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Advances in Nanomaterials for Brain Microscopy

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Abstract

Microscopic imaging of the brain continues to reveal details of its structure, connectivity, and function. To further improve our understanding of the emergent properties and functions of neural circuits, new methods are necessary to directly visualize the relationship between brain structure, neuron activity, and neurochemistry. Advances in engineering the chemical and optical properties of nanomaterials concurrent with developments in deep-tissue microscopy hold tremendous promise for overcoming the current challenges associated with in vivo brain imaging, particularly for imaging the brain through optically-dense brain tissue, skull, and scalp. To this end, developments in nanomaterials offer much promise toward implementing tunable chemical functionality for neurochemical targeting and sensing, and fluorescence stability for long-term imaging. In this review, we summarize current brain microscopy methods and describe the diverse classes of nanomaterials recently leveraged as contrast agents and functional probes for microscopic optical imaging of the brain.

Graphical Abstract:

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TOC Summary: Nanomaterials are revealing new insights into brain structure and function. In this review, we summarize the different optical microscopy techniques and recent advances in nanomaterial engineering that are enabling new methods for visualizing the living brain.

Introduction

Understanding the brain and its susceptibility to injury and disease is a challenge requiring a detailed knowledge of the structure, function, and development of a complex and dynamic system working at numerous length and time scales. Methods to visualize the brain, its neurons, their connectivity, and their neurochemistry are required to provide insight into the underlying mechanisms of brain function in a manner as close to its natural state as possible (Figure 1). Optical microscopy has long been a relatively non-invasive approach used to study the brain with cellular spatial resolution. Recent advances in fluorescent microscopy, including laser scanning confocal, and multiphoton microscopy, among others, have pushed the boundaries of high resolution imaging in brain tissue. The use of fluorescent contrast agents including organic dyes, nanomaterials and genetically encoded proteins have enabled tremendous advances in unwinding the finer structure and connectivity of the brain and its neural circuits [1]. However, large scale, whole brain imaging providing requisite spatial, temporal and chemical resolution of neuronal and non-neuronal activity is necessary to provide a complete picture of brain function. Towards this goal, we review advances towards and inherent challenges in using light to probe the optically dense milieu of the brain, as well as the need for fluorescent probes to explore brain activity. While recent advances beyond the scope of this review have been made to shed light on neuronal activity, including voltage sensitive dyes [2] and genetically encoded calcium indicators [3] to visualize action potentials, and genetically encoded protein sensors for neuromodulators [4, 5] there remains a lack of tools for directly probing the chemical signaling occurring between neurons in the brain extracellular space, as well as the activity of neuroglia.

Nanomaterials stand poised to address many of the challenges facing the study of brain imaging by providing a platform to engineer chemically functional fluorescent probes for neurochemistry. The marriage of neuroscience and nanotechnology over the last decade has seen numerous developments in research and clinical methods for brain science including drug delivery, brain imaging, and neuronal activity readout and stimulation [6, 7]. The advantages of implementing nanomaterials for biological imaging stems from their intrinsic small size, ranging from 1–100 nm, which imparts unique thermal, optical, and chemical properties that differ significantly from the properties of their macroscopic counterparts. The high surface area to volume ratio provides a versatile platform for surface modification to engineer stability and function for targeted delivery and, ultimately, chemical sensing in neurological environments. Furthermore, the tunable band-gaps of semiconducting nanomaterials allows for fluorescent emission to be tuned into the near-infrared (NIR), enabling microscopy methods to explore deep into living brain tissue. In this review, we summarize the latest techniques in both fluorescence- and non-fluorescence-based microscopy techniques for imaging the brain and highlight the burgeoning field of leveraging nanomaterials to shed new light on neurobiology.

Fluorescence Microscopy for Brain Imaging

Fluorescence microscopy is among the most established microscopy techniques to enable imaging of neural systems with molecular specificity. Continued developments for this workhorse technique necessitate a thorough discussion of the latest trends and techniques for imaging in the brain, as these choices influence the spatial and temporal resolution achievable. We first discuss fluorescence excitation mechanisms followed by a comparison of widefield epifluorescence, confocal, multiphoton, and light-sheet microscopy, and complementary techniques commonly used for modern imaging in neural tissues that stand to benefit from the integration of nanomaterial-based fluorophores.

Fluorescence Excitation Mechanisms

Fluorescence microscopy typically makes use of chemical probes embedded within a sample that, when stimulated with light, emit photons of a longer wavelength and lower energy. More recently, particularly for brain imaging, multiphoton excitation (MPE) and upconversion excitation (UCE) of fluorescent probes have been applied to *in situ*, *ex vivo*, and *in vivo* neurological imaging. The mechanism of MPE was first predicted in 1931 [8], experimentally demonstrated in 1961 [9], and introduced to fluorescence microscopy in 1990 [10]. MPE entails excitation of fluorophores, or fluorescent nanomaterials, via simultaneous absorption (Figure 2a) of multiple lower-energy photons which sum to more energy than the emission energy [11, 12]. To date, MPE has been achieved with 2-photon (2PE), 3-photon (3PE) and 4-photon (4PE) excitation modes [13].

Similar to MPE, upconversion excitation (UCE) involves the absorption of lower energy excitation photons than those emitted. (Figure 2a). In contrast to MPE, however, UCE involves a semistable state between the excited and ground state [14–16], while true MPE involves no such states. This long-lived semistable state allows for subsequent absorption of photons, as opposed to the simultaneous photon absorption required for MPE [17]. For this reason, upconverting fluorophores can be efficiently excited at lower excitation densities than MPE, potentially minimizing tissue damage and increasing image penetration depths into specimens such as the brain. Nanoparticles containing a solid-state core doped with rare earth ions are among the most widely used UCE fluorophores, which have already seen numerous applications to *in vitro* and *in vivo* biological imaging [18].

Techniques of Fluorescence Microscopy

Widefield Fluorescence Microscopy—Widefield illumination is the traditional approach to fluorescence microscopy where the imaging plane is typically filled with photons from a defocused excitation source. This optical geometry is achieved with infinity-corrected optics by focusing the excitation source onto the back focal plane of the objective, which is referred to as Köhler illumination. The image of a fluorescent specimen is focused using a tube lens and subsequently collected with a digital camera. This arrangement allows collection of both in and out of focus photons (Fig. 2B), producing high background levels of fluorescence, and reducing imaging contrast with the advantage of capturing a wide field of view. As such, for imaging applications implementing bright and stationary fluorophores, widefield fluorescence microscopy is the preferred imaging modality. Additionally, the

relative low cost for and ease of building a flexible, customizable system compared to other methods makes it an accessible yet powerful technique. For imaging deep into brain tissue, however, absorption and scattering of photons reduces signal strength and introduces aberrations that detrimentally impact imaging quality.

Confocal Microscopy—In widefield geometries, background fluorescence generated from scattered excitation or emission photons outside the imaging focal plane can dramatically reduce signal-to-noise for microscopy, particularly in brain tissue. To eliminate this background fluorescence, a pinhole can be introduced to the excitation and emission paths of the conjugate planes of the imaging system, thereby confining the excitation and collected emission volumes respectively (Figure 2b). This approach, referred to as confocal microscopy, typically features a laser scanning excitation system. A pair of scanning mirrors or an acousto-optic deflector (AOD) scans a diffraction limited laser spot across the imaging plane, while a photomultiplier tube (PMT) or avalanche photodiode (APD) collects photons for each pixel. The reconstructed image can be collected for multiple vertical sections throughout a sample, producing a three-dimensional stack of the sample, limited by the lateral laser scan speed and the resolution of focal scanning. This modality is commonly used for high resolution diffraction-limited imaging if the sample is sufficiently thin. However, high laser powers are necessary to obtain scanning speeds fast enough to temporally resolve certain molecular processes, which can lead to tissue damage. New labeling techniques that improve fluorescence quantum yield, absorption cross-section and photostabilities will greatly improve the flexibility of confocal imaging. Nanomaterial alternatives to organic dyes for confocal imaging have already begun to show significant progress towards improving confocal imaging in brain tissue, including examples using phospholipid nanoparticles [19], quantum dots (QDs) [20, 21], single shell QD [22], multi-shell QD [23], carbon dots [24], UCE nanoplates [16], magnetic nanoparticles [25], gold nanoparticles [26], silver nanoparticles [27], and conjugated polymer nanoparticles [28].

Multiphoton Microscopy—Microscopes designed to utilize multiphoton excitation (MPE) of fluorophores combine the advantages of confocal microscopy with the tissue penetrating ability of near infrared wavelength light, making MPE one of the most commonly used techniques for in vivo and/or deep-brain brain imaging [29]. Because MPE of a fluorophore is a low probability process [12], the excitation and emission volume is small in the z-axis compared to confocal (Fig. 2B). High peak power is necessary to excite fluorophores or luminescent probes, such that a pulsed laser is required to maintain a low average power to avoid sample heating [30]. This approach can be extended further into the near-infrared, thus improving tissue penetration depth by leveraging three- and four-photon excitation modes, but must be met with subsequent increases in laser power [13] and accompanying potential tissue heating and damage.

