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Novel imaging tools for investigating the role of immune signalling in the brain.

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Abstract

The importance of neuro-immune interactions in both physiological and pathophysiological states cannot be understated. As our appreciation for the neuroimmune nature of the brain and spinal cord grows, so does our need to extend the spatial and temporal resolution of our molecular analysis techniques. Current imaging technologies applied to investigate the actions of the neuroimmune system in both health and disease states have been adapted from the fields of immunology and neuroscience and are inherently limited by their semi-quantitative nature, loss of spatial resolution, photobleaching and detection sensitivity. Thus, the development of innovative methods which overcome these limitations are crucial for imaging and quantifying acute and chronic neuroimmune responses. Therefore, this review aims to convey novel and complementary imaging technologies in a form accessible to medical scientists engaging in neuroimmune research.

Introduction

What is Neuroinflammation?

The neuroimmune system is an incredibly diverse and intricate system comprised of endogenous (astrocytes, microglia, neurons and oligodendrocytes) and exogenous (infiltrating monocytes and T cells) immune-functioning cells and their associated signalling factors within the central nervous system (CNS) (table 1) (Hutchinson et al. 2011). These immunocompetent cells are pivotal to both the homeostatic and disease states of the brain and spinal cord (Grace et al. 2014; Ousman & Kubes 2012). For example, following injury or infection, glial and immune cells act to enhance the elimination of pathogens as well as facilitate repair via processes ranging from central immune signalling and neurokine events, to gross "neuroinflammation".

Table 1 Cellular targets historically employed to image, detect and "quantify" neuroinflammation

Neuroinflammation occurs along a graded continuum with each insult displaying its own unique neuroinflammatory signature. While acute neuropathological insults such as ischemia or infection and chronic neurogenically mediated disorders such as hypertension, have robust neuroinflammatory responses, sub-inflammatory neuroimmune responses are now also recognized to be associated with various psychiatric disorders (Hutchinson & Watkins 2014; Najjar et al. 2013; Wu et al. 2012). These sub-inflammatory responses do not function in a traditional inflammatory manner. Rather, this response more closely resembles the discrete and localised nature of neurotransmission and hence requires a separate terminology of neurokine or central immune signalling (Jacobsen et al. 2014; Hutchinson et al. 2011). Consequently, neuroinflammation has profound effects on both human and animal behaviour.

For example, the high levels of inflammatory mediators observed during pathological neurodegenerative disease states such as Alzheimer's and Parkinson's disease cause neuronal cell death, which in turn directly effects an individual's memory, language, and mood (McGeer & McGeer 2010; Rogers et al. 2007). In contrast, although the lower level of neurokine and central immune signalling observed during psychiatric disorders such as depression do not appear to result in the gross loss of cells, instead they subtly alter neuronal function and in turn behaviour (Eyre & Baune 2012). Table 2 highlights the main inflammatory, neurokine and central immune signalling mediators implicated in the neuroimmune system.

Table 2 Molecular targets historically employed to image, detect and "quantify" neuroinflammation.

Current limitations of viewing and "quantifying" neuroinflammation

The most recent technological advances to discern the structure and function of CNS cells have focused predominantly on neurons. For example, significant methodological improvements to viral tracing, development of fluorescent reporter transgenic animals and optogenetics measurement techniques have drastically increased our understanding of neuronanatomy and have identified many specific functional subgroups of CNS cells. However, most of these techniques are unsuitable for imaging neuroimmune cells. Existing techniques are unable to address the most pressing issues within the field of neuroimmunology such as, understanding the complex organization of multicellular systems, the heterogeneity within and between immune cells and how low abundant inflammatory proteins (neurokines) alter behaviour. Researchers in the field attempting to address these issues are left with traditional technological modalities that are fraught with difficulties. The limitations of antibodies is a particular problem for imaging central immune or neurokine

signalling due to the low expression levels of inflammatory mediators. This necessitates the use of high affinity antibodies with a strong signal and long life span in order to view and quantify these cellular responses.

