic rats in which neurotoxic chemotherapy determined mechanical and thermal nociception, TRPV1+ neurons were increased and most TRPV1+ neurons had a small-medium size. A similar pattern has been described for CGRP with a decrease in the co-localization with TRPV1.[82]

Alterations in DRG, SGCs and in neuron-SGCs crosstalk

Although the scenario is huge and conditions variable, SGCs seem to be generally less susceptible than sensory neurons to several kinds of injury. In many cases, the alterations of SGCs do not seem to be due to a direct effect of exogenous insults, rather to the response of these cells to alterations occurring in sensory neurons. Consequently, in most cases, SGCs damage can be described as secondary to sensory neuron damage. The general response of SGCs to toxic-induced neuronal as well as axonal injury is the increase in number (*i.e.*, gliosis) after mitotic division[83-85] to meet the increased metabolic requirements of damaged neurons to attempt axonal regeneration. After this, SGCs undergo a series of structural modifications involving cytoplasmic organelles and some metabolic changes in enzymatic activities. Evident sign of SGCs activation is an increased expression of GFAP, S100, transforming growth factors, neurotrophins, receptors, cell adhesion molecules, interleukins, tumor necrosis factor.[57,59,86-89] Other changes that occur in SGCs after neuronal damage are a marked increase in gap junctions that connect neighbouring SGCs and the formation of bridges from previously separated perineuronal sheats.[90] In physiological conditions, SGCs are connected within the same perineuronal sheats, while in pathological circumstances, the formation of molecular bridges between different perineuronal sheats and the increased gap junction lead to an enhanced coupling among SGCs providing long-distance interconnections between them. These plastic changes in SGCs are trophic for damaged neurons in responding to stress.[91]

Considering that SGCs are the only cells in direct contact with sensory neurons and fundamental in regulating neuronal homeostasis and excitability, they play a role in the development and maintenance of neuropathic pain. Their changes are secondary to nervous alterations, after activation of injured neurons-SGCs signalling pathways. Several molecular mechanisms have been proposed to explain how SGCs can be activated and react to nerve injury and sensory neurons' hyperexcitability acting on the development and maintenance of neuropathic pain. An example is the activation of purinergic receptors on SGCs after ATP release by neurons: this can cause the release of proinflammatory cytokines by SGCs that in turns enhance neuronal excitability.[86]

Since gap junctions between SGCs increase in number and coupling after nerve injury, they can be involved in the generation and maintenance of neuropathic pain. The evidence came from the ability of gap junction blockers in restoring neuronal excitability and reducing pain in several pain models including chemotherapy treatment.[92,93] Several papers also postulated that the expression of connexins, the proteins that assemble together forming the gap junctions, can be augmented in painful pathological conditions.[48,91,94] Morpho-structural abnormalities are also evident in SGCs of animals chronically treated with chemotherapy treatment (bortezomib or cisplatin): they show clear cytoplasmic vacuolation and hypertrophy of the cytoplasm, invaginations of the plasmamembrane and altered chromatin segregation, as well as increased proliferation (gliosis).[69,95,96]

GFAP expression increment, because of gliosis, is widely used as a marker of neuronal damage in both CNS and PNS.[97-101] For instance, some authors have observed an increment in the number of GFAP+ SGCs surrounding stressed neurons (therefore ATF3 positive) in animal models of arthritis evidencing a direct relationship between neuronal insult and SGCs activation.[99,102] On the other hand, Barragán-Iglesias and colleagues[103] and Peters and colleagues[104] have observed a strong ATF3 positivity also on GFAP+ SGCs at late stages of both diabetic- and paclitaxel-induced peripheral neuropathy animal models.