MPE techniques provide greater imaging depth capabilities than widefield epifluorescence microscopy, while preserving the spatial resolution benefits of confocal imaging. The finer depth resolution allows for better resolved z-scanning than confocal or widefield microscopy, while also yielding a lower fluorescence background signal, further increasing MPE signal-to-noise ratio. However, in high power regimes, 2PE can still cause out of plane

background fluorescence, which can be circumvented in 3PE and even 4PE systems [13, 31]. Additionally, higher costs and the limited flexibility of some commercial systems can limit the adoption of MPE for new applications. Despite these limitations, MPE microscopy using nanomaterials as the optical probe has produced numerous examples of novel brain imaging techniques, realizing exceptional imaging depths. These include carbon nanotubes imaged with 2PE [32, 33], K⁺ ion concentrations imaged with 2PE [34], quantum dots imaged with 2PE [35] and 3PE [36], gold nanorods imaged with 3PE [37], organic dye doped nanoparticles imaged with 2PE [38], and luminogens imaged with 3PE and 4PE [39]. Future work will look towards designing new nanomaterials to improve quantum yield and cross section, enabling faster scan rates, as well as to add functionality such as molecular recognition for sensing applications.

Light-Sheet Microscopy—Light-sheet microscopy, introduced in 2007 [40], is an approach to fluorescence microscopy whereby illumination is decoupled from the optical detection pathway, maximizing planar illumination of the focal volume while minimizing potential phototoxicity [41], enabling whole brain and whole organism imaging. The sample is illuminated in a two-dimensional plane orthogonal to fluorescence detection (Figure 2b), providing improved signal-to-noise while minimizing out of focus exposure. While geometrically challenging to engineer, light-sheet microscopy allows for fast scanning of 3D volumes enabling whole specimen imaging and long-term exposures imaged directly onto a camera array. The latest advancements to light-sheet microscopy have shown to dramatically improve resolution and tissue imaging depths by incorporating other imaging modalities, namely structured illumination[42] and MPE[43], to allow for direct probing of brain activity of whole animals such as zebrafish larvae and *C. elegans*.

Although numerous dyes and fluorescent proteins [44, 45] have been used successfully with light-sheet microscopy, nanoparticles have yet to make a significant impact on this imaging modality, presumably as a result of the microscopy technique's relative infancy. It is likely that ongoing developments of highly fluorescent and stable nanoparticle probes will further advance light-sheet microscopy, particularly for whole-brain functional imaging at the micron scale.

Adaptive Optics—The highly scattering nature of brain tissue introduces significant optical aberrations when reconstructing an image, degrading image quality as imaging depths increase due to the warping of excitation photons by the heterogeneous index refraction of brain composition [46, 47]. Adaptive optics circumvents this issue by introducing a deformable mirror, usually micro-electro-mechanical (MEMS) or piezoelectric based deformability, into an imaging feedback loop. The imaging wavefront, either upstream or downstream of sample, can be corrected with the adaptive corrections of this mirror array, increasing performance and minimizing distortions to create high quality images and thus improving spatial resolution for physiological and functional deep-brain imaging. Adaptive optics was developed and implemented primarily with dyes and fluorescent proteins as optical reporters [46, 47], though it can be extended for use with multiphoton microscopy [13] and could prove to be a powerful tool for improving image quality, particularly for imaging nanomaterials deep in brain tissue.

Sample Preparation: CLARITY and Expansion Microscopy—While many of the above approaches aim to minimize the detrimental effects of tissue opacity through optical methods, CLARITY sidesteps the challenges of imaging deep into biological tissue by chemical preparation of the tissue resulting in tissue-transparency of a fixed sample. This method, developed in 2013 [48, 49], dramatically reduces tissue scattering and absorption of photons by degrading lipids, allowing for deeper imaging in relatively intact brain samples. Similar methods for such optical tissue clearing include the passive clarity technique (PACT) and benzyl alcohol/benzyl benzoate (BABB) technique. In the case of CLARITY, the proteins of an organ, such as the brain, are suspended in a gel scaffold (such as acrylamide) after which the lipids of the tissue can be washed out with lipophilic surfactants. As lipids cause the bulk of light scattering in the visible wavelength imaging regime, the resulting optically-clear brain tissue maintains an estimated 92% of the original proteins in their original configuration for high resolution imaging [49]. This technique has been used to prepare samples for confocal, multiphoton, and light sheet [44] microscopy and is particularly well suited for light-sheet microscopy [50, 51].

Another sample preparation technique that improves imaging depth and resolution can be realized by chemically swelling the tissue sample. Termed expansion microscopy [52], this approach physically magnifies tissue prior to imaging by chemically fixing different molecular constituents followed by dialysis with water to swell the sample by over an order of magnitude. This approach has been used to image brain tissue with both light-sheet and MPE microscopy in conjunction with super-resolution techniques to image single synapses in the mouse cortex with 70 nm resolution.

While these approaches allow for molecular imaging at unparalleled depths and resolutions in brain tissue, both are post-mortem techniques, making them incapable of imaging dynamic cellular and molecular processes. While nanomaterials have yet to be integrated in to either CLARITY or expansion microscopy, deeper imaging fields with shorter integration times could be enabled by utilizing band-gap tunable and low-photobleaching nanomaterial-based contrast agents.

Nanomaterials for Fluorescence Microscopy

The CLARITY and expansion microscopy methodologies highlight the state of the art for brain microscopy, yet applications that integrate nanomaterials as contrast agents for these imaging modalities are just beginning to take hold. Engineered nanomaterials for imaging and sensing must exhibit several figures of merit to be compatible with exploring in the brain. First, they must be biocompatible, non-toxic, and colloidally stable in the complex environment of brain tissue, particularly for long-term functional imaging in vivo. Additionally, their surface must be chemically functionalizable to tune stability, even distribution, and binding affinity. Fluorescence emission, either intrinsic or through chemical modification, must be stable and any modulation to fluorescence upon binding a target should be fully reversible to enable dynamic sensing. Lastly, any binding affinity must be sensitive enough to detect endogenous concentrations of the target while maintaining selectivity. Below, we review select classes of nanomaterials (Figure 3) that exhibit many of

the above properties and provide recent examples of promising applications towards imaging brain structure and chemistry at the microscale.

Carbon Allotropes—The discovery of fullerenes in 1985 ushered in a new era of synthetic carbon-based nanomaterials [53]. Fullerenes are a class of molecular carbon allotropes, the simplest of which forms hollow spheroidal structures, such as C₆₀, the buckyball. The unique chemical, electronic and optical properties of fullerenes fueled a “carbon gold rush”, leading to the discovery and synthesis of new geometries of carbon nanostructures, including the zero-dimensional carbon nanodot (CD) and graphene quantum dot (GQD), the quasi-one-dimensional carbon nanotube [54] and two-dimensional graphene [55] among others [56, 57]. Applications of carbon nanomaterials over the last two decades spans numerous disciplines including electronics, photonic, composites, energy storage, environment, and biomedicine [58–61]. Many of the early applications of carbon nanomaterials to biomedicine leveraged their electronic and chemical properties as well as their small size to develop new diagnostics and therapies. Functionalizing carbon nanomaterials towards these ends often entails covalent tethering of polymers and biomolecules or the adsorption of therapeutic drugs to the nanomaterial surface. Since the discovery of the inherent photoluminescence of certain carbaceous nanoparticles, such as carbon nanotubes [62] and graphene [63–65], research focus has shifted to applications of these nanomaterials for fluorescent imaging and sensing in tissues [66–69]. In this section, we present recent advances for some of the most carefully studied fluorescent carbon nanomaterials and discuss their application to visualizing brain structure and function.

Carbon Nanotubes—Carbon nanotubes are a diverse class of nanomaterial, owing to the broad range of structural and chemical forms and their resulting optical and physical properties. The ease with which carbon nanotubes are chemically functionalized has enabled the development of gene and drug delivery platforms [59, 69], whereby carbon nanotubes have also been shown to deliver drugs across the blood-brain barrier (BBB) to the brain [70]. However, an orthogonal strength of carbon nanotubes for brain research is their intrinsic near-infrared (NIR) photoluminescence. Specifically, semiconducting single walled carbon nanotube (SWNTs) exhibit photoluminescent emission (900–1500nm) falling within the NIR-II window (1000–1700 nm), a regime where photons are minimally scattered by brain tissue and minimally absorbed by water, providing a notable advantage for improving imaging signal and resolution at depths approaching several millimeters [71–73]. Additionally, SWNTs do not photobleach, such that images can be collected for timescales well beyond those enabled by organic fluorophores and even quantum dots (QDs) [74]. The infinite photobleaching lifetime of SWNTs enables the possibility chronic or long-term imaging studies in animal models. The first *in vivo* experiments utilizing SWNTs for imaging in neural tissue were demonstrated in *drosophila* larvae by Leeuw *et al.* where SWNTs were incorporated into feed stock and found to distribute throughout different tissues, including the brain, with no observed short-term toxicity [75]. This result paved the way for future studies implementing SWNT to explore brain tissue at the cellular scale.

