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"A picture is worth a thousand words": the use of microscopy for imaging neuroinflammation

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Declaration of interest

The authors declare that they have no conflict of interest

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List of abbreviations

AD	Alzheimer's disease
ALDH1L1	Aldehyde dehydrogenase 1 L1
ALDOC	Aldolase C
ALS	Amyotrophic Lateral Sclerosis
AMCA	Aminomethyl coumarin
APP	Amyloid precursor protein
AQP4	Aquaporin 4
BBB	Blood-brain barrier
BFP	Blue fluorescent protein
BrdU	5-bromo-2'-deoxyuridine
C3	Complement component 3
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CD	Cluster of differentiation
CFP	Cyan fluorescent protein
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
CLDN	Claudin
CNS	Central nervous system
CODEX	Co-detection by indexing
COX-2	Cyclooxygenase 2
CSF	Cerebrospinal fluid
CT	Computerized tomography
CX3CL1	Fractalkine
CX3CR1	CX3C chemokine receptor 1
CXCL1	Chemokine (C-X-C motif) ligand 1
Cy2	Cyanine
Cy3	Indocarbocyanine

DAPI	4′,6-diamidino-2-phenylindole
EAE	Experimental Autoimmune Encephalitis
FITC	Fluorescein isothiocyanate
Gad65/67	Glutamic acid decarboxylase 65/67
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GS	Glutamine synthetase
Iba-1	Ionized Calcium-Binding Adaptor Molecule 1
IF	Immunofluorescence
IFN-γ	Interferon-*gamma*
IHC	Immunohistochemistry
IL	Interleukin
LAMP-1	Lysosomal-associated membrane protein-1
LN3	Monoclonal antibody used as a microglial activation marker
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MERFISH	Multiplexed error-robust fluorescence *in situ* hybridization
MHC II	Major histocompatibility complex II
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MS	Multiple Sclerosis
NFAT	Nuclear factor of activated T cells
NIRF	Near-infrared fluorescence
NLRP3	NOD-like Receptor Protein 3
Nr4a1	Nuclear receptor subfamily 4 group A member 1
OCLN	Occludin
P2RY12	Purinergic receptor P2Y, G-protein-coupled receptor 12
PET	Positron emission tomography
PS1	Presenilin 1

PSD-95	Postsynaptic density protein 95
seqFISH	Sequential fluorescence *in situ* hybridization
SR101	Sulforhodamine 101
TBI	Traumatic brain injury
TLR	Toll-like receptor
TNF-α	Tumor Necrosis Factor-*alpha*
TRITC	Tetramethylrhodamine isothiocyanate
TSPO	Translocator protein
vGLUT-1	Vesicular glutamate transporter-1
YFP	Yellow fluorescent protein
ZO	Zonula Occludens

Abstract

Since the first studies of the nervous system by the Nobel laureates Camillo Golgi and Santiago Ramon y Cajal using simple dyes and conventional light microscopes, microscopy has come a long way – to the most recent techniques that make it possible to perform images in live cells and animals, in health and disease. Many pathological conditions of the central nervous system have already been linked to inflammatory responses. In this scenario, several available markers and techniques can help imaging and unveil the neuroinflammatory process. Moreover, microscopy imaging techniques have become even more necessary to validate the large quantity of data generated in the era of "*omics*". This review aims to highlight how to assess neuroinflammation by using microscopy as a tool to provide specific details about the cell's architecture during neuroinflammatory conditions. First, we describe specific markers that have been used in light microscopy studies and that are widely applied to unravel and describe neuroinflammatory mechanisms in distinct conditions. Then, we discuss some important methodologies that facilitate the imaging of these markers, such as immunohistochemistry and immunofluorescence techniques. Emphasis will be given to studies using two-photon microscopy, an approach that revolutionized the real-time assessment of neuroinflammatory processes. Finally, some studies integrating omics with microscopy will be presented. The fusion of these techniques is developing, but the high amount of data generated from these applications will certainly improve the comprehension of the molecular mechanisms involved in neuroinflammation.

1. Microscopy in the light of the study of the central nervous system

Since the revolutionary studies by Santiago Ramón y Cajal (1852-1934) and Camillo Golgi (1843-1926) (1,2) – whose work is still a foundation of routine techniques of histopathologists and neuroscientists (3–5) – to modern developments of molecule specific labelling and imaging in freely moving animals, microscopy-related technologies have constantly impacted the understanding of the healthy and pathological nervous system, and made clear the importance of understanding the relationship between cell structure, localization, and connections (3,6,7).

The collection of fields known as 'omics' also highlights the importance of connections. The omics fields – such as genomics, transcriptomics, metabolomics, and proteomics – aim to identify and quantify a large number of elements associated with a physiological function, and the connections between them. It has also led to groundbreaking findings, *e.g.,* by identifying targets that could help confirm diagnosis (8). Omics approaches are particularly helpful when studying immunological functions, such as neuroinflammation, as the techniques allow complex interactions to be drawn out. However, the processing of tissue removes the structural connections that are revealed by microscopy. In neuroinflammatory studies, changes in structural connections, cell morphology, and localization of molecules are outcomes of interest, and so a combination of both techniques is key. Several studies have integrated these two approaches to elucidate specific mechanisms, considering the correspondence between omics and microscopy.

In this review, we describe current applications of microscopy to image neuroinflammation, pointing out how changes in the shape and structure of specific cells can indicate neuroinflammatory processes. First, we will address some of the main neuroinflammatory responses and discuss the signs of neuroinflammation, focusing on the involvement of the blood-brain barrier (BBB) and the phenotypic changes in glial cells, as well as the main markers that can be used to study neuroinflammation using microscopy techniques. Then, we discuss basic methodologies, such as immunohistochemistry and immunofluorescence, which allow the microscopic observation of those neuroinflammatory markers. We will also emphasize the importance of two-photon microscopy to the *in vivo*

real-time assessment of neuroinflammatory processes. Studies elucidating the function of the inflammatory components in healthy and disease conditions will be briefly mentioned as examples of the applications of these techniques. Lastly, we will discuss the newest omics techniques and how the molecular and imaging approaches may complement each other and be applied simultaneously to improve the comprehension of the mechanisms involved in neuroinflammation.

2. The concept of neuroinflammation

The term neuroinflammation arose in the 1980s from observations using microscopy techniques, which showed an accumulation of leukocytes around degenerating myelin and brain vessels in samples of multiple sclerosis (MS) (9). Since then, the study of neuroinflammation has expanded greatly, with almost 5,000 articles listed in NCBI PubMed for 2020 alone.

The process of neuroinflammation involves multiple types of cells and factors (**Figure 1**), which play different roles depending on the context of the inflammation, duration, experimental model, or disease, an understanding of which is complicated by inconsistency in terminology (10).

Neuroinflammation is mediated by signaling molecules known as cytokines, which are proteins produced and secreted by different cell types that mediate the immune and inflammatory responses (11). However, it is worth mentioning that some of the “neuroinflammatory components” are also involved in physiological function. For example, cytokines considered to be neuroinflammatory markers, such as Interleukin-1 (IL-1), IL-18, IL-6, and Tumor Necrosis Factor-α (TNF-α), have already been described as key players in physiological mechanisms involved in memory consolidation (12). Glial cells are also involved in mechanisms activated in non-pathological contexts. Microglia can be activated through IL-17 released from hippocampal neurons taking part in synaptic remodeling as part of memory consolidation (13). The recent description of the importance of IL-17 from meningeal-resident γδ T cells for short-term memory (14) has also increasingly opened up the possibilities of studying "inflammatory" cells and mediators in health, not only in disease.