Antibodies, which are the most common method to visualize antigens, are at the centre of the "reproducibility crisis" within biological science. Current estimates suggest approximately 50 per cent of antibodies are either unreactive towards the purported epitope or cross-react with others (Berglund et al. 2008). This problem is amplified by common chemical treatments on the collected tissues that are necessary for many immunohistochemical techniques. Specifically, the potential binding of antibodies is reduced by detergents that can denature proteins and fixatives - required to stabilize fragile tissues in order to survive the harsh staining conditions and subsequent washes, can inadvertently mask antigen-binding sites. Furthermore, the current fluorophores, such as fluorescein isothiocyanate (FITC), and Alexafluors, used to visualize antibody binding, have a limited lifespan and/or spatial resolution, quench in the presence of light and often emit in the same spectrum as the biological tissue making it difficult to differentiate between background fluorescence (autofluorescence) and the signal of the target protein (Figure 1).

Figure 1 The photobleaching effect of traditional fluorophores**.** Traditional fluorophores are readily photobleached during routine microscopy in brain samples (A). Alexfluor488 labelled activated neurons (c-FOS protein, green, black arrows) and Alexafluor555 labelled microglia (OX-42 protein, orange, white arrows) in rat hypothalamic sections following acute stress shown as maximum intensity projection images derived from epifluorescent widefield microscope obtained z-stacks. In a series of five sequential images significant photobleaching results in dramatic loss of detail in fluorescent antibody staining. (B) Zoomed image demonstrating the effects of Alexafluor photobleaching following five sequential images.

Bio-molecular imaging

Conventional labelling and imaging methods

Traditionally, the neuroimmune system has been studied by immunohistochemistry of key proteins and signalling molecules. In brief, immunohistochemistry (IHC; when using peroxidase reaction) or immunofluorescence (IF; when using fluorophores) enables visualization of target antigens within cells or tissue via antibodies labelled with peroxidases, biotin and/or fluorophores. This technique has been extensively used, and has furthered our understanding and appreciation of the topographical relationship between the different cell types and molecules within a defined region of tissue. IHC works well for thin tissue sections (5-50μm), as the light from the microscope remains relatively localized enabling discrete excitation of the fluorophore of interest. Imaging thicker sections of tissue (>50um to mm range), using conventional light microscopes and confocal scanning microscopes increases the scattering of light throughout the tissue, reducing the signal intensity and precise spatial localisation. To overcome this limitation, multiple slices of tissue are processed for IF and the images are digitally reconstructed, via image processing software, to generate a 3D image of the target. This process is time consuming, expensive and prone to artefacts due to difficulties in reconstructing images seamlessly.

Novel deep tissue imaging methods

Owing to the aforementioned constraints of IHC/IF, visualizing complex circuits from a systems perspective has historically been difficult. Many of the limitations, such as difficulty binding to and visualizing antigens, stem from the lipid membrane. The physical properties of the lipid bilayer prevents large macromolecules for example, antibodies, from readily diffusing deep into the tissue whilst the molecular heterogeneity of the lipids themselves alter the refractive index of the tissue, further perturbing the excitation and emission of light from fluorescently-labelled probes (Richardson & Lichtman 2015). To overcome these problems, "clearing" methods such as CLARITY, PARS, CUBIC, SeeDB, Sca*l*A2, iDISCO and

3DISCO have been developed (Susaki et al. 2015; Bin Yang et al. 2014; Renier et al. 2014; Tomer et al. 2014; Susaki et al. 2014; Ke et al. 2013; Becker et al. 2012; Hama et al. 2011). These methods make tissue optically transparent by removing the lipids and bringing the tissue to the same optical refractive index as the surrounding media. This enables light to penetrate deeper, facilitating the imaging of whole organs (Richardson & Lichtman 2015). Conventional IF protocols are subsequently applied to this tissue. However, for optimal staining the incubation time is significantly extended (hours and weeks in tissue slices and whole brains respectively) (Tomer et al. 2014) and the working distance of most microscope objectives limits the capture of whole organs. It is worth noting specialized lightsheet microscopes and CLARITY specific objectives can be employed to circumvent this limitation. However, these systems are expensive and consequently these may not be available to all researchers.

Novel imaging compounds

Nanoparticles

Nanoparticles are specialized inorganic molecules that can be tailored to specific sizes. These particles can be doped with inorganic elements (impurities) within their crystal lattices to generate fluorescent signals (Lu et al. 2015). Much research has been devoted into methods that allow the coating and functionalization of nanoparticles with biomolecules such as antibodies to then allow visualization using widefield light and scanning confocal microscopy or with fibre optics. The main benefits of nanoparticles over traditional organic dyes and fluorophores include their enhanced luminescence signal, reduced background noise, superior stability, excitation ranges in the near infrared (NIR), and the ability to use time-resolved imaging. Time-resolved microscopy allows the collection of the fluorescence signal after a designated time delay to account for the fluorescence decay time of the biological sample.