In pioneering work from Hongjie Dai's laboratory, precise, long term, and noninvasive imaging of the cerebral vasculature through a mouse skull was accomplished with SWNTs

as imaging agents [76]. Biocompatible SWNTs were functionalized with a phospholipid-PEG construct (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethyleneglycol, 5000)]) (DSPE-PEG) and injected intravenously. By collecting SWNT fluorescence emission between 1300–1400 nm, Hong *et al.* dynamically quantified blood perfusion throughout the brains of healthy mice and those with surgically induced middle cerebral artery occlusion, achieving imaging depths in excess of 2 mm. Although restriction to the first several millimeters of brain tissue makes SWNT imaging unsuitable for most human neuro-imaging clinical applications, the non-invasive nature of this approach opens an array of possibilities for neurological imaging of awake and behaving animal models. For instance, placement of SWNTs in brain tissue as contrast agents could enable cellular and potentially molecular resolution imaging in subcortical layers of the brain without the need for a chronic cranial imaging window [77].

Another key example of SWNT fluorescence to study the brain focused on measuring the tortuosity of the brain extracellular space (ECS). Groc, Cognet, and colleagues demonstrated that SWNTs readily distribute throughout the extracellular space of acute brain slices obtained from rat pups [78]. SWNTs non-covalently functionalized with DSPE-PEG were delivered via intra-cerebroventricular (ICV) injections to several brain regions (neocortex, hippocampus, striatum) and imaged as DSPE-PEG-SWNTs diffused through the ECS. With single-molecule localization, the authors obtained super-resolution maps of the ECS morphology with spatial resolution approaching 40 nm. The near infrared emission of the SWNTs enabled imaging depths of 100 μm into the brain slices, avoiding surface layers commonly damaged during acute slice preparation. These results unveiled new complexities in the ECS morphology at the nanometer scale in living tissue, a critical parameter for understanding extrasynaptic volume transmission, the impact of changes to glial cell morphology on brain function, neurodegenerative disease, and the behavior of drug molecules. There is much future potential for new engineered nanomaterials with tunable size, near-infrared fluorescence, and chemical affinity to complement established methods for probing ECS morphology, to devise new strategies for pathology and drug delivery [79–81].

In the above study of SWNTs in acute brain slices to study ECS morphology, SWNTs delivered into hippocampal pyramidal cells by patch-pipette showed a 1000-fold lower diffusion constant compared to the ECS. The large diffusion constants measured for ICV injected SWNTs suggested that these nanoparticles remained primarily in the ECS and were not actively internalized into cells. This is in stark contrast to phenomena observed across other unrelated studies in which SWNT are introduced into biological systems and readily internalized into cells through passive and active mechanisms [82–85], which could potentially pose unintended issues such as fluorescence quenching and cytotoxicity. Additionally, while the morphology of microglial cells, the brain's resident immune cells, was noted as normal in the above ECS study, microglial inflammatory responses could cause changes in cytokine expression levels. These cellular responses should be monitored and carefully quantified at the mRNA or protein level, in addition to morphological changes, for all studies utilizing nanoparticles in brain tissue to ensure minimal impact on homeostatic function. It is also important to note that functionalization of nanoparticles can alter region-specific toxicity mediated by microglia [86]. This strong toxicity dependence on

nanoparticle surface chemistry stresses the importance of further studies on how nanoparticle surface chemistry affects nanoparticle biodistribution, bio- and immune-interactions, and toxicity in living systems. Such efforts will prompt continued research into new non-covalent and covalent chemistries with which to control nanoparticle interactions with biological systems [87].

Another consideration for the implementation of SWNTs, as well as other nanomaterials, for imaging studies is in the choice of excitation source. Continuous wave lasers of powers exceeding 100 mW are most commonly used to excite SWNTs on-resonance at their second order excitonic transition, S_{22} , ranging from 600–800 nm. However, concerns over tissue health under such high fluence have motivated investigations into alternative excitation methods. Recently, a direct comparison between excitation of (6,5) chirality SWNT S_{22} transitions (568 nm) and alternative methods, phonon-sideband excitation (845 nm) and up-conversion (1064 nm), have been demonstrated. Although the use of 1064 nm laser light was shown to provide the best signal-to-noise ratio for SWNT imaging in brain tissue, simulations demonstrated a 15 K increase in brain tissue sample temperature under 1064 nm excitation. As such, authors concluded 845 nm as an optimal SWNT excitation source for in-brain imaging [88].

With increasing confidence in the compatibility of infrared SWNTs for brain imaging, new directions are looking towards incorporating molecular sensing capabilities into SWNTs to realize dynamic imaging of brain chemistry. Towards this goal, a new method for imparting molecular recognition elements onto SWNTs preserves their NIR fluorescence by using non-covalent adsorption of amphiphilic polymers [67]. The method, termed *corona phase molecular recognition*, imparts molecular selectivity of interactions between the nanotube and analyte through the self-organization of the polymer adsorbate phase. To date, this approach has successfully yielded infrared fluorescent sensors for small molecules, nucleic acids, and proteins [89–92]. The creation of a turn-on NIR fluorescent nanosensor for the neuromodulatory neurotransmitter dopamine with a fluorescent response of F/F of 90% shows promise to realize real-time imaging brain neurochemistry with high spatial and temporal resolution [93, 94]. Optical imaging of dopamine with this SWNT-based dopamine nanosensor has thus far been achieved in PC12 cells cultured onto a nanosensor covered surface [95]. Recent results have shown that a highly sensitive SWNT-based catecholamine nanosensor (nIRCats), with a fluorescent response of F/F exceeding 2400% [96], can be used to image dopamine release and reuptake in living brain tissue [97]. In this latter work, authors demonstrated imaging of dopamine release with nIRCats evoked with both electrical or optogenetic stimulation of acute striatal brain slices. Dopamine reuptake in brain slices was also demonstrated both in artificial cerebral spinal fluid, and also in brain slices exposed to antidepressant drug nomifensine, with high spatiotemporal resolution. These results, combined with recent analytical modeling of SWNT nanosensors for dopamine sensing [98, 99], provide promising steps towards utilizing these nanosensors for *ex vivo* and *in vivo* studies investigating neurochemistry in the living brain.

Multi-walled carbon nanotubes (MWNTs), consisting of concentric cylinders of graphene, are also finding applications in brain imaging applications. Functionalized MWNTs have primarily been utilized for targeted delivery of therapeutics in brain tissue [100, 101], where

they are observed to cross the BBB, a highly selective, semipermeable membrane separating circulating blood from the central nervous system. The ability to cross the BBB represents an enormous advantage for delivery of nanoparticles into the brain because it avoids tissue damage associated with intracranial injections. In more recent work, it was discovered that MWNTs also exhibit inherent photoluminescence, where photon emission (450–650 nm wavelengths) could be stimulated using near infrared (750–950 nm wavelengths) excitation through a two-photon absorption process [102]. The photoluminescence of functionalized MWNTs was later leveraged for multiphoton fluorescent imaging in brain tissue [33]. In this work, MWNTs were covalently functionalized with diethylenetriaminepentaacetic dianhydride (DTPA) and humanized MUC1 antibody fragment antigen binding region (Fab') and delivered intravenously into a mouse model. The MWNTs translocated the BBB without inflicting damage and accumulated in the parenchyma within 5 minutes after injection. Although this particular MWNT formulation is considered non-cytotoxic, transient inflammation (~72 hours) was confirmed using a TNF-alpha ELISA assay but was minimized with co-injection of a steroidal anti-inflammatory dexamethasone, an approach that could be adopted for other nanomaterials use in the brain. While these results show promise, the high absorption and scattering of the visible fluorescence of MWNTs compared to the NIR fluorescence of SWNTs limits potential imaging applications deep in tissue.

Carbon Nanodots and Graphene Quantum Dots—CDs and GQDs are two related classes of zero-dimensional carbon nanomaterials receiving increased attention in the last decade. Both CDs, which consist of a quasi-spherical particle of carbon with a diameter below 10 nm, and GQDs, particles consisting on fewer than 10 stacked layers of graphene, exhibit tunable photoluminescence [103]. Additionally, the large surface-area to volume ratio and abundance of sites for covalent modification make CDs and GQDs an ideal platform for chemical modification and functionalization. Combined with properties including low-photobleaching, biocompatibility, and low toxicity, these nanomaterials are seen as promising alternatives to traditional fluorescent dyes for biosensing, drug delivery, phototherapy and bioimaging [59, 104]. Surprisingly, few examples in the literature demonstrate the potential utility of CDs and GQDs for brain imaging.

To demonstrate the advantages of GQDs for deep tissue imaging in the brain, Qian *et al.* performed microinjections of graphene oxide nanoparticles conjugated with 2000 MW polyethylene glycol (PEG) into the brain of mice [105]. They found two photon excitation of the nanoparticles using 810 nm fs laser pulses enabled imaging at depths reaching 300 μ m into the brain tissue. Additionally, the authors note that the efficient three photon excitation observed for of these nanoparticles could, in theory, enable even deeper tissue imaging depths and lower background, higher contrast images. Although the nanoparticles were found to have negligible cytotoxicity confirmed via cell proliferation assays and long term histological comparisons, it should be noted that later studies suggest that similarly synthesized nanoparticles can downregulate neuronal signaling without affecting cell viability [106].