In addition, Peters and colleagues[104] reported the presence of CD68+ macrophages in rat DRGs and sciatic nerves after 6 days of paclitaxel treatment that, besides SGCs activation, represents as well a sign of tissue damage. Painful neuropathies induced by paclitaxel activate the expression of monocyte chemotactic protein 1 (MCP-1) triggering macrophage infiltration in the DRG, which cells, in turn, express inflammatory cytokines.[105,106] Interestingly, this event seems to happen in concomitance with the onset of the neuropathy, suggesting an important role of CD68+ macrophages in the development of the pathology in DRG. This observation is validated, somewhat, by Zhang and colleagues[106] who describe that the administration of anti-MCP-1 neutralizing antibodies reduced macrophage infiltration in the DRG and prevented the development of mechanical hypersensitivity (paw withdrawal threshold) in the paclitaxel treated animals. Therefore, macrophage infiltration in DRG during painful neuropathy, more than simply helping remove degenerating neuronal debris and contribute to subsequent regeneration, as previously suggested,[106,107] seems to contribute to the onset of the pathology and maintenance of chronic pain.[106,108]

DRG insult can also trigger the expression of pro- and anti-apoptotic molecules. In fact, in a 10-month rat chronic model of diabetes, even though Kamiya and colleagues[109] observed an increment in pro-apoptotic protein expression (caspase-3) against a decrement (HSP70) or no alteration (Bcl-xL, HSP27) in the anti-apoptotic ones, they did not discern the presence of apoptotic cells (DRG neurons, mitochondria or endoplasmic reticulum did not show any morphological alteration). DRG neurons seemed to be kept in a sustained stressed state. Only the Golgi apparatus appeared highly vacuolized and fragmented, and this could have been one of the reasons for the progressive DRG cells loss in these animals.[109] On the other hand, in chronic (12 months) experimental diabetic rats several authors reported apoptosis (Caspase-3 and TUNEL positive) as soon as 1 and 3 months after streptozotocin treatment together with the presence of swelled mitochondria with disrupted inner cristae structure and condensation of chromatin.[74,110] It has also been implied the existence of a direct association between oxidative injury (8-OHdG expression) and neuronal apoptosis in these animals.[74] Mitochondria of DRG neurons also happen to be a target in platinum-based peripheral neuropathies as demonstrated *in vitro*[111] and *in vivo*[112] and when damaged ROS rise and calcium homeostasis becomes altered.[113,114] In fact, platinum-

based drugs form DNA adducts not only in the nucleus of the DRG neurons,[96,115,116] but also within mitochondrial DNA where profound functional modifications such as replication and transcription interference take place, leading to a progressive mitochondrial vacuolization and degradation.[117] Moreover, Corsetti and colleagues[118] described mitochondrial and cytoplasmic alterations also in SGCs after cisplatin treatment and propose that neuronal damage is subsequent to SGCs damage, therefore attributing to SGCs a main role on platinum-based peripheral neuropathies.

Another marker for SGCs functional changes under stress conditions is Connexin 43. Its expression in gap junctions increases in SGCs after taxane treatment,[93] indicating an increment of gap junction-mediated coupling between SGCs that contributes to the establishment of chronic pain as reported in different neuropathic pain models.[54,90,119,120]

Moreover, SCG purinergic P2 receptors are the target of the ATP and TNF-α released by stressed or damaged neurons that triggers a gap junction-dependent Ca^{2+} waves between the SGCs.[94,12] Interestingly, in diabetes animal models, the purinergic P2X7 receptor is upregulated in SGCs which results in a hyperexcitability of DRG neurons.[122]

In keeping, peripheral neuropathy induces a variety of alterations in both DRG neurons and glia and in their reciprocal interactions that can be investigated by standard histological procedures on DRG sections, combined with immunohistochemistry. These classical approaches provide an effective tool to address cellular and subcellular alterations and still represent the gold standard to detect neurodegenerative processes. However, these classical methods, while favoring the access to individual cell bodies, disrupt the overall organization of the DRG, thus resulting in loss of some crucial information. Optimizing the technical approach to investigate the entire DRG is the necessary premise to fully evaluate the impact of internal organization on function. In recent times, innovative whole-mount methods, discussed below, have been developed to investigate large areas of the nervous system without slicing and allowing the evaluation of cell populations in their intimate relationship, close to physiological conditions.