Nevertheless, in pathological contexts, the inflammatory signaling that characterizes neuroinflammation might become detrimental and ultimately result in neuronal death (15,16). This process involves different cytokines, chemokines, as well as reactive oxygen species synthesis by microglia, astrocytes, infiltrated immune cells, and endothelial cells. These mediators can potentially lead to harmful outcomes, such as edema, tissue damage, and cell death (10,11,17,18). Glial cells orchestrate an inflammatory response in a very context-dependent manner, presenting different neuroinflammatory phenotypes based on which pathways are activated, insult context, and the degree of inflammation in experimental models, developmental stages, and in health and disease conditions (10,11,19–22). The situation is complicated somewhat by the lack of clear terminological definitions of individual cells in the central nervous system (CNS) such as terminology concerning astrocyte responses in physiological and pathological conditions (23). **Table 1** summarizes a variety of targets commonly used in light microscopy studies to describe different aspects of the neuroinflammatory process.

3. What to observe: the signals of neuroinflammation

In this section, we introduce the cell markers used to study BBB damage in neuroinflammatory contexts and the brain clearance 'glymphatic' system. We also discuss the cells involved in the neuroinflammatory responses, along with the immunological synapse that comprises the interaction between nervous and immune cells. These are the main targets to be observed when using a microscope as a tool to study neuroinflammation.

3.1. Damage to the blood-brain barrier

Although seen as a structure separating the nervous system from the periphery, the BBB also regulates the passage of substances into and out of the nervous system, and damage to the BBB is a key point in several inflammatory processes.

The BBB is essential to maintain the microenvironment of the CNS distinct from the periphery, regulating the entry and exit of solutes and the entry of peripheral leukocytes (24). Its function is performed by specialized blood vessel endothelial cells, which have limited

vesicular transport and are connected through highly specialized tight junctions, preventing paracellular transport (25,26). The tight junctions are formed by the claudins (CLDN), occludin (OCLN) and the zonula occludens protein family (ZO-1, -2, and -3) (27). Pericytes and astrocytes are cellular components in close contact with the endothelium, influencing and participating in the complex maintenance of the BBB (26,28).

The BBB is disrupted in several neuroinflammatory contexts (29) such as cerebral ischemia (30,31), Alzheimer's disease (AD) (32,33), and MS (34), and it is also affected by systemic inflammation, *e.g.*, induced by systemic injections of lipopolysaccharide (LPS) (35). Loss of BBB integrity can also affect neurodevelopment, as shown in a model of prenatal exposure to valproic acid in rats (36) and neonatal hypoxic-ischemic events (37). Sophisticated imaging techniques such as computerized tomography (CT), magnetic resonance imaging (MRI), and near-infrared fluorescence (NIRF) are widely used in these studies, but the limitation of analysis to macroscopic scales places microscopy as an important tool to elucidate the molecular mechanisms involved in the neuroinflammatory process (27).

Immunohistochemistry (IHC) was used in brain samples from both epileptic patients and rats to detect increased anti-albumin labeling in the hippocampus (38). Since albumin is not expected to cross BBB under normal conditions, this suggests a loss of barrier integrity (24). The paper by van Vliet *et al.* (38) demonstrated similar results in chronically epileptic rats using immunofluorescence (IF) techniques. This finding was subsequently confirmed by injecting fluorescein dye in the tail vein of the animals and visualizing the infiltrated dye with a confocal microscope. Since fluorescein does not penetrate the CNS under normal conditions and can bind to albumin, it offers a confirmatory method to analyze the infiltration. Albumin signal was also co-labeled with astrocyte, neuronal and microglial markers to demonstrate the relationship of these cells with invading molecules (38).

In addition to analyzing the leakage of some substances through the BBB, targeting junction proteins can provide relevant data on BBB integrity. Decreased expression of junction proteins CLDN-5, OCLN, ZO-1, α-catenin, and vascular endothelial cadherin, was detected by IF in pre-reactive inflammatory lesions from MS human brain samples (34).

One-time and repeated imaging was performed in a mouse model of ischemic stroke with CLDN-5 gene coupled with green fluorescent protein (GFP) reporter gene, in which green fluorescence was detected using two-photon microscopy, thus providing evidence of dynamic changes in tight junctions in this pre-clinical ischemic stroke model (39). Also, using GFP expression under the control of CLDN-5 promoter, two-photon microscopy revealed remodeling of the tight junctions preceding the invasion of a peripherally injected dye that normally does not cross the BBB in an experimental autoimmune encephalitis (EAE) model. Interestingly, this dynamic remodeling of the tight junctions precedes the EAE onset (40).

In a blast-induced traumatic brain injury (TBI) model, a decrease in the fluorescence intensity of ZO-1, CLDN-5, and OCLN suggested a BBB leakage that was confirmed by analyzing the fluorescent tetramethylrhodamine isothiocyanate–dextran (TRITC-Dextran) dye invasion into the brain (41). An interesting way to quantitatively analyze the damage to the BBB is the use of dyes of different sizes. In a model of sustained inflammation induced by systemic injections of LPS, only dextran-Texas red (10 kDa) crossed BBB, while dextran-tetramethylrhodamine (40 kDa) and dextran-fluorescein (70 kDa) did not (42). Thus, as described so far, microscopy can assist in fundamental issues related to BBB integrity, from differences in immunoreactivity and location of junction proteins involved in BBB integrity to the degree of barrier breakdown assessed through fluorescent dyes.

IHC and IF allow the colocalization of inflammatory cytokines with different cell markers to determine the involvement of specific cell types in the neuroinflammatory process. Four hours after systemic LPS administration, stromal cells niches in leptomeningeal and choroid plexus, and also epithelial cells from the choroid plexus and hippocampal vessels were the main producers of chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, and IL-6. Interestingly, after 24 hours the main source of mediators such as CCL11 and CXCL10 were astrocytes, evidenced by co-labeling with glial fibrillary acidic protein (GFAP). The use of histological techniques also showed that the expression of cytokine receptors following systemic LPS administration occurred specifically in the astrocytic end-feet, a part of the cells that is in contact with capillaries and which plays a role in BBB maintenance (43).

Visualizing fluorescent tracers in the cerebral cortex using two-photon microscopy allowed the elucidation of a brain waste clearance system: a perivascular pathway that enables the transport of cerebrospinal fluid (CSF) into and out the brain parenchyma. The same study showed that aquaporin 4 (AQP4) in astrocyte foot processes is fundamental to fluid dynamics (44). The glial-based clearance system was termed the “glymphatic system”. The same research group later used *in vivo* two-photon and *ex vivo* fluorescence microscopy to find that cerebral arterial pulsatility is also critical to CSF transport into and through the brain (45). Also, light-sheet fluorescence microscopy has been applied as a new way to study the glymphatic system, reducing some of the limitations, such as the need for antibodies, due to the use of CSF tracers (for details, see reference (46)). The wide range of approaches in these studies demonstrates that microscopy was a fundamental tool for describing this important clearance system in the brain.

3.2. Cell types involved in the neuroinflammatory response

Glial cells - mainly microglia and astrocytes - and invading leukocytes orchestrate the neuroinflammatory response following BBB disruptions. Microglia cells are the CNS macrophages, and assume different phenotypes within a spectrum ranging from M1 (pro-inflammatory phenotype) to M2 states (anti-inflammatory phenotype) (47,48). The M1 phenotype is characterized by secretion of pro-inflammatory cytokines such as TNF-α, interferon-γ (IFN-γ), and IL-6 (49), whereas the M2 phenotype is related to anti-inflammatory cytokines such as IL-4 and IL-10 (49). Besides differences in the cytokines released by each microglial phenotype, the shape of the microglia themselves following stimulation also varies, and the different shapes reflect their differing function. For example, following LPS challenge, M1 microglia show a profile with an ameboid shape (50), aiding its ability to phagocytose pathogens in the CNS. In contrast M2 morphology presents ramified branches that enhance its surveillance role (51). It is important to point out here that oversimplifications of these phenotypes should be avoided when describing not only the state of microglia but also the outcomes of a particular neuroinflammatory event (52).

Microglia polarization analyzed by immunostaining and further confirmed by flow cytometry has been employed in middle cerebral artery occlusion (MCAO) models (53). Flow cytometry has been employed to confirm the inflammatory response and microglial polarization using F4/80 labeling, which is a glycoprotein found on the surface of macrophages and resting microglia (53).