In another study, Zheng *et al.* synthesized CD to enable targeted imaging of brain cancer. The resulting aspartic acid-functionalized CD were found to be water soluble, non-cytotoxic, and exhibited excitation dependent photoluminescence emission ranging from 475–635 nm.

Additionally, CD-Asp delivered by tail injection were found to readily pass through the BBB and preferentially accumulate in C6 glioma cells in the brains of mice, serving as a glioma-targeting imaging agent. Although the precise mechanism both for selective uptake and BBB passage remain unclear, the results show promise for future applications incorporating functional CDs and GQDs for *in vivo* fluorescent sensing of neurochemistry, such as Cu^{2+} , Ca^{2+} and neurotransmitters [24, 107, 108].

Nanodiamond—Nanodiamond is another carbaceous nanomaterial that shows potential for developing contrast agents and fluorescent probes for brain imaging and neurochemical sensing. Nanodiamonds are crystalline nanoparticles between 2–10 nm in diameter with tunable surface chemistry, excellent optical and mechanical properties, and lower toxicity compared to other nanoparticles [109]. The introduction of nitrogen-vacancy centers into the nanodiamond lattice produces stable luminescence and a long spin-coherence time, making them the most commonly used variant for applications involving high-resolution magnetic sensing and biomedical imaging. To date, these materials have been used to quantify diseased-induced changes of intraneuronal transport in cultured mouse hippocampal cells [110] as well as intraneuronal temperature mapping [111]. Additionally, simulated experiments show promise for using nanodiamond to measure changes in magnetic fields generated by the axon transmembrane potential for imaging neural activity with high spatial and temporal resolution [112]. Although these early studies are restricted to working in culture and *in vitro*, new vacancy engineering approaches that push nanodiamond luminescence into the near infrared [113] may aid in adapting these technologies for imaging and sensing in brain tissue.

Inorganic Nanoparticles—Inorganic nanoparticles were discovered earlier than carbon-based nanoparticles, with the first examples of gold nanoparticles in ancient Roman times [114], the first mention of semiconducting quantum dots in 1986 [115], and have since been more extensively developed for optical imaging [116]. Two major classes of inorganic nanoparticles that have been applied to brain imaging include quantum dots and noble metal (gold and silver) nanoparticles. Both classes of nanomaterials lend themselves to optical imaging in the brain, with properties including band-gap tunable fluorescence with minimal photo-bleaching, and facile chemical functionalization for targeting specific physiological and biochemical regions of a specimen. We start by first reviewing the chemical and optical features of these nanoparticles followed by recent highlights in their application to visualizing brain vasculature, brain diseases, and neuronal structure.

Quantum Dots—Quantum dots (QDs) are nanoparticles composed of fluorescent semiconducting material [117]. The nanoscale size of the QD leads to strong electronic confinement of electrons, resulting in fluorescence properties that are tunable by altering the size or composition of the nanoparticle. QDs typically consist of a semiconducting core stabilized by either one (core-shell) or two (core-shell-shell) layers of semiconducting materials. These semiconducting layers are often hydrophobic and need to be further passivated with ligands and polymers to enable their aqueous dispersion. QDs are ideal candidates for biological sensing owing to their broadband optical absorption for ease of excitation at multiple wavelengths, narrow emission bands for selective multicolor imaging,

high photostability for biological imaging, and polyvalency for multifunctional applications [116, 118–122]. To better enable imaging in brain tissue, recent efforts have focused on pushing fluorescent emissions further into the near-infrared by exploring new semiconducting materials for band gap tuning.

Several near-infrared fluorescent QDs have been synthesized exhibiting wavelengths in the NR-II optical window. Lead sulfide (PbS), silver selenide (Ag₂Se), and silver sulfide (Ag₂S) QD emit in the NIR, or short-wavelength NIR (swNIR), and have been used for *in vivo* imaging of mouse vasculature [123–125]. Ag₂Se and Ag₂S QDs have also shown remarkable biocompatibility. Recently, Franke and colleagues developed a method to synthesize indium arsenide (InAs) quantum dots using a continuous injection method [126]. These InAs QDs showed tunable fluorescence from 900 to 1600 nm, with this upper limit beyond the NIR window and spanning the full spectral response for contemporary NIR detectors. swNIR emitting InAs QDs exhibit greater chemical stability than PbS, Ag₂Se, and Ag₂S QDs in aqueous media because of their unique zinc blende crystal structure, allowing for a more straightforward ligand exchange and coating. This stability is particularly necessary for applications where long-term fluorescence imaging in the complex biological environment of the brain are required. The selection of ligands, such as small targeting peptides and polyethylene glycol (PEG), can enable the delivery of QD to specific brain regions and extend the circulation time of quantum dots [22].

InAs QDs have high quantum yields (typically 10–20%) and narrow size distributions (core sizes of 4.3 ± 0.4 nm), allowing for deep tissue imaging, high spatial resolution, multicolor imaging, and fast image acquisition speeds, all of which are critical to quantifying dynamic biological processes such as neurotransmission. InAs QDs are easily functionalized and have been incorporated into phospholipid micelles, lipoproteins, and composite particles for delivery into living animals [127]. These InAs QDs (excitation: 785 nm, emission: 900–1350 nm) were both photo- and bio-stable, and used to image glioblastoma, a particularly malignant form of brain cancer, in mouse brains, allowing the detailed reconstruction of tumor vasculature. Imaging was acquired through a cranial window after tail-vein injection using one-photon microscopy for minutes at a time. This demonstrated the use of InAs QDs to image large volumes of vasculature for the pathological study of brain diseases, such as stroke, or the detection of brain activation by changes in blood flow as a response to external stimuli.

In consideration of QD applications for brain imaging, many imaging experiments with QDs have been limited to the study of neural vasculature. Several obstacles prevent the delivery of QDs from blood vessels to brain tissue for the study of the brain's morphology or the neuronal activity at synapses. First, QDs, like other nanoparticles, are hindered by their small size from migrating to the walls of the blood vessels. The QDs experience multiple collisions with larger red and white blood cells that hinder movement to the blood vessel walls [128], a necessary step for the diffusion of particles from blood vessels into tissues through a process termed margination. Entering brain tissue from the circulatory system poses the additional challenge of traversing the BBB [129], however chemical methods for functionalizing QDs can be employed to overcome this barrier. After crossing the BBB, probes or theranostics must also travel through the ECS. The strong fluorescence, small size,

and photostability of QDs has allowed for precise optical measurements of ECS in living rat neocortex, providing details relevant to understanding endogenous neurotransmitter spread and diffusion of exogenous agents [130].

Non-invasive imaging of brain tumors has recently been accomplished with QDs decorated with targeting peptides that traverse the BBB. Huang and colleagues showed functionalization of CdSe/ZnS QDs (excitation: 488 nm, emission: 525 nm) with asparagine-glycine-arginine (NGR) peptide and polyethylene glycol (PEG) to create NGR-PEG-QDs that localized to glioma brain tumors [22]. The NGR peptide specifically targets a type II transmembrane glycoprotein (CD13) overexpressed on the surface of glioma cells, whereby the PEG surface functionalization increases the circulation time of the QDs. These NGR-PEG-QDs were injected into mice through tail-vein injection and shown to localize in the brain tumor after 8 hours. This localization into brain tumors is driven by targeting ligands and potentially the increased permeability of tumor vasculature. For future work, the development of a more general delivery method will be necessary to bypass the BBB for brain imaging in cancer-free systems towards imaging homeostatic brain activity [131].

Ligand surface functionalization can also be used to distinctly tune the physical properties of QDs that affect the localization and application of these nanoparticles [132]. Recent work has shown the importance of negative surface charge of QDs which, on a timescale of minutes, exhibit specific membrane localization of QDs to neuronal cells over glial cells [133, 134]. Surface charge as opposed to QD size, shape, or composition was shown to be a prevailing factor for internalization due to the QDs interaction with the bioelectrical surface of the neuron. This recent work does contradict previous findings that show nonspecific uptake of QDs by glial cells independent of surface charge and functionalization [135, 136]. More research into the physical properties and subsequent function of QDs is necessary, but these findings point to ways for future design of targeting QDs for brain imaging.

To circumvent the barrier of diffusion through the BBB, QDs can also be injected directly into the brain. Using high osmolar solutions to temporarily open BBB junctions or by performing direct intracerebral injections [137, 138], imaging contrast agents can be introduced into brain. Although intracerebral injections are used ubiquitously for the administration of drugs and therapies to brain tissue, precise localization is a challenge and limited diffusion can impact distribution. Additionally, injections can locally activate glial cells, triggering an inflammatory response that may complicate experimental results. Alternatively, injections directed into the brain ventricle, the cavity of the brain where cerebral spinal fluid is housed, show relatively low activation of microglia [20]. The ventricular area is one of the more permissive barriers for delivery into the brain, making it a promising approach for introducing QDs and other nanomaterials into functional regions of the brain [129]. With ventricular injections, antibody-QD conjugates (excitation: 561 nm, emission: 655 nm) have been used to label single-dopamine receptors for single-receptor diffusion tracking in acute brain slices. This method of direct injection is not unique to QDs and can be exploited for delivery of other nanoparticles directly to the brain without having to pass the BBB.