Whole mount morphological approaches for studying neuron-to-glia structural association in DRGs

Most whole-mount 3D methods aim to convert large opaque pieces of tissue in transparent specimens by reducing light scattering (tissue clearing methods, for review[123,124]). Different technical approaches have been proposed, based on either the use of solvent (hydrophobic methods, such as BBAB, DISCO), aqueous reagents (hydrophilic methods, such as SeeDB), or hydrogels (such as CLARITY).[124]

Initial attempts to clarify nervous tissues were chiefly based on solvent-based approaches to solvate lipid content in the samples, thus allowing the tissues to become translucent. Although relatively easy to implement, these methods were typically hampered by high levels of autofluorescence, incapacity to preserve endogenous fluorescence and limited compatibility with macromolecule-based phenotyping techniques.[125] Nevertheless, hydrophobic clearing methods have been successfully applied to certain whole-mount preparations of peripheral sensory neurons.[123] Indeed, a combination of transparency and immunophenotyping has been recently reported in intact DRGs from both embryonic[126] and adult mice.[127]

On the other hand, the innovative hydrogel based methods proposed by Karl Deisseroth's lab in 2013, known as CLARITY, paved a novel path for combining whole-mount preparations and immunostaining.[128] The clearing method originally consisted in an active lipid extraction procedure allowing the conversion of entire pieces of brain into light-permissive macromolecule-permeable specimens.[128] Hydrogel reinforcement allows excellent transparency of the specimen (as obtained by classical hydrophobic methods) with minimal loss of macromolecules and antigenicity.[124] Moreover, unlike hydrophobic approaches, CLARITY does not affect endogenous fluorescence and can be conveniently adopted in transgenic animals. The initial method was, however, somehow labored and changes have been subsequently proposed to improve reproducibility, reducing costs, and mitigate the overall duration of the clearing/staining protocols (see for instance, PACT, SHIELD, PARS).[129]

Recently, Bernal and colleagues[130] have proposed a cost-effective variation of CLARITY (inCLARITY) based on passive lipid clearing protocol to image DRGs. The method consisted in a first phase of hydrogel polymerization in acrylamide/bisacrylamide, followed by a long lipid clearing phase in sodium dodecyl sulphate (>7 days). This method was used for combining immunophenotyping of sensory neurons and retrograde fluorescent tracers. While inexpensive and technically simple, the method requires long incubation times spanning over two weeks for both clearing procedures and antibody incubation.

In 2016, we proposed an alternative whole-mount method for 3D DRG processing without a clearing approach.[131] Indeed, tissue clearing is instrumental to minimize light scattering in large specimens. However, rodent (and particularly mouse) DRGs are relatively small (<1 mm) and light permissive. The main obstacle to obtain multilayer immunostaining in entire DRGs is thus represented by antibody penetration. To overcome this problem, we proposed to incubate freshly dissected DRGs with collagenase (5-10 mg/ml) to digest the connective tissue forming the DRG capsule. Collagenase was previously used to permit patch clamp recordings in entire rat DRGs.[132,133] Indeed, we applied this approach for recording functional properties of sensory neurons without dissociating, thus preserving local anatomical interactions. After recording, and following a short fixation in paraformaldehyde, DRGs were processed for immunohistochemistry in order to phenotype the recorded neurons. Surprisingly, we found that collagenase treatment significantly improved antibody penetration in the DRGs, as compared to untreated DRGs.[131] Representative 3D reconstructions are shown in Supplementary material.