This removes the tissue autofluorescence from the signal of interest (Diamandis 1988). Interference from autofluorescence is a major issue in CNS tissues when imaging neuroinflammation due in part to high expression levels of endogenous amino acids and lipofuscins (Lakowicz 2006; Marmorstein et al. 2002).

Among the different categories of nanoparticles, nanodiamonds, nano-rubies and lanthide-based up conversion nanoparticles show great promise for future applications in in vitro microscopy (see table 3). Nanoparticles have wide applicability including *in vitro* microscopy, small animal imaging, multimodal imaging, high sensitivity bio-sensing and photodynamic therapy (PDT) (Mochalin et al. 2012; Chatterjee et al. 2008). Although there have been recent advancements facilitating functionalization, due to the complexity of the surface functionalization methodology, the use of antibody-conjugated nanoparticles has yet to be fully exploited for bio-imaging (please see Bradac et al. 2015; Lu et al. 2015; Edmonds & Sobhan 2013 for review)..

Table 3 Characteristics of key nanoparticles with specific utility for bioimaging.

An important next step in using these nanoparticles for bioimaging is refining the synthesis and surface modification strategies including the non-specific binding potential of the functional groups used for conjugation (mainly amino, carboxy and thiol; for details see Wang et al. 2011). By changing the overall charge of the particle, the functional groups can negatively impact colloidal stability and facilitate particle aggregation. Fortunately the resulting particle aggregation can be resolved by chemically engineering suitable functional groups, such as polyethylene glycol, for bio-conjugation (Wang et al. 2011). Although an active area of research, the use of nanoparticles bio-conjugated to proteins, peptides or polynucleotides that target biomarkers involved in neuroimmune signalling promises better

resolution of *in vivo* or in cleared CNS tissue. This will allow for greater imaging of low protein expression levels associated with central immune and neurokine signalling.

Complementary RNA based probes

RNA-based probes have contributed greatly to our understanding of RNA distribution throughout the brain. Visualization of RNA is achieved by hybridising complementary RNA sequences conjugated to fluorophores or other labelled molecules (RNA probes) to nucleic acids within tissue. There are a variety of molecular labels available for hybridisation including; hydrazine derivative of tetramethyl rhodamine isothiocyanate (TRITC); biotin-tagged nucleic acids; and digoxigenin (DIG). A major advantage of both DIG-based in situ hybridisation (ISH) and fluorescent in situ hybridisation (FISH) is the possibility of simultaneously investigating multiple neuroinflammatory molecules with multiplexed fluorescent RNA probes, proteins and/or fluorescent tracer molecules in the brain and spinal cord (Parker et al. 2013; Jeong et al. 2010).

Despite significant advances, as with IHC/IF, the current commercially available fluorescent conjugates used for FISH are not photostable for extended periods of time and can be difficult to differentiate from background autofluorescence in tissue samples. It is therefore essential to develop and utilise a new generation of highly sensitive, non-toxic and photostable nanoparticles for ISH-based imaging applications in brain and spinal cord tissues.

As mentioned earlier, nanodiamonds are impervious to environmental changes and achieve visible fluorescence by exploiting intrinsic defects and impurities that are deep in a crystal lattice independent of their surface structure (Hui et al. 2010). This makes them ideal for visualizing RNA in live cells or in fixed tissues as they prevent loss of information due to

tissue bleaching. Increased photostability is essential for improving neuroimaging capabilities of cleared brain tissues. Imaging whole organs require long exposure times owing to repeated z-stacking and are thus readily susceptible to bleaching with traditional fluorophore tagged probes or antibodies. Nanodiamonds are additionally non-toxic, emit beyond the range of cellular autofluorescence (with nitrogen or silicon vacancy centres) and have the ability to be utilised as efficient drug carriers (Merson et al. 2013) making them an ideal particle to incorporate into RNA based ISH techniques for whole organ imaging. Alternatively, the conjugation of improved fluorescent lanthanides such as the europium chelate BHHST (4,-4'-bis-(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl) sulfonylamino-propyl-ester-N-succinimide-ester-o-terphenyl) to RNA probes would also be advantageous compared to conventional fluorophores. Lanthanide chelates including BHHST have greater signal detection sensitivity compared to traditional organic dyes and importantly also have the ability to discriminate against high levels of cellular background fluorescence for more accurate imaging confidence when used in association with time resolved microscopy (Connally et al. 2004).