Usually, microglia alterations are widely used to suggest a neuroinflammation condition in the CNS using the ionized calcium-binding adaptor molecule 1 (Iba-1) marker (54–56). Some authors applied double-labeling of the Iba-1, as a marker for all microglial cells, labeling the soma, with LN3 (a monoclonal antibody used as a microglial activation marker since it recognizes the major histocompatibility complex II - MHCII), to evaluate microglial hypertrophic and ameboid morphology (57). Another interesting marker, P2Y purinoceptor 12 (P2RY12), was double-stained with Iba-1 to enhance the labeling of the microglial ramifications (57,58). Also, CX3C chemokine receptor 1 (CX3CR1) fractalkine receptor is expressed on the surface of macrophages and microglial cells and responds to CX3CL1 (fractalkine) (59–61), and is considered a potential marker for macrophages and microglial cells.

Although it can lead to confusion with neutrophils, cluster of differentiation 11b (CD11b) is used as a marker for microglia, as these cells express this protein (56,62). On the other hand, myeloperoxidase protein (MPO) can be used as a marker for neutrophils (63), allowing discrimination of neutrophils from microglia. As mentioned before, Iba-1 is widely considered a microglia marker; however, this antigen does not differentiate microglia from monocytes. CD45 could help in this case, as it is more expressed in monocytes than in microglia. So, both Iba-1 and CD45 positive cells – particularly round-shaped ones – are likely monocytes, while Iba-1 positive cells and CD45 negative cells with processes are microglia (62,64). Another helpful marker is CD68 (a macrophage/phagocytic activity marker), which appears after ischemia in the border zone of the lesion, but invades the ischemia core 7 days after the insult (56,62).

The microglial inhibitor AZD8797 (a CX3CR1 inhibitor) was used in a study designed to observe microglial-dependent neuroinflammatory responses. A significant increase in the

percentage of IL-1β+ and IL-6+ cells (observed by IHC) caused by hypertension was mitigated by blocking microglia with AZD8797 (59). In a model of brain ischemia, immunofluorescent staining was used to co-label cells with anti-Iba-1 and anti-TNF-α, demonstrating that microglia were the main cell type responsible for the TNF-α increase observed in the brain tissue. As IF was performed after 12 hours, 1 day, 3 days, and 7 days following ischemia induction, it allowed a sequential analysis of the role of microglia in establishing the neuroinflammatory process (65).

Due to the extensive range of possible microglial phenotypes, double labeling may differentiate the pattern of the response of these cells in a particular situation. Moreover, studies should consider not only the number or optical density of cells immunoreactive to these markers but also cell morphology and time course of the event. These factors further reinforce the need to use microscopy techniques to study cellular responses in neuroinflammation (64,66).

Astrocytes also play an important role in different CNS diseases (67) such as stroke (68), TBI (69), AD (70–72), amyotrophic lateral sclerosis (ALS) (73), and other neurodegenerative diseases (67,72,74). Astrocytes and microglia act in a coordinated way to maintain homeostasis (75), influencing each other in the pathophysiology and inflammatory responses (76–79). Astrocytes are also responsive to physiological and pathological stimuli, becoming reactive and presenting functional and morphological changes (70,80). While morphological analyses are useful to describe astrocyte remodeling in different contexts, the techniques are less helpful for understanding functional changes such as metabolic and transcriptional modifications (23).

Nevertheless, morphological changes are extremely important to understand the role of astrocytes both in health and disease. One of the main markers for analyzing astrocyte morphology is GFAP, the principal intermediate filament (81), a member of the cytoskeletal protein family of these cells (82–84). Due to its unique and highly immunogenic epitopes, GFAP application in IHC has been suggested since 1994 (85). Reactive astrocytes show increased GFAP immunoreactivity (23), as well as an increase in the number and length of processes leaving the soma as well as an increase in the thickness of the branches (74,80).

Imaging techniques across a range of studies have found changes in important features of astrocytes in a variety of models of neuroinflammation. Following LPS injection into the substantia nigra of adult rats, astrocytes changed morphology, exhibiting long, thick branches compared to the small soma and few thin branches in basal conditions. LPS-injected animals also showed increased GFAP+ cell number and GFAP immunoreactivity (86). Astrocyte-linked inflammation was also seen via increased GFAP immunofluorescence following experimental TBI (87). Astrocyte morphology is altered in distinct conditions. In a model of epilepsy, astrocytes in brain slices presented hypertrophy and significant overlap of their processes (88). An increased astrocytic arborization was also seen after intermittent exposure of rats to heavy consumption of alcohol (89). While no alteration was found in hippocampal astrocytes after acute hypoxia exposure, neuroinflammation was observed through increased microglial markers (Iba-1 and CD86) (90). GFAP upregulation was also evidenced in ischemic injury, and different functional significance was found according to sex (91). In young rats, GFAP fluorescence intensity was increased after toluene exposure together with an increase seen in both the number of CD11b+ cells and the mRNA levels of the proinflammatory cytokine IL-1β (92). Even aging was associated with GFAP upregulation. In a model of perioperative cognitive impairment, an increase in the number of GFAP+ astrocytes – which led to the production of proinflammatory cytokines and was suppressed through NOD-like receptor protein 3 (NLRP3) inflammasome inhibition – was observed in the hippocampus of aged mice (93).

It has already been demonstrated that, despite the various structural modifications, even when reactive, astrocytes did not present alterations in the total area occupied (84), nor in the cellular division process of mature astrocytes, except for a specific subpopulation of these reactive astrocytes, identified by the GFAP expression (94). The GFAP positive subpopulation of cells originates from neural stem cells in specific regions, such as the subventricular zone (94) and the subgranular layer of the hippocampus (95). The region-specific reactive astrocytes may be used in treatment as multipotent cells that could be applied within the injury site to support damaged or dysfunctional neurons in important and specific regions (96), a discovery in which the use of microscopy to visualize fate-mapping

astrocytes in adult mice was key (96). Markers of proliferation such as BrdU (5-bromo-2'-deoxyuridine) or Ki67 (97) and aldehyde dehydrogenase 1 L1 (ALDH1L1), glutamine synthetase (GS), or aldolase C (ALDOC) (23,98) co-labeled with GFAP might robustly characterize remodeling and modifications in the number of astrocytes (23). Thus, when testing a therapy based on multipotent cells or any other therapy involving CNS, evaluating astrocyte response is a valuable approach.

In addition to the structural changes in the resident glial cells of the central nervous system described so far, the invasion of peripheral cells is also an important part of the neuroinflammatory response. The term immunological synapse was coined to designate a cell junction between a lymphocyte and an antigen-presenting cell (99). Microglia are the most important antigen-presenting cell in the brain, and genes involved in microglia and T cell interactions are upregulated in neurodegenerative disease (for review, see 100). Imaging this interaction is a challenge. In the periphery, the immunological synapse has been studied using super-resolution microscopy to allow single-molecule imaging (101). In the CNS, Flügel and colleagues studied autoimmune encephalomyelitis, an animal model for multiple sclerosis (102). The study used a retroviral vector system to combine fluorescently labeled nuclear factor of activated T cells (NFAT) with histone protein H2B. The combination of both sensors allowed imaging T cell activation in real-time. In intravital experiments using two-photon microscopy, the authors showed that contact with phagocytes from leptomeninges is important to activating T cells entering the CNS. They were also able to show contact of T cells with non-activated and activated microglia along with the progression of the disease. These double-fluorescent T cells could be used in other neuroinflammation models to allow real-time monitoring of the pattern of interaction between peripheral immune system cells and CNS cells.

In summary, astrocytes and microglia (and their structural changes) are key players when evaluating neuroinflammation (103). However, the interaction between brain cells and invading peripheral immune cells (104) is becoming an important topic of study to unveil the mechanisms involved in neuroinflammation. Moreover, most of the studies discussed in this review showed that the fundamental mechanisms, which served as the basis for the highly

advanced techniques currently used in the study of neuroinflammation, have been described using basic histological techniques, highlighting the importance of microscopy.