Another challenge in the study of neuronal activity occurs once the QD is delivered in the brain. The large hydrodynamic size of many commercially available and/or colloiddally suspended QDs (~30–100 nm) relative to the size of neuronal synapses (~30 nm) often prevent imaging of synaptic features. However, recent methods in QD synthesis enable tunable synthesis of small-diameter QDs that can access synaptic clefts and are now available commercially with PEGylation [139]. Cai and colleagues synthesized stable small quantum dots (sQD, excitation: 488 nm, emission: 527 or 615 nm) that have 9 nm diameter cores and thin ligand layers [140]. These sQDs can be labeled with streptavidin and were shown to bind biotin-labeled AMPA receptors in two live rat primary neuronal cell lines. The sQDs were imaged using super-resolution microscopy photoactivated localization-fluorescence imaging with one-nanometer accuracy (PALM-FIONA) system, and diffusion of the AMPA receptors were quantified by tracking sQDs within synapses. Single quantum dot tracking experiments have been used to study a myriad of different synaptic receptors types [141, 142], providing insight into changes in synaptic plasticity, a first step towards visualizing dynamic processes associated with neural activity. In another example of particular importance to fundamental neurobiology, QDs loaded into cultured hippocampal vesicles were used to visually confirm and quantify the controversial concept of kiss-and-run versus full collapse fusion of neurotransmitter-loaded vesicles at pre-synaptic boutons [143]. Although powerful, these methods remain limited to indirectly visualizing neurochemistry in cultured cells, with future work likely targeting their implementation *in vivo*.

Another approach to inferring neural activity is through quantifying changes in neuron membrane potential, which governs neurotransmitter release and chemical communication between cells following an action potential. QDs offer several advantages over alternative methods, namely voltage sensitive dyes and genetically encoded fluorescent proteins, because QDs are brighter, more photostable, and can be engineered to exhibit large fluorescence changes as a function of voltage with rapid temporal responses [144, 145]. Recently, work in further tailoring these QDs to localize into plasma membranes has resulted in the superior fluorescent responses to changes in membrane potential in HeLa cells, PC12 cells, and in anesthetized adult mice *in vivo* [146]. CdSe/CdS/ZnS nanoparticles (emission 600–650 nm) between 600–650 nm functionalized with peptide and fullerene molecules were introduced into the brain by intracortical injection into the parietal cortex and imaged at a depth of 100 μ m using 460–520 nm light stimulation. Electrical stimulation was used to modify cell membrane potentials while monitoring the fluorescence response of the QDs. Although the QDs provided a superior fluorescent signal compared to available voltage dyes, it remains to be seen whether this method can be used to study endogenous neuronal firing *in vivo*.

Many studies focus on neuronal activity, but the function and activity of non-neuronal structures remain an important component to understanding overall function and health of the brain. Neuroglial cells, comprising primarily of microglia and astrocytes, are the resident immune cells of the brain and function in concert with neurons to modulate brain activity. QDs have been implemented to track the glutamate transporter GLT-1 on the membrane of astrocytes, revealing that the diffusion of GLT-1 dictates glutamate clearance rates near synapses, thus shaping neurotransmission [147]. Such long-term fluorescent imaging experiments that are enabled by the robust photostability of QDs and other nanomaterials

could provide important insights into dynamic interactions between neural and glial cells at a molecular level, opening new avenues of research into their role in neurological and psychiatric disease.

Although QDs have made tremendous strides for *in vivo* imaging of brain tumors, neuronal vasculature, neuronal receptors, and neural electrical activity, challenges remain. QDs as synthesized are not biocompatible and require ligand functionalization that is not necessarily straightforward, particularly if the combination of multiple functionalities are required. There is also a concern of heavy metal toxicity from QD for long term *in vivo* imaging [148, 149]. Accumulation of QDs in the body has shown to result in oxidative breakdown of the nanoparticle in the lysosome of the cell, causing reactive-oxygen species induced toxicity [150–152]. These challenges in functionalization and stability need to be addressed before incorporation of quantum dots for imaging spatiotemporal dynamics of brain electrical (action potential) and chemical (neurochemical and neuromodulatory) activity can be achieved.

Noble Metal Nanoparticles—Noble metal nanoparticles, including gold and silver nanoparticles, exhibit numerous physical and chemical advantages that lend themselves to biological and imaging applications. Owing to their lower toxicities, these nanoparticles have been extensively studied to create probes for imaging, therapeutics, and theranostics [153, 154]. However, noble metal nanoparticles are less frequently studied for use with fluorescence microscopy due to their weak intrinsic photoluminescence [155] and their propensity to quench fluorescent dyes. Despite this limitation, several applications for brain imaging with noble metal nanoparticles have been realized, highlighting their potential application as multifunctional fluorescent probes. Gold nanoparticles (AuNPs) exhibit strong surface plasmon resonance, the coherent oscillation of the surface conduction electrons excited by the electromagnetic field of incoming light. Surface plasmon resonance leads to powerful fluorescence and surface enhanced Raman scattering properties in AuNPs for optical imaging applications as enumerated in previous reviews [156]. Surprisingly, fewer applications of AuNP have been translated to optical brain imaging, although AuNP have been implemented in several non-optical methods for detection such as magnetic resonance imaging (MRI), positron emission tomography (PET), and computed tomography (CT) not discussed in this review [157–159]. Due to this multitude of attractive AuNP features for imaging, many reports that study AuNPs involve theranostics, the coupling of therapeutics and diagnostics to the same nanoparticles [160, 161]. AuNPs often do not have intrinsic fluorescence, but are readily used for imaging and brain imaging after conjugation with fluorescent moieties and stabilizing or functional ligands.

Delivery of AuNPs is critical for eventual adoption for brain imaging. A study of the biodistribution of colloidal AuNPs after intravenous administration shows that AuNPs smaller than 50 nm in diameter diffused through the BBB [162]. Alternatively, the delivery of AuNPs to the brain through the olfactory pathway is also a successful strategy.[163] AuNP labeled with fluorescein (excitation: 490 nm, emission: 525 nm) were fluorescently imaged in the central nervous system of locusts upon olfactory delivery. The AuNPs, with a mean diameter of 13.22 ($\pm$ 1.19 nm) were shown to passively enter through the BBB within 1 hour of olfactory exposure. This method of delivery to the brain is less invasive compared to

injection methods described in the previous sections. However, more studies need to be conducted to determine how physical attributes such as size, charge, and shape govern the successful delivery of AuNPs and other nanomaterials to the brain by this olfactory pathway.

AuNPs can quench the fluorescence of fluorophores in close proximity to the nanoparticle's surface through energy transfer, enabling the design of functional fluorescent sensors. Phthalocyanine 4 (PC4) is an intrinsically-fluorescent hydrophobic photodynamic therapy drug for glioblastoma multiforme, one of the deadliest forms of brain cancers, that can be adsorbed on the surface of AuNP through a terminal amine group [164]. Upon adsorption to the surface of AuNP, the fluorescence of PC4 at 685 nm is quenched, and only drug release from the nanoparticle upon illumination with light recovers the fluorescence. This approach has enabled fluorescent quantification of drug release in tumors from brain slices excised from mice models.

AuNPs are easily surface-modified with ligands to improve colloidal stability and to target specific organs or tumors, a useful approach adaptable to targeting particular neural subsets or brain regions. The gold surface readily makes strong bonds with thiolated chemicals that enable the tethering of targeting moieties, such as antibodies, targeting peptides, and fluorophores. AuNPs were shown to have higher retention in brain tumors after *in situ* enzyme-induced aggregation.[26] Ala-Ala-Asn-Cys-Lys peptide conjugated AuNP and 2-cyano-6-amino-benzothiazole modified AuNPs were hydrolyzed by legumain, a protein involved in hydrolysis and found at disproportionately high concentrations in brain tumors, to form fluorescently labeled aggregates. These aggregates were prelabeled with Cy5.5 fluorophores which enabled selective fluorescence imaging of brain tumors.

Gold nanorods (GNR) have many of the same attractive properties of AuNPs but with a strong longitudinal localized SPR (LSPR) band tunable from visible to NIR wavelengths. Additionally, they exhibit strong plasmon-enhanced multi-photon luminescence with a brightness that rivals common organic fluorophores [165]. To adapt GNRs for deep in vivo brain imaging, Wang et al. modified a conventional seed-mediated synthesis to create large aspect ratio GNRs (100 nm length) having LSPR peaks at 1000 nm, within the tissue transparency window for imaging the brain [37]. Using a femtosecond pulsed laser, the GNRs were found to emit broadband multiphoton luminescence (420–630 nm) owing to a combination of two-photon and three-photon excitation without any observe photobleaching. After passivation using PEG, the GNRs were intravenously injected into the mice and high-resolution imaging of brain blood vessels was achieved at depths reaching 700 μ m into brain tissue with extremely low background fluorescence. The highly sensitive nature of LSPR and the ease in which their surface can be functionalized make GNRs a promising platform for future work aimed at imparting molecular recognition to design functional chemical probes to investigate brain function in living brain tissue.