Novel imaging modalities

MALDI imaging

Mass spectrometry plays an important role in biomarker analysis due to its unique advantages, such as, high sensitivity, detection of a wide range of molecules, molecular specificity and multiplexed analysis on a single platform (Norris & Caprioli 2013a). Amid the various mass spectrometry ionisation techniques used to analyse tissues directly, Matrix Assisted Laser Desorption Ionization (MALDI) is the most widely used imaging mass spectrometry (IMS) method in biological and clinical applications (Aichler & Walch 2015).

Figure 2 MALDI imaging mass spectrometry. Schematic outline of a typical workflow for fresh frozen or formalin fixed formalin-fixed, paraffin-embedded tissue samples. The tissue section is mounted on a conductive (indium-tin oxide (ITO)) glass slide. Suitable matrix is applied across the tissue section and mass spectra are generated by a MALDI-time-of-flight (TOF) instrument. Ion (m/z value) densities of various molecules can be spatially displayed with their relative intensity in the tissue section. MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; m/z, mass to charge ratio.

Since its introduction in 1987 (Karas et al. 1987), MALDI-IMS has provided unique advantages in the analysis of tissue specimens. A broad spectrum of analytes ranging from proteins, peptides, glycans, lipids, small molecules, pharmaceutical compounds, endogenous and exogenous metabolites can be analysed *in situ* from tissue sections through this technique (Barry et al. 2015; Gustafsson et al. 2015; Patel et al. 2015; Quiason & Shahidi-Latham 2015; Uzbekova et al. 2015; Wang et al. 2015; Rebours et al. 2014; Touboul & Brunelle 2014; Zemski Berry et al. 2014). The data from such analysis is a pictograph that can be overlaid on the actual tissue sample; the location and intensity of the signal corresponds to the relative abundance of the analyte ions (Figure 2). Any tissue section could be used for such analysis, although typically these sections are covered with a suitable organic matrix material that co-crystalizes with the sample molecules. A laser beam irradiation ablates the matrix surface with the formation of charged molecular species (Karas & Krüger 2003). These resulting ions traverse through a time-of-flight (TOF) analyser, and their mass-to-charge (*m/z*) is determined. Using different matrices and technology, several analyte classes can be identified (Norris & Caprioli 2013b). Typically a raster-scanned image of a tissue section provides a spatial resolution ranging from approximately 200 μm down to 20 μm generating a mass spectrum for every individual measuring spot (Aichler & Walch 2015). Recent technological improvements have contributed to imaging phospholipids, neuropeptides, and drug

compounds at a pixel size between 5 and 10 μm (Aichler & Walch 2015; Anderson et al. 2014; Römpp & Spengler 2013; Römpp et al. 2011; Römpp et al. 2010).

A pronounced advantage offered by this technique is the subsequent analysis by other traditional histological technologies of the same tissue section, for example, by haematoxylin and eosin (H & E) staining or IHC. The mass signals (*m/z* values) detected from MALDI-IMS can be further visualised as ion intensity maps assigning molecular patterns to the tissue sections. The intensity of the unique *m/z* signals that correspond to the molecule of interest represents its distribution, localisation and relative abundance in the tissue section. This visualization is similar to IHC however, it allows multiple molecules to be examined with one measurement. The multiplexed mapping of relevant biomolecules such as cytokines and neurotransmitters, can provide valuable knowledge and significant insights into neurobiology. For example, IMS enables the spatial quantification of numerous relevant biomolecules in healthy and diseased states and thus can potentially identify novel disease biomarkers.

Raman spectroscopy

Optical spectroscopy techniques offer a number of unique opportunities for *in vitro* and *in vivo* sensing applications. By using light to interrogate a sample, and seeking to correlate a change in the observed optical signal to a biological phenomenon, optical methods allow minimally-invasive measurements to be performed on sensitive samples. Different sensing modalities have been utilized for measurements of biologically relevant parameters, such as fluorescence sensing (Tsiminis et al. 2014), resonant techniques such as surface plasmon resonance (Francois et al. 2011), Bragg gratings which can be utilized either for biological

sensing or physical parameters (Warren-Smith & Monro 2014), or alternative label-free methods such as Raman spectroscopy (Movasaghi et al. 2007).