4. How to observe: the methodologies for visualizing neuroinflammation

Several pathways are activated during neuroinflammatory events (19,105) and numerous techniques were developed using specific dyes or antibodies against suitable targets (106,107). However, it is difficult to determine a specific marker to unambiguously detect a neuroinflammatory response is underway (23), and imaging technologies assist the identification and localization *in situ* of CNS cells.

4.1. Conventional light microscopy

The Golgi technique stains the plasma membrane of neurons using a reaction of silver nitrate and potassium bichromate (107), and modifications of the method of Golgi are still used today to describe the morphology of individual neurons (108–110) and generate information about changes in morphology under neuroinflammatory circumstances. In a model of TBI, Golgi staining was used to quantify the decrease in the size and number of general and apical ramifications of pyramidal neurons in the cerebral cortex during neuroinflammation induced by injury (111). The Golgi technique was also used to show dendritic spine changes in hippocampal neurons in radiation-induced neuroinflammation (112) and to assess cortical and hippocampal neurons under systemic LPS injection, where they showed decreased branching and dendritic spines compared to animals without inflammatory stimulus (113). A decrease in the density of dendritic spines was also found in the medial posterodorsal amygdala in animals subjected to prolonged stress (114). Using a model of influenza infection, a modified Golgi staining showed a decrease in the density of dendritic spines and the branching of specific neuronal subpopulations in the hippocampus after infection (115). The density of dendritic spines was also negatively influenced in a transgenic animal model of AD, and treatment with valproic acid partially ameliorated it, demonstrated by modified Golgi staining (116). The Golgi technique remains a widely used

method for analyzing changes in dendritic spines, in part because of the simplicity of the technique and equipment involved.

Despite the ease of use and wide applications of histological stains, their specificity is limited. The ability to target a protein of interest with a labelled antibody allowed the development of new techniques such as IHC and IF and opened up the neuroscience field (117). The main techniques used for imaging neuroinflammation will be elucidated below, from immunohistochemical to *in vivo* approaches (**Table 2** describes the advantages and limitations of basic types of preparation used to view specimens with a light microscope).

4.2. Immunohistochemical techniques

The application of IHC in neuroscience as a tool to assist in the localization and identification of proteins, has remodeled and reorganized the clinical diagnosis as well as day-to-day bench techniques (118). Antibodies provide an extensive map of the CNS by pinpointing antigens to specific locations and subpopulations of cells in the brain. In the context of neuroinflammation, immunohistochemical analysis can be very helpful to indicate the exact location of antigens and suggest cell-to-cell communication in a specific disease or condition, elucidating the mechanisms involved in a unique way (118). This technique enables the detection of a protein of interest using a specific antibody against the protein's epitopes. In IHC, the antibody (usually a secondary antibody) is conjugated to an enzyme whose product forms a precipitate which can be visualized using conventional microscopy (119). Co-labelling with multiple markers, for example the commonly used Iba-1 and GFAP described above, can provide greater information than each marker alone but studying the intensity of immunoreaction, cell counting, and morphological analysis of the cell (15,120–124). As for astrocytes, IHC labelling of GFAP can reveal an increase in the length of cell processes in neuroinflammatory contexts such as animals subjected to a high-fat diet (119), alcohol-induced neuroinflammation (125), and hypoxia (121).

Microglia cells acquire an amoeboid shape and proliferate during neuroinflammation. In a study using avidin-biotin-peroxidase complex to visualize microglia, the count of Iba-1+ cells and the area occupied by microglial cell body relative to the total area occupied were

used as indices of microglial activation in a model of postsurgical neuroinflammation (126). In a hypoxia model, several parameters such as the size of the cell soma and branches were used to indicate microglia activation (127). A proliferation marker like BrdU can also be used to assess microglial proliferation during neuroinflammation (120).

Sholl analysis (128) is another useful tool to analyze morphological alterations after neuroinflammation. This analysis is applied to astrocytes (129,130), microglia (121,131–133) and also, neurons (111,115). Sholl analysis consists of applying a series of concentric circles on marked cells, allowing quantification of some features of cell processes in a systematic, simple, and relatively inexpensive way. After concentric circles are applied, two central and two lateral quadrants can be delimited (**Figure 2**). Parameters such as the number of intersections of processes in each quadrant, the number of primary processes leaving the soma, and the largest branch of each quadrant might provide useful insights indicative of cell activation during neuroinflammation (130,131). Sholl circles have been used to elucidate astrocyte activation in different contexts of neuroinflammation, such as neuroinflammatory activation caused by intermittent exposure to alcohol (89) or neonatal excitotoxicity (134). The same goes for microglia, whose neuroinflammatory activation can be studied under classical stimuli such as systemic LPS administration (135) or in hypoxia (121). In a model of TBI, Sholl circle analysis evidenced microglia with a more ameboid/reactive morphology that was reverted after neutralizing IL-1β (136). Last but not least, Sholl circles also provide a tool for neuronal morphology quantification, evidencing a decreased number of neuronal branch intersections after TBI (111) and altered dendritic tree in hippocampal neurons after influenza infection in mice (115).

4.3. Fluorescence techniques

IF is an alternative to the enzymatic system used in IHC, taking advantage of primary or secondary antibodies conjugated to a fluorochrome, excluding the need for a period of incubation with an enzyme substrate necessary to produce color in IHC. The broad range of fluorophores currently available allows the use of multiple fluorophores excited by different wavelengths and therefore the detection of multiple targets simultaneously. Some of the

most used fluorophores in histology are fluorescein isothiocyanate - FITC (green/yellow), TRITC (red), rhodamine (red), Texas red (red), cyanine - Cy2 (green), indocarbocyanine - Cy3 (red), and aminomethyl coumarin – AMCA (blue) (118). Red and green fluorescent dyes are broadly applied due to their sensitivity and fast responses in optical imaging systems (137). In this sense, fluorescein and rhodamine can be broadly used to evaluate cells, tissues, and whole animals (138), considering their excellent optical properties due to the long excitation wavelength and high fluorescence quantum yield (137). Usually, the cell DNA is also stained with a fluorescent substance such as DAPI (4′,6-diamidino-2-phenylindole) (139), allowing nuclear visualization to indicate that fluorescent emission comes from a real cell, not from an artifact.

Iba-1 and GFAP are targets widely used in IF to assess the spectrum of activation and morphology of astrocytes and microglia under neuroinflammatory settings such as astrocytes subjected to oxygen and glucose deprivation *in vitro* (140), Parkinson's disease model (141), EAE model (142) and, AD model (143). The areas covered by Iba-1+ and GFAP+ cell fluorescence were determined under immunological activation through systemic LPS administration (144) and in a mouse model of tauopathy (145) and used as a signal indicative of astrogliosis in both contexts. Besides quantifying immunoreactivity through the intensity of the signal, the number of Iba-1+ cells (relative to the total number of cells in the area) has been used to quantify microglial proliferation after a neuroinflammatory stimulus (146). To accurately assess microglial proliferation in the face of neuroinflammatory stimuli, markers such as Iba-1 and DAPI can be colocalized with BrdU, a proliferation marker (120). Stolz and colleagues used a computer-aided method to perform an unbiased and automatic analysis of astrocytes and microglia in different models of EAE. They programmed the analysis of several parameters, including particle density and mean intensity, and found differences in microglia and astrocyte activation in different models of EAE (147).

Quantifying the fluorescent co-labeling of GFAP+ and C3+ (complement component 3), used as a marker of astrocytic activation, astrocytes in the spinal cord and the motor cortex of patients with MS were positively labelled, indicating the occurrence of astrogliosis (73). IF can also be useful to analyze targets involved with neuroinflammation within cells,

for example, double-marking Iba-1+ or GFAP+ and an enzyme producer of inflammatory mediators such as cyclooxygenase 2 (COX-2) (125). Fluorescence-coupled antibodies can be used to mark specific proteins of certain models and diseases involved in neuroinflammation, such as amyloid-beta (146) and alpha-synuclein (148). Also, labeling specific proteins from neurons such as vesicular glutamate transporter-1 (vGLUT-1) and Homer-1 to identify excitatory neurons and glutamic acid decarboxylase 65/67 (Gad65/67) and Gephyrin to identify inhibitory neurons, Jafari and colleagues demonstrated a preferential synaptic loss in excitatory neurons in a model of MS (149).