Silver nanoparticles (AgNPs) are photostable, non-photobleaching, and highly scattering nanoparticles with few, but recently increasing, applications to brain imaging. A particular class of AgNPs are silver nanoclusters (AgNCs), which are ultrasmall (with dimensions < 1 nm), biocompatible, photostable NIR fluorescent probes, with some recent progress towards adaptation for *in vivo* imaging [166, 167]. Several key optical imaging methods have been

employed for brain imaging with AgNPs as the optical probe. In one application, highly scattering AgNPs (initially $35.4 \text{ nm} \pm 5.3 \text{ nm}$) were imaged *in vivo* in glioma brain tumors using darkfield microscopy on a confocal microscope.[27] AgNPs were entrapped in a poly(lactic-co-glycolic acid) (PLGA) polymer nanoparticle and conjugated to choleroxin peptides that selectively bound to glioma cells. The AgNP-PLGA constructs with a final size of $114 \text{ nm} (\pm 2 \text{ nm})$ were injected to a mouse through the tail-vein, permeabilized through the BBB, and were shown to aggregate in glioma tumors. The AgNPs within the PLGA nanoparticle served as a contrast agent, which eliminated the need for nanoparticle surface modifications that might impact the behavior of the nanoparticle *in vivo*.

Recently, DNA-stabilized silver nanoclusters (DNA-AgNC, excitation: 495–575 nm, emission: 500–800 nm) have also been shown to be effective fluorescent sensors of neurotransmitters, such as dopamine [168]. The fluorescence of select DNA-AgNCs was found to either increase or decrease upon the addition of dopamine either pre-synthesis, for *in vitro* sensing, or post-synthesis for potential *in vivo* sensing applications. These results highlight the potential of using DNA-AgNCs as a platform for engineering selective, sensitive, ratiometric fluorescent sensors for direct fluorescent sensing neurotransmitters. With reversibility studies and further validation of their biocompatibility in brain tissue, these small AgNCs could enable *in vivo*, fluorescence imaging of neurotransmission at and around synaptic connections.

Noble metal nanoparticles, including AuNPs and AgNPs, have been well characterized for optical imaging in biological systems, and have found some applications in brain imaging. AuNPs have been studied extensively, optimized for biological delivery, and can be coupled to fluorescent drugs or fluorophores for fluorescent or darkfield imaging of brain tissue. Additionally, recent efforts focused on using noble metal nanoparticles as neurotransmitter sensors has suggested the possibility of developing methods for *in vivo* imaging of neurochemistry. Although widely regarded as biocompatible, more studies need to be conducted to characterize longevity and clearance of noble metal nanoparticles for long-term brain imaging studies.

Soft Organic Nanoparticles—Organic nanoparticles such as lipid-based nanoparticles [169] and polymer nanoparticles [170], dubbed “soft” nanomaterials, have recently gained popularity in fluorescent imaging applications [171]. Since the discovery of the liposome in the mid-1960s, these amphiphilic nanoparticles have ushered major advancements in therapeutic drug delivery through the stable encapsulation of highly hydrophobic drug molecules. Organic nanoparticles are accompanied by fewer biocompatibility issues than their inorganic counterparts, as they are composed of biodegradable monomers that can be cleared from the body [172]. These organic nanoparticles can be intrinsically fluorescent, as in the case of fluorescent conjugated polymers, or through the encapsulation of a fluorophore. Although there are benefits in implementing fluorescent soft organic nanoparticles for bioimaging, particularly for brain tissue [173], there are few examples pertinent to their implementation for brain imaging. Here, we review two major classes of organic nanoparticles in their potentials for brain imaging: lipid-based and polymer-based nanoparticles.

Lipid-based Nanomaterials—Lipids have the ability to encapsulate organic dyes and fluorescent nanoparticles to create biocompatible fluorescent nanoparticles and nanodroplets. The liposomes and micelles created in lipid formulations are often robust against different pH and chemical environments [174]. Lipid encapsulation localizes multiple organic fluorophores into the nanoparticle core, resulting in a particle with higher fluorescence signal than a singular fluorophore. Lipid encapsulation can also circumvent the insolubility of many organic fluorophores, by encapsulating fluorophores in a hydrophilic particle with a hydrophobic core [175].

This technique of lipid encapsulation was employed in the NIR fluorescent imaging of brain tumors using a hydrophobic heptamethine cyanine (IR780) fluorophore (excitation: 745 nm, emission: 815 nm) [19]. The fluorophore was encapsulated into liposomes and phospholipid micelles, with the size of the particles selectively and accurately tuned by changing the lipid formulation by the ratio of different phospholipids and cholesterol. IR780 has inherent brain tumor-targeting abilities, thus encapsulating in lipid nanoparticles allowed accumulation of micelles in brain tumors after tail-vein injection. Hong and colleagues used a similar technique to encapsulate fluorescent conjugated polymer nanoparticles with a quantum yield of 1.7% and tunable emission from 1050–1350 nm, falling within the NIR-II region of biological transparency window. They demonstrate high resolution, deep tissue imaging of mice vasculature, improving upon image quality compared to traditional ultrasound and computed tomography methods. The fluorescence emission of these nanoparticles makes them well suited deep tissue brain imaging, particularly if they can be engineered as chemical probes.

Polymer-based Nanoparticles—Polymer-based nanoparticles are comprised of two forms: fluorescent conjugated polymer nanoparticles and polymer-encapsulated fluorescent moieties. To-date, only fluorescent conjugated polymer nanoparticles and dendrimer-encapsulated fluorophores have been used for brain imaging.

Fluorescent conjugated polymers have been shown to self-assemble into nanoparticles used for neurotransmitter sensing. Conjugated copolymers of fluorene and benzothiadiazole were coupled to phenylboronic acid tags (PFPBA) to create highly sensitive, selective, and photostable fluorescent dopamine sensors (excitation: 309 nm and 419 nm, emission: 529 nm) [28]. The phenylboronic acid acts as a recognition ligand that specifically binds to dopamine over other neurotransmitters. This copolymer formulation was shown to self-assemble into spherical nanoparticles by solvent exchange which promotes formation of a hydrophobic core surrounded by a hydrophilic shell, resulting in nanoparticles of 120 nm in diameter. The authors observed no significant cytotoxicity or cell death with the concentrations of nanoparticles used in the study. These nanoparticles were used to image dopamine both in PC12 cells *in vitro*, and in zebrafish larvae *in vivo* with confocal microscopy. However, this polymer nanoparticle is unable to detect endogenous dopamine levels, owing to the lack of a fluorescent baseline, but can successfully measure exogenous dopamine added to zebrafish larvae brains. Further improvements to recognition chemistry may provide future fluorescent sensors for other neurotransmitters, expanding the range of compatible neural probes.

Dendrimers are monodispersed multibranched polymers with recent applications in brain imaging. Although not inherently fluorescent, dendrimers have been used to encapsulate or covalently tether fluorescent molecules for imaging. Of note, PEG-functionalized poly(amidoamine) (PAMAM) dendrimers have been used to encapsulate ion sensitive fluorescent dyes for the detection of important neuronal ions including potassium and sodium in brain slices [34, 176]. Dendrimers are ideal candidates for optical imaging in the brain since certain formulations can traverse the BBB. Once internalized into the brain ECS, dendrimers can be uptaken by neurons and glial cells, while the dendrimeric formulation protects the fluorescent dye cargo from triggering immune responses or interfering with receptor-antagonist interactions of neurotransmitters [177–179]. More research into ligand decoration of dendrimer surfaces and better controlled growth of dendrimer structure could further promote their use in optical imaging.

Organic nanoparticles provide a facile scaffold to create fluorescent imaging probes. The monomeric components of organic nanoparticles are smaller than the hydrodynamic cutoff of 5.5 nm for effective renal excretion from the body [180]. However, these nanoparticles often encapsulate fluorescent loads, such as hydrophobic dyes and quantum dots, that cannot be excreted renally, which poses concerns for long-term cytotoxicity. Fluorescent conjugated polymers can also suffer from loss of fluorescence originating from the chemical coupling of a ligand required for molecular recognition or targeting. Although polymeric nanoparticles are difficult to synthesize, they enable tunable surface properties such as hydrophilicity and hydrophobicity, grafting of targeting moieties, and have sizes that are amenable to brain imaging.

Non-Fluorescence Imaging Methods in the Brain

Imaging Vibrations: Surface Enhanced Raman Scattering (SERS)

In addition to fluorescence, a number of other optical processes have begun to gain popularity for brain imaging applications, including vibrational spectroscopies such as Raman scattering and infrared (IR) absorption. Vibrational spectroscopies are optical techniques that measure the vibrational modes of molecules, typically through either direct absorption of IR light or inelastic scattering of incident light (Raman). Vibrational modes are bond and functional group specific, thus provide information about the chemical composition of a sample. Vibrational imaging is therefore attractive as it provides spatially resolved and chemically specific information often without the need for molecular labelling with fluorescent dyes. However, vibrational spectroscopies, like Raman scattering and IR absorption, come with some disadvantages when applied for imaging. IR absorption comes with the drawback of low spatial resolution due to the long wavelengths used (3–100 μm), and difficulties arising from the strong absorption of water in this wavelength region. For this reason, Raman scattering, which is the inelastic scattering of photons due to vibrations in the sample, is often preferable as it can be performed with visible or near IR illumination. However, this process has very low scattering cross-sections requiring higher laser powers and long image acquisition times. One method to circumvent challenges of low signal to noise implements plasmonic nanomaterials and a process known as surface enhanced Raman scattering (SERS).