Raman spectroscopy is particularly interesting for biomedical sensing, as it is a label-free technique that can uniquely identify chemical species present in a sample. Raman spectroscopy is a form of vibrational spectroscopy that uses excitation from a monochromatic laser light to interrogate samples by analysing the shift in energy of a small part of these excitation photons due to interactions between the excitation light and the vibrational modes of the chemical bonds in the molecule (Ferraro et al. 2002). Specifically, the laser light sets the bonds' vibrational modes in motion, producing light at certain optical frequencies; much like hitting a tuning fork with a small rod sets vibrations in motion with specific frequencies in the audible spectrum, Raman spectroscopy produces a vibrational spectral signature that can be used to determine the chemical composition and therefore the type of analyte present in a sample (Carey 1978). This is illustrated in in Figure 3 for the simple case of hydrogen peroxide (H_2O_2).

Figure 3 Simulated Raman spectrum of hydrogen peroxide (H2O2), showing the different peaks corresponding to the vibrational modes of the chemical bonds in the molecule.

Since this method does not require labelling of the targets before measuring it has the potential to allow for *in vivo* analysis of biological samples (Freudiger et al. 2008; Vo-Dinh et al. 2005) and has found applications in brain lipid identification (Krafft et al. 2005).

A historical limitation of Raman spectroscopy is the low signal intensities observed due to the weak nature of the interactions involved. This results in optical signals that are significantly lower than those observed from other optical detection techniques. Techniques

such as surface enhanced Raman spectroscopy (SERS) (Le Ru & Etchegoin 2009), or coherent anti-Stokes Raman spectroscopy (CARS) (Begley 1974) can mitigate this issue, resulting in renewed interest in Raman spectroscopy for sensitive detection in biology and medicine (Hanlon et al. 2000). CARS in particular has found widespread adoption when it comes to tissue imaging as it enables the mapping of specific chemical species in 2 or 3 dimensions in tissue samples (Evans & Xie 2008).

In addition to the measurements of biomarkers and chemical species on samples, Raman spectroscopy has also found extensive use for *in vivo* applications (Romeike et al. 2015). Typically, Raman spectroscopy is used to identify different chemicals, such as lipids (Nan et al. 2003), proteins (Uzunbajakava et al. 2003) and myelin (Wang et al. 2005), found in tissue. Changes in these parameters can be linked to histopathological observations, for example the identification of metastatic brain cancer (Fullwood et al. 2014). Raman spectroscopy and imaging has, through its ability to identify subtle changes in tissue composition, found applications in the fields of early-stage inflammatory apoptosis of adult hippocampal stem cells (Ladiwala et al. 2014), cancer identification in human brain tissue (Jermyn et al. 2015; Ji et al. 2013) and changes in blood vessels associated with heart disease (Matthaus et al. 2012).

Concluding remarks: future of 'neuroinflammation' imaging

Neuroinflammation is a dynamic and complex process involving many cell types and macromolecules. Current techniques employed to visualise these processes are insufficient due to major limitations in their temporal and spatial resolution. These issues are compounded by the inherent complexities associated with antibody use but can be aided by the utilisation of ISH probes for some targets. In this review, we have highlighted the

potential of novel bio-imaging molecules, for example, nanoparticles, which can be incorporated into existing methods to circumvent problems associated with traditional fluorophores. Given that nanoparticles do not photobleach and provide discreet signals, it is logical to apply these technologies to cleared tissue. By combining these two techniques we will be able to clearly view deep brain regions that will, undoubtedly, enhance our understanding of this elaborate system in diseased and non-diseased states.

Collaboration is key, and by adopting a transdisciplinary approach, technologies readily available to researchers in the proteomics and physics disciplines can be incorporated or adapted to develop label-free methods for elucidating sample composition. MALDI, for example, is a very powerful method for identifying the protein, lipid or small molecule composition of tissues or even whole organs. Importantly, this information can be retrieved and analysed with respect to spatial and potentially temporal resolution, something that is currently not possible with traditional imaging methods. Raman spectroscopy is another such example where the chemical composition of a sample can be identified without the need for exogenous labels. Such label-free technologies will prove to be very powerful for addressing major challenges in the imaging field, such as the imaging of neuroinflammation in real-time in live animals.