The fluorescence technique also has its limitations, such as fading of fluorophores, cross-reaction, as well as autofluorescence of some biological materials that could produce false-positive results. Even so, the technique allows several possibilities of use and analysis and must be considered when planning a study on neuroinflammation.

Some of the issues of immunolabelling, such as the fluorophore permeability and cross-reaction can be overcome by using transgenic animals expressing fluorescent reporter genes. Briefly, reporter genes expressing fluorescent proteins are inserted into the DNA chain under the control of the promoter of the gene of interest. When the gene of interest is expressed, both protein of interest and fluorescent protein are produced. Thus, the fluorescent signal enables real-time imaging of the dynamics involving the expression and distribution of a certain cell protein (150).

The first fluorescent reporter protein used was GFP, which was also used primarily in the CNS to differentiate astrocytes (expressing GFP under the control of the GFAP promoter) from other cells (151). Mutations in the GFP gene created other reporter genes with different fluorescent wavelengths: BlueFP (BFP), CyanFP (CFP), and YellowFP (YFP). One of the advantages of this method is the ability to visualize proteins without the use of synthetic compounds or labeled antibodies (152). Reporter genes for fluorescent proteins from other sources have also been characterized, such as AmCyan1, DsRed1, AsRed2, HcRed1, increasing the diversity of analysis and targeting possibilities (153).

Transgenic animals expressing fluorescent proteins are a helpful tool to clarify the role of molecular pathways, communication, and interplay between different CNS cells

related to neuroinflammation. Double transgenic mice can be used to express fluorescent proteins in more than one cell group, for example, neurons expressing YFP and microglia expressing GFP could elucidate microglial responses after laser-evoked spinal cord injuries (154).

Alterations in astrocyte gap junctions were demonstrated in GFAP-GFP animals with an induced abscess, leading to a pronounced inflammatory response. In this study, a group of astrocytes was specifically stained either with sulforhodamine 101 (SR101) acid chloride or double-labeled with GFAP-GFP+ and SR101+, whereas another expressed only one of the markers, describing the astrocytic heterogeneity. Also, a difference in the prevalence of these cells populations was found between control and abscess-induced animals (155). Triple transgenic amyloid precursor protein, presenilin 1 and Nestin-GFP (APP/PS1/Nestin-GFP) mice were used to elucidate hippocampal neurogenesis in a model of AD. Nestin-GFP was used to assess neuronal progenitors in different regions of the hippocampus. A decrease in the number of Nestin-GFP+ cells in the dentate gyrus of AD animals was observed, indicating reduced hippocampal neurogenesis (156). This decrease in Nestin-GFP+ cells was less pronounced in animals treated with valproic acid (116).

Nuclear receptor subfamily 4 group A member 1 (Nr4a1)-GFP transgenic mice were used to elucidate a fundamental role of the Nr4a1 transcription factor in the development of EAE*: intravital* microscopy showed an increase of fluorescence in the spinal cord after the transference of myelin-specific activated lymphocytes (157). In a model of MS, CX3CR-1-GFP and Chemokine (C-C Motif) receptor 2 (CCR2)-RedFP transgenic animals were used to visualize microglia and invading macrophages, respectively. Pre-synaptic (Synapsin-1) and post-synaptic (Homer1 and PSD-95 - postsynaptic density protein 95) proteins and lysosomes (Lysosomal-associated membrane protein 1 - LAMP-1) were also marked to allow the colocalization with microglia and/or macrophages. Results showed that both microglia and infiltrated macrophages participate in the synaptic engulfment occurring in the cortical layers in this model of MS (149).

4.4. The benefits of two-photon microscopy

In vivo studies are important to understand neuroinflammation since most responses are transient and dynamic. Thus, recent methods and techniques used to study neuroinflammation include real-time analysis of living samples or animals. Live cell imaging provides more than a snapshot of the cell state, it allows for the observation of specific processes as they occur over time. The essential requirement to successfully conduct this type of analysis is to have live and healthy specimens. Microscopes are usually inverted and enclosed in a microcell incubator to keep cells alive **(Figure 3)**. However, this process may cause cellular damage when applied to living cells (158) since it requires high-intensity light exposure. Two-photon microscopy may address this problem – allowing more exposure time with less damage (158). Two-photon even allows the acquisition of intravital imaging, one of the most powerful tools for tracking processes *in vivo* and in real-time. Intravital imaging allows the visualization of cellular responses in living organisms (159,160).

Two-photon microscopy is a technique in which two photons, with longer wavelength than the emitted light, cooperate to simultaneously excite a fluorophore. In contrast, in traditional fluorescence microscopy, the excitation wavelength is shorter than the emitted one. Using two-photon, the light detection is more efficient, the tissue penetration is deeper, and the photobleaching and sample damage is reduced **(Figure 4)**.

Two-photon microscopy is particularly helpful for thick specimens. It appears to be one of the most appropriate techniques to describe inflammatory mechanisms in different models of CNS diseases in animals (154,158,160–162). The highly localized excitation of the two-photon absorption process and the reduced light spreading in the sample reduce tissue damage, and two-photon is a suitable tool when working with living samples (live cells and intravital imaging in small animals) (154,160,162,163). Two-photon studies show that microglial cells use their processes to continually scan the brain parenchyma as a mechanism of surveillance of the microenvironment (10,164). It is also a useful tool to elucidate mechanistic aspects of neuroinflammatory diseases: in a study by Jafari and collaborators (149), calcium waves were visualized chronologically in apical dendritic spines as a signal for synaptic pruning. The authors also demonstrated that the synaptic loss in MS

is mostly mediated by microglia and invading macrophages, suggesting the important role of these cells in this condition.

Two-photon microscopy was also key in the *in vivo* study that described the brain waste clearance system now known as “glymphatic system” (44), reported earlier in this review, and which has a potentially large impact on future neuroinflammation studies.

4.5. Omics and spatial information

An important limitation of the imaging techniques described so far is the small number of targets visualized in cells simultaneously (165). On the other hand, advances in bioinformatics and system biology have contributed to the "omics" era – enabling the identification of thousands of targets simultaneously in a single cell, generating data regarding their molecular signatures.

Omics analysis – including genomics, transcriptomics, metabolomics, and proteomics – have emerged and integrated different knowledge areas to identify genes, mRNA, metabolites, and proteins, respectively, in a biological sample. Omics analyses are extremely important for the understanding of gene expression, single-cell evaluations, and activated signaling pathways. However, the techniques used in omics analysis – such as next-generation sequencing, microarray, RNAseq, mass spectrometry, and nuclear magnetic resonance spectroscopy – require the cells to be removed from the original tissue or site, losing crucial information related to the spatial distribution and heterogeneity of cells (166,167).

To overcome this barrier, *in situ* fluorescent techniques such as sequential fluorescence *in situ* hybridization (seqFISH), multiplexed error robust FISH (MERFISH), and codetection by indexing (CODEX) have been adapted to enable the integration of microscopy to the era of omics data **(Figure 5)**.

The technique seqFISH uses fluorescence probes complementary to the RNA sequences but with several rounds of hybridization and probe removal, creating a temporal barcode for each transcript, which is subsequently decoded using computer software, allowing for the identification of multiples targets in a single cell in its native tissue (168,169).

Although in seqFISH the number of targets can grow exponentially according to the hybridization rounds, it can also significantly increase the chances of error (170). MERFISH is also an imaging method capable of simultaneously measuring the number of copies and the spatial distribution of hundreds to thousands of RNA species in single cells, the main difference being a system to detect and/or correct errors, ensuring a higher efficiency (170).

In addition to transcript identification, other techniques using antibodies against proteins of interest are also part of the new era of microscopy. In CODEX, tissue sections are subjected to a mixture of antibodies in a single round. However, only a few of these antibodies are imaged in each round of image capture, as DNA probes are turned on and off from the antibodies in several rounds until a barcode is established, providing a multiplex cytometry technique (171,172).