The protocol was subsequently optimized for 3D morphological analysis and the method allowed to uncover specific spatial arrangements of sensory neurons belonging to different cell populations.[51] Namely, non-peptidergic neurons were found to form cell clusters in which cell somata lie in close apposition, while peptidergic neurons do not. In the same study, we also analyzed the relationship between SGCs and sensory neurons taking advantage of the possibility to analyze the contact between glia and neurons all along the neuronal surface in whole mount-preparation (Figure 2). Interestingly, whole-mount preparation allows us to appreciate how different neuron-glia arrangements can be detected for different populations of sensory neurons.[51]

As compared to clearing-based whole-mount approaches, our proposed protocol is fast, cost effective and highly reproducible and can be easily combined with functional approaches (*i.e.*, addressing the phenotype of recorded neurons).

Major limitations are common to all whole-mount approaches, and specifically:

1) Technical limitations to image large 3D specimens. The increased interest in whole-mount approaches has been accompanied in recent years by a parallel development of optical systems to see deeper in the sample, such as lightsheet microscopy and multiphoton confocal microscopy.[134] Although these devices are becoming more and more accessible, yet standard one-photon

scanning confocal microscopes are by far the more common set-up found in many laboratories. The use of one-photon confocal microscopy allows accurate 3D reconstruction of small volumes or of restricted areas of large samples.[135] This approach is suitable for mouse DRGs as their diameter falls below the millimeter and long working distance objectives are adequate to fully scan the specimen. In our study, we successfully acquired 3D images with a standard one-photon laser scanning confocal (Leica SP5). Interestingly, a one-photon microscope was also used to image cleared DRG by inCLARITY.[130] The overall yield in terms of image definition and thickness of 3D reconstruction are comparable in the two studies thus suggesting that the major limiting factor for Z-scanning in DRGs is not due to the use of clearing approaches but, rather, to the technical limitations of the optical devices.

2) Technical issues for analyzing 3D images. 3D reconstruction of biological samples is providing novel insight in the understanding of the organization of the nervous system. However, to properly analyze and extract quantitative information from these samples traditional 2D methods are limited and appropriate software-based methods are required. To this aim, we have developed a software (3DRG), that provides a fully-automated segmentation of positively labeled nociceptors in whole-mount DRG preparations.[136] 3DRG has been conceived to characterize the sample in a fully-automated way, allowing the visualization of identified cell populations to highlight spatial relations. Recently, Bennett's group has proposed an image analysis tool (StereoMate) to develop image segmentation protocols in DRGs and extract unbiased information at the cell level.[127] The tool can be combined with a machine learning classifier to facilitate correct object recognition. Other machine learning approaches are emerging[137] and represent a promising avenue for addressing cell-to-cell interactions in a 3D environment.

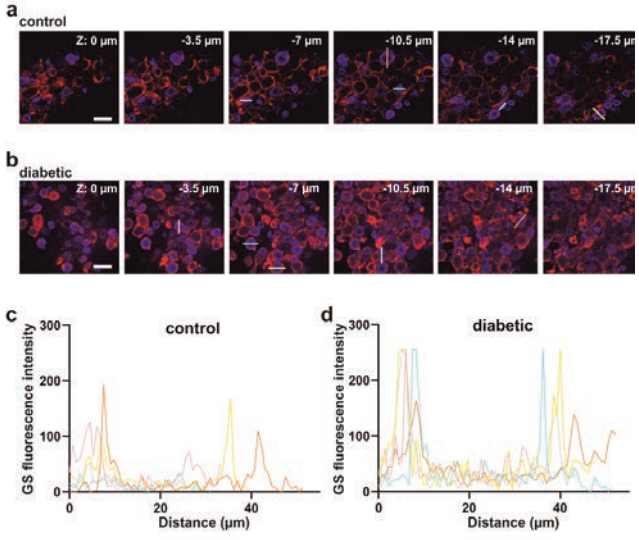

Figure 2. Whole-mount mouse DRG staining after collagenase treatment. a) CGRP+ neurons (in blue) and GS+ satellite glial cells (in red) in lumbar DRG from a control mouse; from left to right, sequential z-stacks are shown; a viable approach for GS fluorescence quantitation is also represented; the intensity of GS staining is measured across the maximum diameter along the Z-axis of identified CGRP+ cells (coloured lines).(b) CGRP+ neurons (in blue) and GS+ satellite glial cells (in red) from a diabetic mouse. c-d) GS fluorescence intensity per pixel along the coloured lines (distance in microns) in (a) and (b). Note the fluorescence peaks at the perimeter of the sensory neurons where SGCs are located. Higher fluorescent signals are detected in diabetic mice (d). Scale bar: 50 µm.