In spontaneous Raman scattering, light of frequency ω_i , incident on a molecule, excites that molecule to a virtual excited state whereupon it relaxes to an excited ground vibrational state, releasing a photon of lower frequency $\omega_i - \omega_v$, where ω_v is the frequency of the vibration (Stokes scattering) (Figure 4a). If the molecule was already in an excited vibrational state, the incident photon can gain energy from the vibration, and be released at frequency $\omega_i + \omega_v$ (anti-Stokes scattering), returning the molecule to the ground state. In SERS, the same process takes place; however, a large cross-section increase occurs as the effective local electric field around a nanomaterial is increased owing to excitation of surface plasmons [181]. Note that the scattered photon energy loss or gain still occurs in response to the vibrations of the molecules in the vicinity of the nanomaterial and not the nanomaterial itself. The nanomaterial functions solely as an “antenna,” enhancing the vibrational signals coming from its local environment.

Historically, SERS has been used to study the chemical composition of samples or their vibrational properties; however, as applied to neural imaging, SERS probes are frequently functionalized with a Raman-active probe, and/or a molecule to specifically localize the probes to a cell type. Often, Raman-active probes are developed for bio-imaging purposes with the introduction of functional groups containing vibrational frequencies in the “cell-silent” region of the spectrum, corresponding to $\sim 1800\text{--}2800\text{ cm}^{-1}$. This window is chosen as the bonds present in biological molecules vibrate at either higher or lower frequencies, providing a considerable spectral region where exogenous signals can be introduced without biological background. The probe is then imaged by specifically looking for a Raman shift in this window. However, this is less of a concern when labelling SERS nanoparticles, as the SERS signal enhancement allows for the fast collection of the entire Raman spectrum of a probe, with high spectral resolution. As a result, nanoparticle-Raman-probe conjugates for brain imaging are developed to have unique Raman spectra, but not necessarily contain modes within the cell-silent region.

One potential obstacle of SERS when applied to neural imaging is that the local field enhancement only occurs in the direct vicinity of the nanoparticle, and as such signal uniformity can vary greatly if imaging over large sample areas. Lithographic techniques for generating periodic arrays of structures exist but are technically challenging to incorporate into brain imaging. Addressing this drawback, Yamazoe et al. [182] synthesized gold nanoparticles on a random nanostructure of boehmite. The resultant random “gold nanocorals” featured $\sim 125\text{ nm}$ diameter AuNPs spaced at 10 nm or less, sufficient for maintaining local field enhancement, while greatly increasing the homogeneity of signal collection over a large surface area. Yamazoe and colleagues demonstrated the utility of this system in ischemic mouse brain slices, where ischemia was induced by middle artery occlusion. The authors determined the boundaries of the affected area with SERS imaging at two vibrational peaks, with one corresponding to adenosine (736 cm^{-1}), since ischemia is known to cause breakdown of ATP into constituent metabolites.

As with above-mentioned discussion of nanoparticles for brain imaging, similar concerns of cytotoxicity and distribution apply to SERS probes when implemented for *in vivo* imaging. In 2010, Wang et al. [183] demonstrated the feasibility of using SERS probes in zebrafish embryos, with AuNPs conjugated to a non-fluorescent Raman label, mercaptobenzoic acid,

which has unique vibrational signatures. The authors injected a 5 nM concentration of SERS probes with conjugated labels into the one-cell stage of the embryo and tracked embryonic development over 7 days. At a fully developed stage, the authors could recover SERS signals from the fore and hindbrain of the zebrafish. Importantly, this study demonstrated that the morphological development of zebrafish injected with SERS probes was indistinguishable from wildtype. Furthermore, the authors demonstrated the ability to simultaneously track SERS probes with two different Raman labels, mercaptobenzoic acid and mercaptopyridine, with vibrational signatures differing by $\sim 50 \text{ cm}^{-1}$, which is less than the linewidth of a typical fluorescent signal.

As an alternative strategy, Diaz et al. [184] used MRI guided focused ultrasound to deliver silica encapsulated AuNPs across the BBB for the purpose of studying the cancer glioblastoma in rat brains (Figure 4b). Specific targeting of SERS probes to glioblastoma cells was accomplished by functionalization with anti-EGFR (epidermal growth factor receptor) antibody, since EGFR is shown to be highly expressed in the cancer. Nanoparticles injected via the rat tail vein could be visualized in the brain parenchyma after focused ultrasound, but not solely after injection. Specific uptake of anti-EGFR SERS probes by tumor cells was verified in cultured cell lines, and particle retention was also demonstrated in human glioblastoma cells after xenograft into the mouse brain. These methodologies and results indicate the potential utility of SERS nanoparticles in studying brain cancer models.

The use of SERS nanoparticles in the study of the brain cancer glioblastoma has been further explored by other groups. Karbeber et al. demonstrated the potential clinical application of the technology with silica encapsulated AuNPs to guide glioblastoma tumor resection from mice using a hand held Raman scanning device [185]. Tumor uptake of the nanoparticles was confirmed by TEM and immunohistochemistry. The resection success rate was found to be higher than simple white light visualization and comparable to the detection abilities of traditional Raman microscopy, although the use of the handheld scanner improved acquisition speeds by orders of magnitude, making it a viable alternative for real-time brain tumor imaging in a clinical setting.

The Kircher group extended this work by developing and implementing surface enhanced resonant Raman scattering (SERRS) probes [186]. SERRS relies on the linking of a SERS nanoparticle, in this case gold nanospheres or nanostars, to a Raman label which is on electronic resonance with the excitation laser, in this case IR792 perchlorate (for the nanospheres) or IR780 (for the nanostars). The electronic resonance of the Raman reporter provides multiple additional orders of magnitude in signal enhancement leading to detection sensitivities in the femtomolar range. The IR792 construct was integrin targeted (RGD-SERRS), and the IR780 construct served as a control (RAD-SERRS). The constructs were injected in equimolar ratio into the mouse tail vein prior to imaging, and both were found throughout the brain, although the RGD-SERRS showed orders of magnitude higher signal intensity in tumor regions than the RAD-SERRS. Additionally, the RGD-SERRS allowed detection of microscopic glioblastoma invasions beyond the site of the main tumor, where RAD-SERRS was not detected.

Imaging Vibrations: Nonlinear Raman Microscopy (SECARS)

Nonlinear optical techniques require expensive pulsed lasers and additional optics to enable label-free imaging. However, nanomaterials used in conjunction with nonlinear optical techniques can enable additional functionality over label-free counterparts. One such example is a nonlinear variant of SERS, surface enhanced coherent anti-stokes Raman scattering (SECARS), which has been applied to brain imaging using iron oxide nanoparticles [187]. SECARS is based on coherent anti-stokes Raman scattering (CARS), a four-wave mixing technique which uses two incoming pulsed lasers of frequencies ω_p & ω_s such that $\omega_p - \omega_s = \omega_v$, the frequency of some molecular vibration in the sample, with the emitted photon being released at frequency $\omega_{as} = 2\omega_p - \omega_s$. This process already provides strong signal enhancement compared to spontaneous Raman scattering but can be surface enhanced as before when any of the three frequencies, ω_p , ω_s , ω_{as} , excite plasmon resonances in a nearby nanomaterial.

Machtoub et al. made use of ultra-small paramagnetic iron oxide particles (USPIO) labelled with anti-CD4 in a rat amyotrophic lateral sclerosis model. USPIO are a well-known MRI contrast agent, allowing for direct image comparisons with SECARS. SECARS imaging results indicated enhancement of lipid associated vibrations in the vicinity of the USPIO, correlated with areas of inflammatory response, due to the anti-CD4 labelling of the USPIO. The data show lipid and iron accumulation in areas associated with amyotrophic lateral sclerosis.

Imaging Absorption: Photoacoustic Imaging (PAI)

While optical imaging tools have high spatial resolution and can have high chemical specificity, optical imaging typically suffers from low penetration depths in biological tissues due to the highly scattering nature of biological tissues. Photoacoustic microscopy and tomography (PAM and PAT; or photoacoustic imaging, PAI) are recently-developed hybrid biomedical imaging tools which make use of optical excitation, typically with ns pulsed lasers, and ultrasonic detection [188]. The technique measures thermoelastic expansion due to optical absorption, providing molecular specificity based on absorption, and has deep tissue penetration capabilities due to low scattering of the released ultrasonic waves. While the spatial resolution of PAI is not as high as purely optical techniques, the penetration depth is greatly increased over fluorescence microscopy, and whole- brain images can be collected in rodents (Figure 4c). Additionally, spatial sensitivity can be improved by using certain classes of nanomaterials as contrast agents.

In 2010, Lu et al. implemented PAI with hollow gold nanospheres (HAuNS) to image the brain vasculature [189]. The HAuNS were 45 nm spheres with a hollow interior and gold shell thickness on the order of 4 nm, which absorbed at 800 nm in the NIR to maximize the optical penetration depth. The hollow interior increased absorption coefficients while decreasing scattering coefficients. When coated with PEG, the HAuNS could be injected intravenously and used to visualize brain vasculature. HAuNS enabled resolved imaging of 100 μ m diameter blood vessels in whole mouse brains. Of note, limited toxicity to the liver, spleen, and kidneys was observed via immunohistochemistry in animals injected with HAuNS.