In the brain, seqFISH may avoid some of the previously mentioned 'omics era' challenges since it accurately captures (through a confocal microscope) the mRNAs for 10,000 genes in single cells with a lower diffraction-limit resolution (169). Other spatial transcriptomics combines the CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing) (173) data with ultra-high multiplex immunohistochemistry, obtained with CODEX (171,172).

MERFISH, SeqFISH, CODEX are raising many possibilities to be explored in the field of imaging neuroinflammation by assessing spatial and localized information, combining the pioneering approach (histology) with omics. The high amount of data generated from these applications will considerably improve the comprehension of the molecular mechanisms involved in neuroinflammation, although the high cost and the system knowledge required are significant limitations to the broader employment of these techniques.

Considering the different phenotypes the same cell type could adopt, and the demonstrated heterogeneity (i.e., astrocytes and microglia), it may be important to evaluate the spatial information regarding neuroinflammatory genes, metabolites, and proteins in several CNS pathological conditions. For that reason, future studies should explore the fusion of microscopy and omics.

5. Human neuroinflammatory studies: still a long way to go

Up to this point, the vast majority of neuroinflammation findings were described in useful animal models (for details, see **Table 1**), as well as live cell and real-time assessments that allowed the better comprehension of specific inflammatory mechanisms (75,120,174). In humans, immunohistochemistry has been applied to identify microglia polarization and phenotype in postmortem brain tissue following deep subcortical lesions linked to cognitive impairments and depression (175), drug-abuse-related neuroinflammation (123), the association of amyloid plaques and neurofibrillary tangles in AD (98), and to describe the existence of the P2Y12 receptor (176). Microscopy was the basis of neuroinflammatory-related descriptions, and even the postmortem human brain studies made it clear that a combination of different microglial markers is required for phenotype identification (175).

However, beyond postmortem studies, approaches to imaging neuroinflammation in humans– such as MRI – are limited by their spatial resolution, although the 18 kDa translocator protein (TSPO) binding evaluated by positron emission tomography (PET) has been considered an *in vivo* marker of neuroinflammation at the cellular level (177). Notwithstanding that, it is still a challenge to identify real-time inflammatory processes in human patients due to the invasive aspect of existing procedures.

The pinpointing of neuroinflammatory features in humans would significantly improve the prognosis, diagnosis, and possible further treatments for CNS neuroinflammatory conditions.

6. Concluding remarks

This review summarizes the current and developing approaches for better understanding neuroinflammation using microscopy. It is clear that even with a conventional light microscope, it is possible to study many aspects of neuroinflammation. Nonetheless, it seems that two-photon and *in vivo* approaches emerge as some of the most appropriate techniques to evaluate neuroinflammation in real-time. Regardless, we still have a long way

to go to untangle the details of neuroinflammation in different CNS conditions and diseases, mainly when considering clinical assessments at the cellular level.

The emergence of *'omics'* technologies has enhanced the understanding of the diagnosis and mechanisms of certain neurological diseases and conditions. Nevertheless, microscopy techniques are still needed to provide spatial and temporal localization, in addition to allowing *in vivo* and real-time studies.

As omics and microscopy advance together and the production of more data is possible, more details of this complex picture called neuroinflammation will be unraveled.

7. Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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10. Author contributions

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Figure captions

Figure 1. Neuroinflammation is characterized by several cellular and molecular processes, leading to different cell phenotypes, mainly in astrocytes and microglia. These neuroinflammatory features (production and release of pro-inflammatory mediators and BBB leakage) can drive morphological changes in glial cells, which can be assessed through histological techniques with different markers (see the main text for details). BBB: blood-brain barrier.

Figure 2. Simplified illustration of Sholl analysis of cell arborization. (A) In this technique, the cell can be imaged using immunofluorescence or immunohistochemistry. (B) Concentric circles are drawn over the cell, with the body at the center. (C) Two diagonal lines can be drawn to establish two lateral quadrants and two central quadrants. (D) Analysed parameters can include: number of branches starting from the soma; number of times branches cross the circles in each quadrant; the length of the longest branch. Parameters can be reported in total or by quadrant (128,130,131).

Figure 3. *In vivo* microscopy can be performed in cell cultures or tissue slices. The microscope is coupled to an incubation system that maintains controlled conditions such as temperature, atmospheric gases (O_2, CO_2), pH, and osmolarity, to keep cells/tissue alive for as long as possible.

Figure 4. Two-photon microscopy versus one-photon microscopy. Two-photon microscopy uses the simultaneous excitation of a fluorophore by two photons. The excitation wavelength is longer than the emission wavelength. Longer wavelengths are lower energy, reducing sample damage caused by phototoxicity. In traditional one-photon fluorescence microscopy shorter excitation wavelengths are used (for example, ultraviolet light), reducing cell/tissue viability.

Figure 5. Omics analyses are a range of approaches that examine the totality or a broad selection of features in a biological sample, for example genomics, transcriptomics, metabolomics, and proteomics, identifying respectively, genes, mRNA, metabolites, and proteins. However, omics analysis uses techniques which requires the cells to be removed from the original tissue or site, losing crucial information related to the spatial distribution and heterogeneity of cells. The fusion of microscopy and omics allows the analysis of a large amount of data without losing spatial information, which is extremely important in the study of neuroinflammation.

Table 1. Common targets and related outcomes regarding neuroinflammation markers

Target	Description	Comments	Histological-evaluated responses in different neurological conditions
Damage to the Blood-Brain Barrier (BBB)			
CLDN	Claudin, a transmembrane protein family component of the tight junctions at the BBB	In neuroinflammatory contexts, a BBB impairment may occur, weakening tight junctions and allowing peripheral molecules and cells to invade the CNS. In this sense, evaluating such proteins can be useful to relate its findings with other BBB integrity assays (39,42,178–180).	CLD5 remodeling and changes in localization after stroke (39); ↑ microglia-associated CLDN5 in a model of SLE and after LPS stimuli (42); ↓ CLDN5 in a model of EAE (179); ↓ CLDN3 in MS (180).
OCLN	Occludin, together with CLDN, is one of the main protein families that are part of the tight junctions of the BBB		↓ in stroke (39); ↓ in models of inhaled cigarettes and e-cigarette (178).
ZO	Zonula occludens, a family of intracellular proteins that provides anchoring sites for the tight junctions of the BBB		↓ in stroke (39).

Table 1. continuation...

Microglia			
Iba-1	Ionized calcium-binding adaptor molecule 1	Expressed in myeloid cells. In the CNS is highly and selectively expressed by microglia and widely used to visualize the morphological spectrum between the classic M1 (amoeboid) and M2 (ramified) states. Also used to quantify soma size, perimeter, and to distinguish these two phenotypes (usually associated with another marker e.g., CD68) (54,103,144,175,181–186).	↑ 7 days after global ischemia (181); ↑ 24h after LPS challenge (144); ↑ 24h after HI (182); ↑ 3 days after TBI (54); ↑ after LPS challenge (183); ↓ in the white matter in DSCL (175).
CX3CR1	Fractalkine receptor or G-protein coupled receptor 13 (GPR13)	A chemokine receptor involved in leukocyte adhesion and transmigration (59,187).	↑ in the brainstem in hypertensive rats (59).
P2RY12	Purinergic receptor or G-protein coupled receptor P2Y	A purinergic receptor used as an indicator of ramified microglia (used as a marker of microglial branches) (188).	Microglial P2RY12 deficiency ↓ neurotoxicity after OGD (188).
OX-42	Cluster of differentiation 11b (CD11b), a surface protein from the integrin superfamily	Expressed in myeloid cells (↑ in neutrophils and monocytes in the presence of pathogens). In the brain, it is	↑ 8h after reperfusion in ischemic stroke (131).

		a specific marker of microglia. Due to its rapid shift, CD11b could be considered an early marker of infection. It was blocked in experiments to evaluate microglial phagocytosis of newborn astrocytes (131,189,190).	

Table 1. continuation...