The technologies discussed in this review have the potential to enhance our understanding of the neuroimmune system and facilitate the advent of new diagnostic tests and potential treatment targets. This is particularly relevant for humans given that our current attempts to image neuroinflammation in real time is substantially hampered by our lack of "neuroinflammatory" markers. For example, there has been an over reliance of using translocator protein (TSPO) and the peripheral benzodiazepine receptor (PBR) as biomarkers

of neuroinflammation in PET scans. Unfortunately, the reproducibility and reliability of these markers is often questioned, thereby limiting our understanding and insight of human neuroinflammatory diseases (Stefaniak & O'Brien 2015). By using novel techniques such as Raman or MALDI, we aim to include functionally informative measurements that will translate to more accurate imaging and identify potential therapeutic targets of neuroinflammation-associated pathologies. The combination of established and emerging techniques across different scientific fields will no doubt aid in the prognosis and treatment outcomes of individuals suffering from neuroinflammatory-related diseases and disorders.

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Table 1

Table 1 Cellular targets historically employed to image, detect and "quantify" neuroinflammation

Cell type	*Phenotypic markers*	*Functionally derived markers*	*Function*	*Neuroimmune function*
Microglia	CD11b, CD68, and IBA-1.	CX_3CR1.	Macrophage-like behaviour.	Primary immune effector cells.
Astrocytes	GFAP, S100β.	Aquaporin 4, EAAT, (Humans), GLAST-1, GLT (Rodents).	Contribute to CNS homeostasis.	Secondary immune effector cells.
Neurons	Beta III tubulin, MAP2, NeuN, Neurofilament, NSE.	nNOS, Tyrosine Hydroxylase, ChAT.	Chemical/electrical messengers of the brain.	Secondary immune effector cells.
Endothelial cells	e-selectin, ZO-1.	VCAM-1.	Create a semipermeable barrier surrounding the brain.	Secondary immune effector cells.
T Cells	CD4, CD8.	FOXP3, IL-17.	Adaptive immune response within the CNS.	Adaptive immune response cells.
Oligodendrocytes	MOG.	MBE.	Ensheath CNS neurons in myelin for support and protection.	Limited immune capabilities.

CD, cluster of differentiation; ChAT, choline acetyltransferase; EAAT, excitatory amino acid transporters; FOXP3, forkhead box P3; GFAP, glial fibrillary acidic protein, GLAST-1, glutamate aspartate transporter-1; GLT, L-glutamate transporter-1; Iba-1, ionized calcium binding adapter molecule-1; IL, interleukin; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; MOG, myelin-oligodendrocyte glycoprotein; NeuN, neuronal nuclei or feminizing locus on X-3; nNOS, neuronal nitric oxide synthase; NSE, neuron specific enolase; VCAM-1, vascular cell adhesion protein; ZO-1, zona occuldens-1.

Table 2 Molecular targets historically employed to image, detect and "quantify" neuroinflammation.

Inflammatory mediator	**Structure**	**Function**	**Neuroimmune Function**	**Examples**
Cytokines/ chemokines	Small 5-20kDa proteins.	Cell signalling proteins.	Activate/dampen immune responses from cells.	CCL2, IL-1β, TNFα.
Danger associated molecular patterns (DAMPS)	Purine metabolites (ATP); DNA and RNA; protein chaperones and chromatin binding proteins.	Endogenous warning molecules.	Initiate and perpetuate an inflammatory response.	ATP, DNA, heat shock proteins, HMGB1.
Matrix Metalloproteases	Enzymatic proteases.	Tissue remodelling and regulation of intracellular signalling cascades.	Break down proteins.	MMP-1, MMP-2.
MicroRNAs	Single-stranded noncoding RNA sequences (approximately 21 nucleotides).	Regulation of gene expression.	Regulate inflammatory pathways.	miRNA-155.
Reactive Oxygen Species	Small free oxygen radicals.	Produced in response to threat; can function as neurotransmitters by influencing intracellular calcium signalling.	Kill invading pathogens,	NO.

ATP, adenosine triphosphate; CCL2, chemokine (C-C motif) ligand 2; HMGB1, high-mobility group box 1; IL-1β, interleukin1β; miRNA, micro RNA; MMP, matrix metalloprotease; NO, nitric oxide; TNFα, tumour necrosis factor α.

Table 3 Characteristics of key nanoparticles with specific utility for bioimaging.