Application of a 3D DRG approach in pathological settings

While traditional approaches based on classical tissue slicing techniques are still the gold standard for fine subcellular analysis of morphological and molecular alterations in pathological DRGs (Figure 1), yet whole-mount methods represent a powerful and complementary alternative to explore changes in cell-to-cell relationships. Main advantages of 2D *vs* 3D approaches are reported in Table 1.

We have recently applied our whole-mount technique to interrogate the impact of diabetic neuropathy in altering neuron-neuron and neuron-glia interactions.[51] An example of this application and its potentiality is shown in Figure 2. In agreement with previous studies,[98] major differences were mainly detected in the relationship between SGCs and neurons. Specifically, we used our 3D method to analyze the distribution of SGCs marker GS around identified populations of sensory neurons, such as CGRP neurons (blue in Figure 2 a,b). The possibility of changing the z-axis allows to standardize GS quantification around the largest diameter of the identified neurons, which would not be possible in standard cryostat or microtome-based sections, thus making comparisons between groups more robust. Our analysis demonstrated that GS staining is significantly increased around $CGRP^+$ neurons from diabetic mice (Figure 2 c,d).

3D approaches have also been successfully applied to detect DRG alterations following peripheral nerve injury. Indeed, whole mount DRG preparations were successfully used to quantify the loss of neurons in a murine model of spared nerve injury.[127] A similar result can be also obtained by standard stereological approaches, which are, however, time consuming and more exposed to sampling errors.[127]

In perspective, other potential applications of 3D DRG specimens in pathological conditions may include:

- analysis of DRG infiltrations of immune cells;
- structural plasticity of fluorescent-tagged populations of sensory neurons (*i.e.*, change in size or in cell-to-cell relationship);
- alterations in the somatotopic organization of afferent inputs (*i.e.*, by retrograde labelling of sensory neurons innervating restricted peripheral territories);
- identification of functional interaction between neighbouring

Table 1. *Pros* and *cons* of whole-mount methods *vs.* classical section-based approaches.

PROS AND CONS	2D methods	3D methods
Costs	+	++ > +++
Time consuming	++	+ > +++
Neuronal phenotyping	++	+++
Neuron-glia relationship	+/-	+++
Cell count	++	+++
Volumetric analysis	+/-	+++
Membrane localizations	+++	+++
Subcellular localizations	+++	+
Nuclear alterations	+++	+
Coupling with functional studies	+/-	++
Data extraction and analysis	++	+

+++, highly appropriate; ++, appropriate; +, appropriate (but not first choice); -, inadequate.

sensory neurons by using markers of neuronal activation *(i.e.*, ERK1/2 phosphorylation);[138]
- combining functional and morphological analysis in entire DRGs.[131]

Conclusions

In keeping, 3D analysis of sensory neurons is a growing and promising method that can be adopted together with or as alternative to standard histological 2D approaches for tackling specific questions around the impact of DRG structure on sensory function (Table 1). The anatomical organization of the DRGs suggests that neuron-neuron as well SGC-SGC or SGC-neuron interactions influence neuron physiology. This "interactive" homeostasis can be lost once the DRG are processed with tissue and/or cell preparation standard protocols for conventional morpho-functional analysis. In the whole 3D DRG, instead, the cyto-architecture of the organ as well as the anatomical relationship between the different cell populations are preserved.

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