CD45	Cluster of differentiation 45	Surface tyrosine phosphatase protein, a protein expressed in the membrane of hematopoietic cells. It is highly expressed in CNS-associated macrophages, and expressed in low levels in microglia, allowing to differentiate macrophages from microglia. Expression levels may also indicate amoeboid or more branched morphology. CD45 is increased in disease-related conditions (64,191–195).	↑ around cortical plate lesions in a model of neonatal excitotoxic brain damage (191); ↑ after acute brain injury (192).
CD68	Cluster of differentiation 68	Transmembrane glycoprotein expressed in monocytes and in macrophages with phagocytic activity, usually suggesting microglia with ameboid morphology (M1 phenotype) (54,175,196).	↑ after TBI (54); Involved in white matter damage (175); ↑ in morphologically activated microglia after cranial irradiation (196).
CD86	Cluster of differentiation 86	Microglia/ameboid morphology marker, a transmembrane glycoprotein constitutively expressed in immune cells. Usually, double staining for CD86 and Iba-1 is used to identify microglial	↑ in microglia after subarachnoid hemorrhage (197); ↑ after acute hypoxia (90); ↑ in a model of sepsis-associated encephalopathy (198).

		phenotype (90,197,198).	
CD206	Cluster of differentiation 206	Mannose receptor. Its expression provides anti-inflammatory and immunosuppressive responses of microglia, suggesting M2 phenotype polarization (62,90,197,199–201).	↑ after ischemia (62); Apparently, not expressed in acute sepsis (201); ↑ in $TLR4^{-/-}$ mice after subarachnoid hemorrhage (197); ↓ after acute hypoxia (90).
Arg1	Arginase 1	Alternative phenotype marker of microglia, which expression might be upregulated in the alternative activation phenotype (184).	↓ after ischemic stroke (184).

Table 1. continuation...

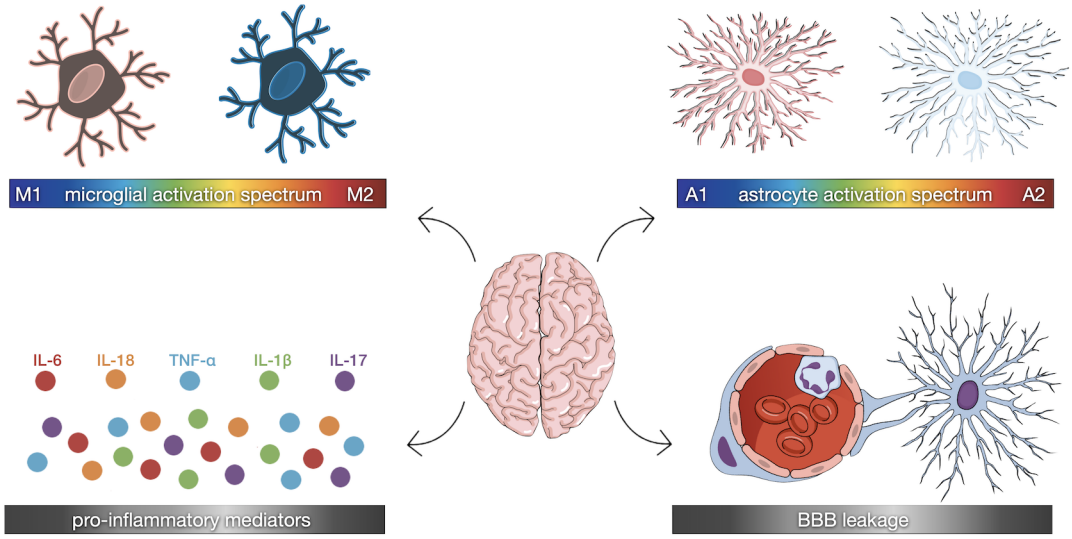
Astrocytes			
GFAP	Glial fibrillary acidic protein	Intermediate filament protein of astrocytes. The cytoskeleton of reactive astrocytes may undergo hypertrophy and other structural changes, affecting GFAP expression (54,119,202,203).	↑ in obese rats after an HFD diet (119); ↑ expression in TBI and contusion trauma (202); ↑ after OGD (203).
S100B	Calcium-binding protein	Primarily produced by astrocytes and mostly expressed in the cytoplasm of these cells, but oligodendrocytes also express S100B. This protein is involved in calcium homeostasis, and since glial cells are sensitive to CNS disturbances, S100B is usually regarded as an indirect biomarker of neural injury (103,204–207).	↑ in a model of stroke-prone spontaneously hypertensive rats (204); ↓ in a model of diabetic ketoacidosis brain injury (205); ↑ in an *in vitro* model of MS (206).
Cytokines			
IL-1β	Pro-inflammatory cytokine	Inflammatory mediators usually produced by glial cells in neuroinflammatory events	↑ in hypertension (59); ↑ in MS (208).

IL-6	Usually considered as a pro-inflammatory cytokine, but may present pro or anti-inflammatory roles depending on the context	(59,65,162,208,209).	↑ in hypertension (59); ↑ in AD senile plaques (209); ↑ after stroke (162).
TNF-α	Pro-inflammatory cytokine		↑ in cerebral ischemia (65); ↑ after stroke (162).

AD: Alzheimer's disease; Arg1: Arginase 1; BBB: Blood-brain barrier; CD: Cluster of differentiation; CLDN: Claudin; CNS: Central nervous system; CX3CR1: CX3C chemokine receptor 1; DSCL: deep subcortical lesions; EAE: Experimental Autoimmune Encephalopathy; GFAP: Glial fibrillary acidic protein; HFD: High-fat diet; HI: Hypoxia-ischemia; Iba-1: Ionized calcium-binding adapter molecule 1; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; KO: Knockout; LPS: Lipopolysaccharide; MS: Multiple Sclerosis; OCLN: Occludin; OGD: Oxygen-glucose deprivation; OX-42: Anti-Integrin αM, CD11b antibody; P2RY12: Purinergic Receptor P2Y, G-protein-coupled receptor 12; S100B: S100 calcium-binding protein B; SLE: systemic lupus erythematosus; TBI: Traumatic brain injury; TLR-4: Toll-like receptor 4; TNF-α: Tumor necrosis factor-alpha; ZO: Zonula Occludens.

Table 2. Main types of specimen's preparation used for imaging neuroinflammation

	Fixed tissue	Live cell	Intravital
Advantages	Relatively low-cost and low complexity technique. It allows the visualization of the spatial localization and general morphology of labeled targets (118).	Over time evaluation of the cell responses with time-lapse. It can also be combined with fluorescence techniques. Some microscopes allow to track cell proteins dynamic, as well as their responses in time and space (210).	Evaluation of living cells in the body over time. Real-time evaluation of physiological and pathological responses in the live animal. Some microscope adaptations should be performed to keep the animal at an adequate temperature, increasing the time for image acquisition (159,211).
Limitations	It is not possible to observe the live cells in action, neither time-dependent responses in real-time. Possibility of alterations in the structure of the tissue during the preparation of the specimens (118).	Expensive and complex process that usually requires trained professional and highly specialized equipment for image acquisition. Microscopes need a set-up with controlled atmosphere/medium and temperature to keep cells alive (174,210).	Impaired resolution due to tissue motion and motion of artifacts in small processes of individual cells. Depending on the specific approach, it can also be expensive (211).