Fluorophore/ Nanoparticle	**Key functional characteristic**	**Excitation/ emission spectra**	**Fluorescent life-time**	**Advantages**	**Imaging method**
FITC	Fluorescein functional group	Excitation: 495nm Emission: ~ 517nm	4ns	Biocompatible, small molecule, stable	Confocal microscopy
TRITC	Conjugated rhodamine group	Excitation: ~ 557nm Emission: ~ 576nm	2ns	Biocompatible, small molecule, stable	Confocal microscopy
Nanodiamonds	Carbon-based nanoparticles doped with an impurity (nitrogen or silicon).	Excitation of nitrogen impurity: 560/580nm Emission: 680nm*	20ns	Biocompatible, low toxicity, surface functionalizatio n ability.	Confocal microscopy, Raman microscopy, STED microscopy.
Nano-rubies	Alpha aluminium oxide nanoparticles doped with Chromium (Cr^{3+}).	Excitation: 400/532nm Emission: 692/694nm	1 - 4ms	Long emission time, surface functionalizatio n ability.	A laser-based time-gated confocal microscope
Lanthanide-based upconversion nanoparticles	Trivalent lanthanide doped nanocrystals.	Excitation: 800/980nm Emission: 400 – 900nm*	100 - 500ms	Biocompatible, do not bleach or blink, their emission wavelength can be altered reflecting the crystal dopant, sharp emission bands.	Optical microscope with a xenon lamp adapted to a diode laser.

* reflects the elemental dopant

FITC, fluorescein isothiocyanate; TRITC, tetramethyl rhodamine isothiocyanate.

Figure 1

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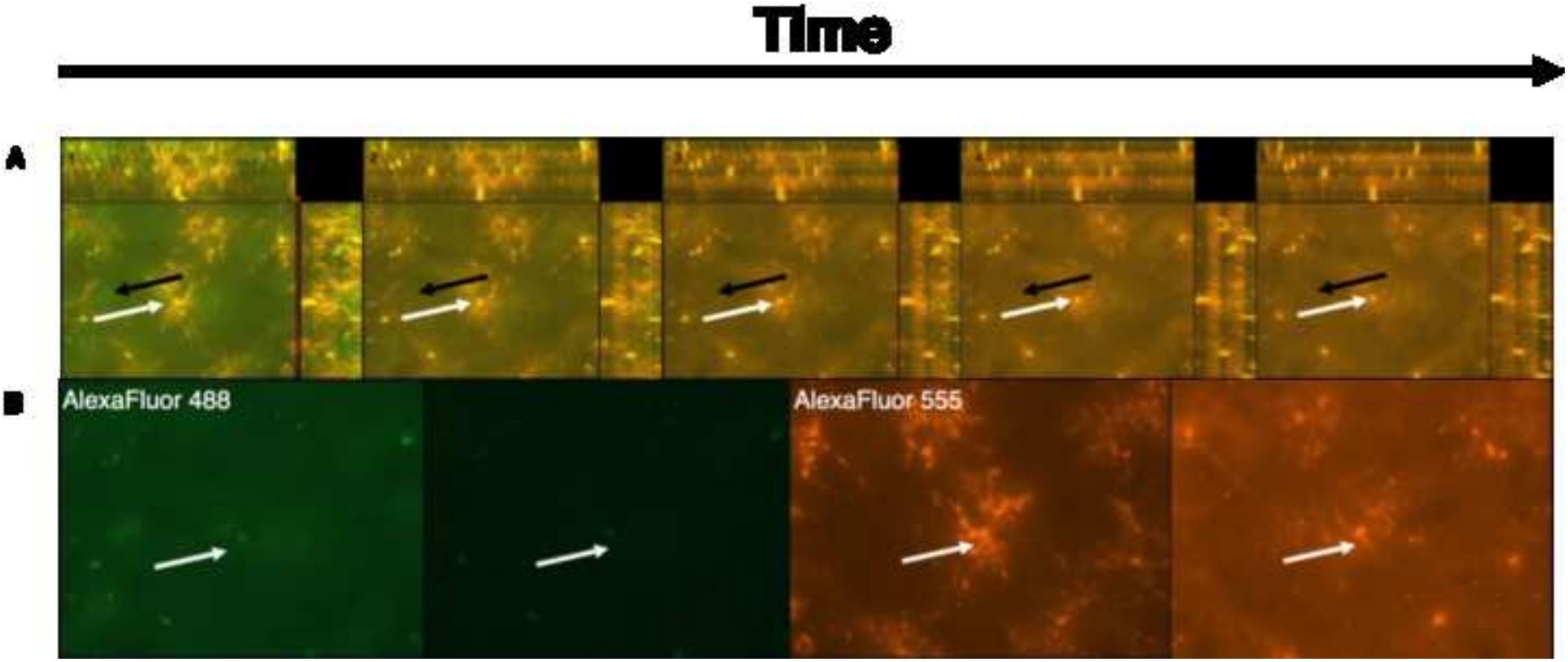