PAI has also been used in clinical contexts for imaging brain tumor boundaries. Ray *et al.* developed nanoparticles synthesized from polyacrylamide hydrogels with conjugated Coomassie Blue dye, and further functionalized with a tumor-targeting peptide for this purpose [190]. *Ex vivo* effectiveness of PAI-based imaging with these nanoparticles was demonstrated in brains excised from rats implanted with 9L glioma, where the tumor targeted nanoparticles provided clear PAI contrast in the implantation regions. However, it was noted that Coomassie Blue, with a 590 nm maximum absorption wavelength, might not be the best dye for *in vivo* applications because of the strong absorption of blood in the visible wavelength range.

In another example of tumor imaging, Fan *et al.* developed perylene-diimide based nanoparticles for NIR PAI of tumors in the brain [191]. The perylene-diimide molecules were encapsulated with DSPE-mPEG5000 creating nanoparticles of ~48 nm in diameter, and with a maximal absorption wavelength of 700 nm. The nanoparticles were demonstrated to be chemically stable in PBS solution and mouse serum, and largely photostable under an hour of continuous illumination. Detection sensitivity was established through tests with subcutaneous injection of the nanoparticle into the lower back of mice, and a linear concentration dependence was observed for the signal with a tissue background detection limit of sub nM concentrations. Interestingly, when testing brain tumor imaging after tail vein injection of nanoparticles, increased imaging depth was achieved over time: 1-day post-injection, signals were observed from a maximum 3 mm tissue depth, whereas 2 days post-injection, signals could be observed from a 4 mm tissue depth, presumably due to increased tumor penetration of the nanoparticles over time.

To merge the strengths and balance the limitations of various imaging technologies, there has been an effort over the past decade to develop novel nanomaterials that can be imaged with a number of different modalities. An example of this effort in nanoscience is in the development of upconverting nanoparticles (UCNPs) gaining popularity as both fluorescent reporters and strong MRI contrast agents. In this vein, Kircher *et al.* developed a three imaging modality nanoparticle: MRI, PAI and SERS (MPR nanoparticle) applied to glioblastoma tumor imaging in the mouse brain [192]. The MPR nanoparticle is composed of an Au core with a Raman active label, trans-1,2-bis(4-pyridyl)-ethylene, coated successively in silica and gadolinium (Gd) for MRI contrast. *In vitro* sensitivity tests in agarose showed that the MPR nanoparticle displayed pM sensitivity when imaging with MRI and PAI, and sub pM sensitivity with SERS. To test the effectiveness of MPRs as a brain tumor imaging agent, human glioblastoma cells were transplanted into mice, and MPRs were injected via the tail vein. All modalities co-localized the MPRs within the brain tumor, as verified by immunohistochemistry. Authors further implemented the MPRs to guide tumor resection, including microscopic tumor extensions detected by Raman and not visible to the eye. As a clinical tool, probes like MPRs can leverage modalities like MRI and PAI to identify tumors and guide early resection, and leverage the higher sensitivity of SERS to verify microscopic clearance of smaller tumors.

Conclusions and Outlook

A comprehensive understanding of brain structure, physiology, activity, and neurochemistry hinges on future efforts pushing the boundaries of imaging neurons, their structural connectivity, their long-range activity, and their chemical communication and modulation. Improving our understanding of these systems through new microscopy techniques and functional probes stands to inform the study of complex brain functions and aberrations thereof [193]. Deep-brain imaging requires (i) overcoming microscopy challenges inherent to imaging targets within a highly optically scattering medium, and (ii) concurrent development of probes that respond to selective neurochemical stimuli in the neuronal environment (Figure 5). The tunable optical, chemical, and structural properties of nanomaterials make them amenable to the multifunctional requirements for visualizing the physiology, structure, electrical activity, and chemical activity of the brain. Methods to design nanomaterials with the chemical and optical properties to concurrently provide the aforementioned information are needed, as it is becoming increasingly apparent that the presumed relationship between action potentials (electrical), readily quantified by modern techniques, and neurotransmission (chemical) cannot be directly inferred. For instance, recent work has shown that each action potential has a mere 6% probability of releasing neurotransmitter dopamine [194], a key neurotransmitter implicated in decision-making, learning, and motor function, whereby aberrations in dopamine signaling result in anxiety, depression, and loss of motor function. Thus, when relying solely on the brain's electrical signals to study the brain, we impose boundary conditions on the three primary dimensions with which we can study the brain: structure, electrical activity, and neurotransmission (Figure 1). Furthermore, if we are blind to the brain's neurochemical signals, we lack the capability to link the role of neuromodulators to the other 'dimensions' of the brain: structure and action potential activity. Thus, there is much opportunity for nanomaterial-based optical probes to elucidate the 'missing optical dimension' of the brain: neurochemistry. Toward this end, the continued development in the design and applications of nanomaterials for neuroimaging must focus on overcoming the challenges inherent to neurochemical imaging in such a complex environment, including colloidal and chemical stability, biodistribution and targeting, molecular specificity, and near infrared fluorescence. Such challenges look to be achieved through continued engineering of the many classes of nanomaterials and may be met with parallel advances in fluorescence microscopy with the combined goal of deeper imaging of brain structure, activity, and higher-order neurochemical function.

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Figure 1. Understanding brain function requires continued improvement and development of imaging methods to visualize brain

i) physiology and structure, ii) activity and connectivity of neurons and neural circuits, and iii) dynamics of neurochemistry with requisite spatial (nm) and temporal (ms) resolution.

Figure 2. Modes of optical microscopy for brain imaging.

(a) Mechanism of excitation: In multiphoton excitation (MPE) simultaneous absorption of photons allow an electron to be excited directly to a higher excited state, without an intermediate electron between ground and excited states. Shown here are examples of two photon excitation (2PE) and three photon excitation (3PE), though four photon excitation (4PE) has also been demonstrated. Up-conversion excitation (UCE) involves an intermediate state that is lower than the emission energy, whereby the remaining excitation energy is typically provided by a phonon. (b) Light path diagrams of imaging modalities: In widefield microscopy the beam fills the field of view (FOV) and the entire volume is subject to excitation and emission photons. In confocal microscopy, pinholes limit the excitation volume and the collected emission volume in the z-axis. The FOV is limited to a diffraction limited spot, scanned across the region of interest to generate an image. In multiphoton microscopy, the light path is scanned similar to confocal, however the MPE mechanism

limits the excitation volume to be smaller in z than can be achieved with the confocal pinholes. In light-sheet microscopy, the light path travels in the same plane as the FOV providing a roughly two-dimensional sheet shaped excitation and emission volume.

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Figure 3. Schematic representation of nanomaterials used for fluorescence imaging of the brain ranging from whole-brain, to brain slice, to single-cell.

Current and potential brain imaging applications include (i) carbon nanomaterials, including single-walled carbon nanotubes (SWNTs), multi-walled carbon nanotubes (MWNTs), graphene quantum dots (GQDs), and carbon nanodots (CDots); (ii) inorganic nanomaterials, including quantum dots (QDs), noble metal nanoparticles (NPs), and noble metal nanoclusters; and (iii) soft organic nanomaterials, including lipid nanoparticles and polymer nanoparticles.

Figure 4. Raman-Based Brain Imaging.

(a) Raman Scattering and Surface Enhanced Raman Scattering: Raman scattering is the inelastic scattering of light due to molecular vibrations. At room temperature, since most compounds are in their ground vibrational state, the inelastically-scattered light is of lower energy than the incident light, with the energy difference corresponding to the energy of the molecular vibration (left). Raman scattering is an infrequent process; however, the use of plasmonic nanomaterials (right) results in a local field enhancement through the process of Surface Enhanced Raman Scattering (SERS), resulting in a signal increase and faster imaging rates. For brain imaging applications, Raman-active probes are usually affixed to plasmonic nanoparticles (e.g. AuNPs) (right). (b) SERS Probes for brain cancer imaging: SERS probes, labelled with specific Raman active molecules, can be applied for brain imaging to detect brain tumors. Cancer cells are specifically targeted through functionalization of SERS probes with targeting molecules, or through exploitation of the ‘enhanced permeability and retention’ effect, where cancer cells can more readily uptake and retain nanomaterials. Detection of the Raman active molecule’s signature indicates cancer (top spectrum), whereas lack of signal indicates healthy tissue (bottom spectrum). (c) Photoacoustic Imaging: The photoacoustic effect, where absorption of light results in thermoelastic expansion, can be exploited for Raman imaging. Certain nanomaterials, such as gold nanoparticles (top), have large photoacoustic efficiencies which provide strong imaging contrast over large areas. Introduction of nanoparticles can therefore be used to

construct tomographic reconstructions of large segments of brain morphology, e.g., the vasculature (bottom).

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Figure 5. The final frontier in brain imaging aims to image neurochemistry with high spatial and temporal resolution deep within the living brain.

Such methods will more tightly couple and quantify the relationship between brain activity, such as neuronal firing, and neurochemical release events, providing important insights for understanding chemical modulation as it relates to function, behavior, and disease.