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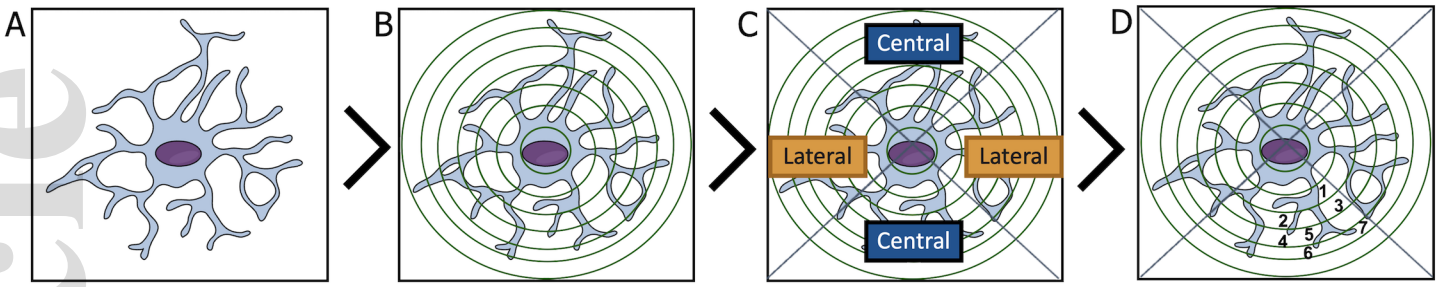

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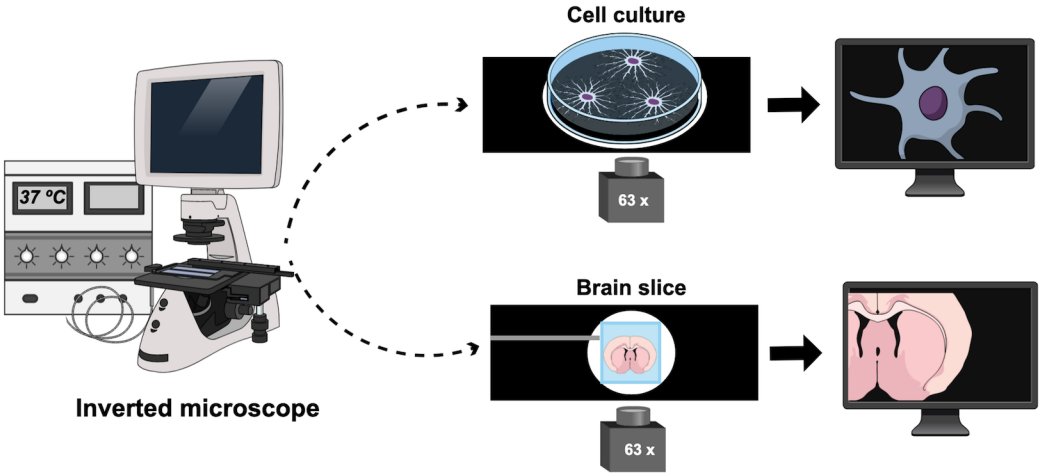

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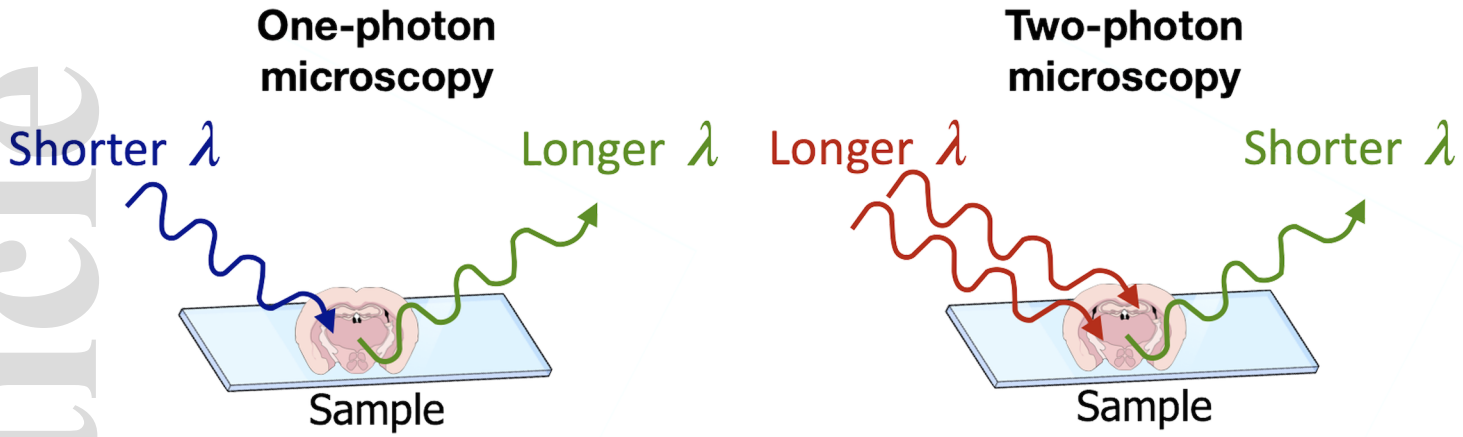

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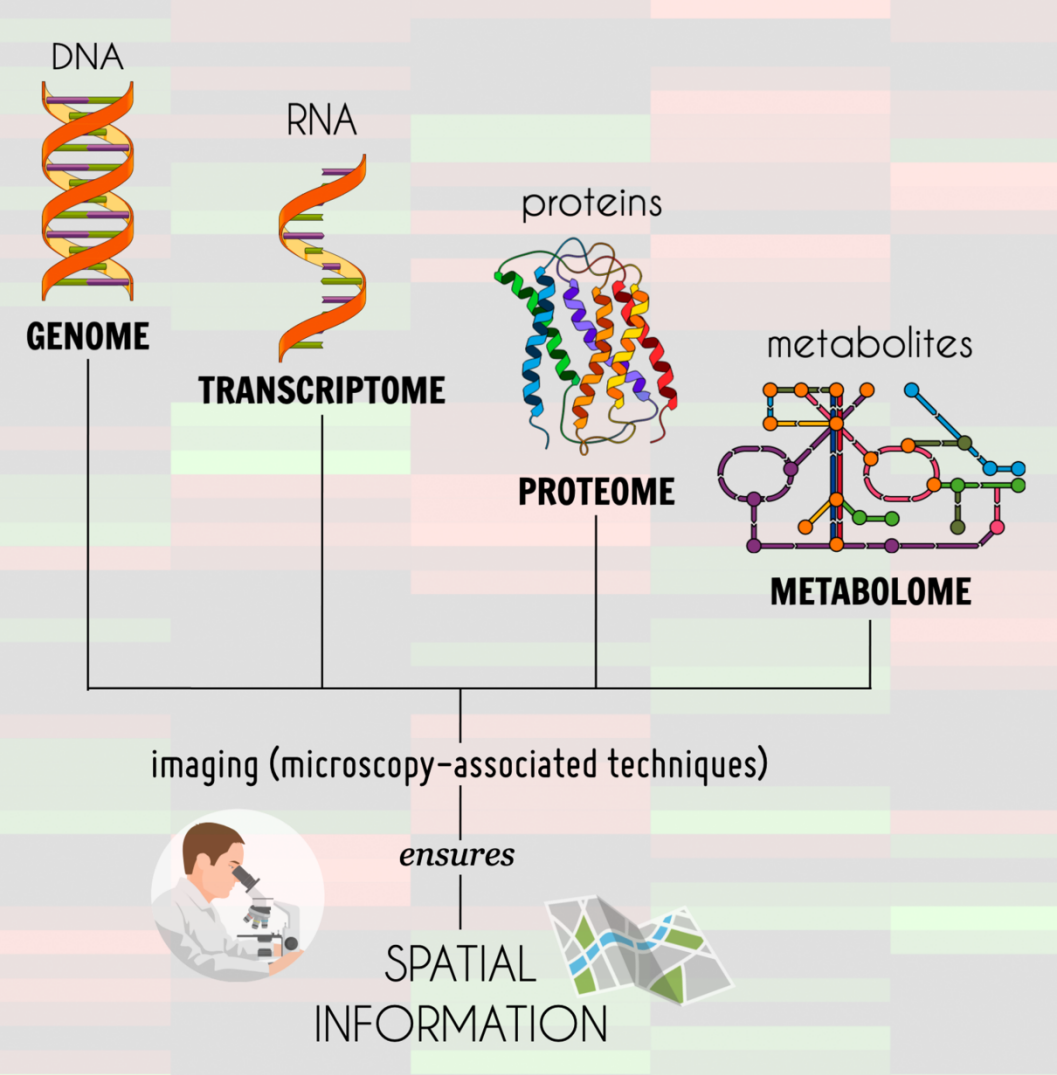

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