Figure 2

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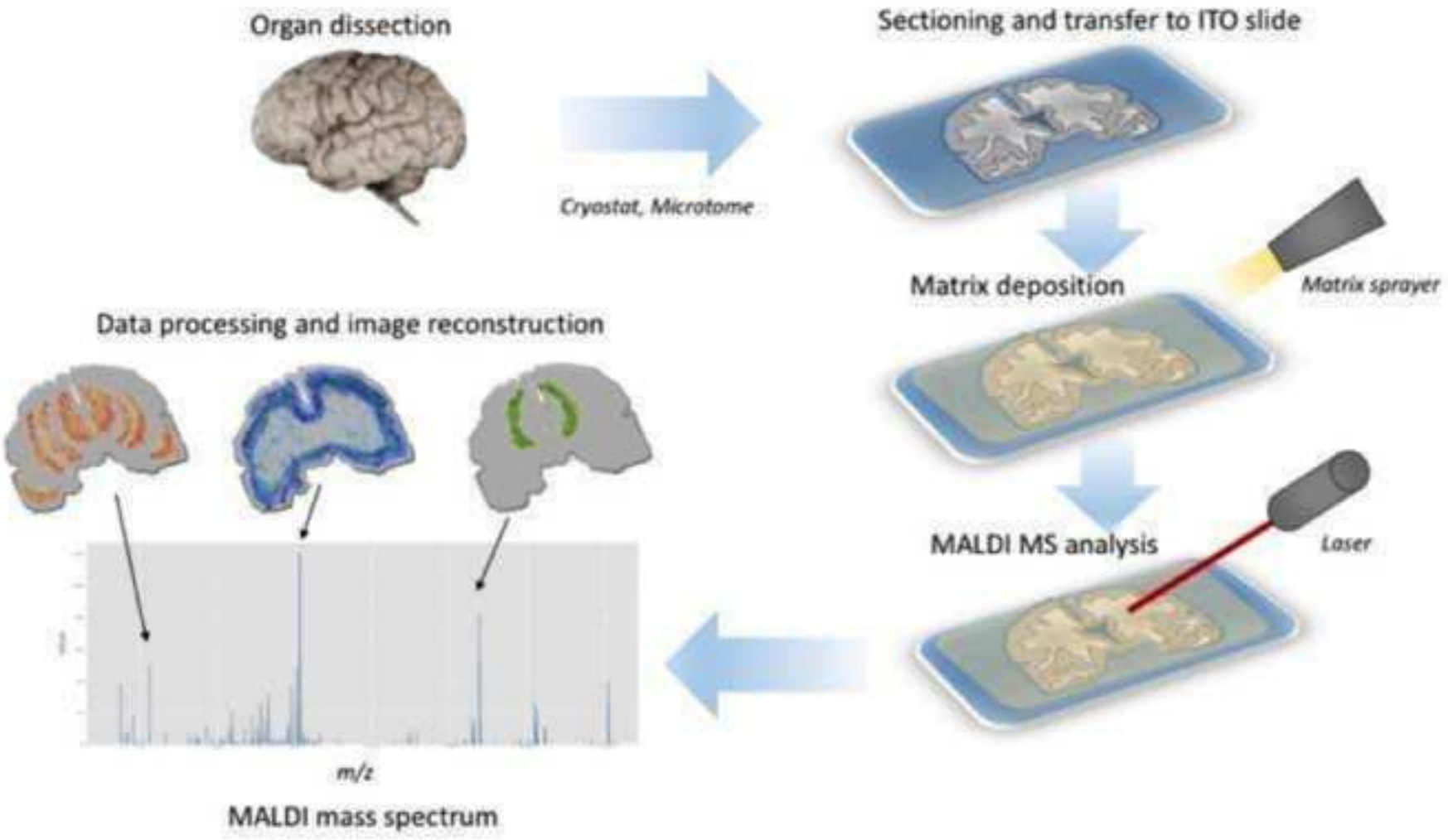

Figure 3

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