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Measuring glymphatic function: assessing the toolkit

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From the Contents

Abstract

Glymphatic flow has been proposed to clear brain waste while we sleep. Cerebrospinal fluid moves from periarterial to perivenous spaces through the parenchyma, with subsequent cerebrospinal fluid drainage to dural lymphatics. Glymphatic disruption is associated with neurological conditions such as Alzheimer's disease and traumatic brain injury. Therefore, investigating its structure and function may improve understanding of pathophysiology. The recent controversy on whether glymphatic flow increases or decreases during sleep demonstrates that the glymphatic hypothesis remains contentious. However, discrepancies between different studies could be due to limitations of the specific techniques used and confounding factors. Here, we review the methods used to study glymphatic function and provide a toolkit from which researchers can choose. We conclude that tracer analysis has been useful, *ex vivo* techniques are unreliable, and *in vivo* imaging is still limited. Finally, we explore the potential for future methods and highlight the need for *in vitro* models, such as microfluidic devices, which may address technique limitations and enable progression of the field.

Key Words: aquaporin-4; cerebrospinal fluid; efflux; glymphatics; imaging; influx; methods; microfluidics; parenchyma; periarterial; perivenous; tracer

Introduction

The existence of a perivascular cerebrospinal fluid-interstitial fluid (CSF-ISF) exchange system in the brain has been known since the 1980s (Rennels et al., 1985). However, the inability to replicate these initial findings led to the conclusion that CSF only inconsistently entered perivascular spaces (Ichimura et al., 1991). Due to *in vivo* microscopy advancements, this waste exchange system was rediscovered in 2012, and the term "glymphatic system" was coined (Iliff et al., 2012; **Figure 1**). This glymphatic system can be divided into five distinct segments: (1) CSF is produced by the choroid plexus (and is also likely generated from extrachoroidal sources, including capillary influx and metabolic water production); (2) CSF enters periarterial spaces, potentially driven by arterial wall pulsatility; (3) CSF enters the brain parenchyma via advection, termed "influx." This is likely paracellular, though water molecules may also enter transcellularly via aquaporin-4 (AQP4) channels on astrocytic end-feet; CSF mixes with ISF; (4) the combined CSF-ISF fluid is transported to the perivenous circulation, termed "efflux"; (5) The fluid drains out of the brain primarily along meningeal and cervical lymphatics, as well as along nerve sheaths, with additional drainage via arachnoid granulations and arachnoid cuff exit points. It has since been shown that glymphatic dysfunction is associated with several neurological conditions such as stroke, Alzheimer's disease, and traumatic brain injury (Rasmussen et al., 2018).

As the glymphatic field is relatively new, many subjects of debate remain, such as whether AQP4 is necessary for CSF transport (Smith et al., 2017; Mestre et al., 2018b), the relative importance of diffusion or advection in the interstitial space (Quirk et al., 2024); whether arterial pulsation (Iliff et al., 2013b) or neuronal network activity (Jiang-Xie et al., 2024) drive CSF flow; and whether solutes can travel from the parenchyma through the periarterial basement membrane to enter the bloodstream (Albargothy et al., 2018). Additionally, there has been a recent debate on whether glymphatic flow increases or decreases during sleep (Miao et al., 2024). Such controversy may result from methodological differences, as techniques have varying degrees of reliability, or experimental design. This review will discuss the reliability of historical and current techniques used to measure glymphatic function, confounding factors, and future methods, such as miniature microscopy, genetic manipulations, and *in vitro* models. Altering glymphatic flow using ultrasound (Lee et al., 2020) or transcranial magnetic stimulation (Liu et al., 2017) will not be discussed here, nor mathematical modeling of the glymphatic system, which has been extensively reviewed elsewhere (Bohr et al., 2022).

Search Strategy

This review article examined publications from PubMed and Google Scholar with searches up to August, 29, 2024. The exact keywords used were: glymphatic system, glymphatics, influx, efflux, CSF, perivascular, aquaporin-4, glymphatic techniques, and glymphatic imaging. No restrictions were set on publication year or authorship.

Tracers

To characterize the physiology of CSF and waste transport, tracers are traditionally injected into the cisterna magna (Iliff et al., 2012), directly into the brain parenchyma (Cserr et al., 1977), or into the periphery (Iliff et al., 2013a; **Table 1**). These tracers are often conjugated to a fluorescent molecule such as Texas Red (Iliff et al., 2012) or fluorescein isothiocyanate (Iliff et al., 2012), so they can be visualized either *in vivo* or *ex vivo*. Tracer administration is needed for nearly all the methods used to study glymphatic function, which are summarized later (**Table 2**, **Figure 2**, and **Figure 3**).

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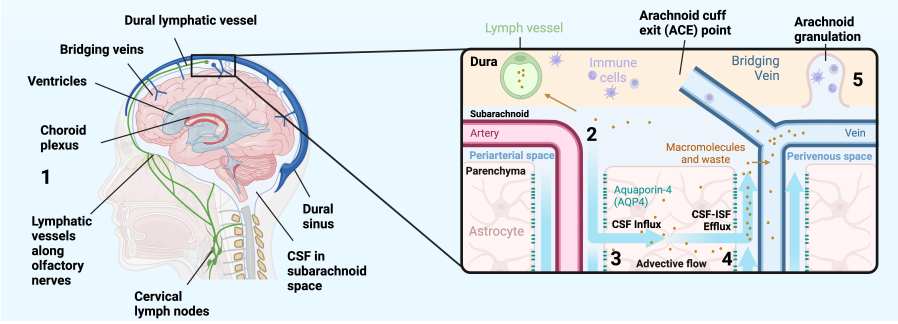

Figure 1 | **Structure of the glymphatic system.**
(1) Cerebrospinal fluid (CSF), produced by the choroid plexus, is found in the ventricles and subarachnoid space; (2) CSF enters periarterial spaces; (3) CSF flows from the periarterial space to the brain parenchyma, termed "influx"; (4) Here, CSF mixes with interstitial fluid (ISF), and the CSF-ISF fluid is exported into the perivenous space, termed "efflux"; (5) The CSF-ISF fluid can drain into dural lymphatics via arachnoid cuff exit (ACE) points, which facilitate direct communication between the dura and parenchyma. Some fluid also drains directly into the blood via arachnoid granulations, which contain dense populations of immune cells for surveillance. The CSF-ISF fluid is eventually exported to extracranial lymphatics. Created with BioRender.com.

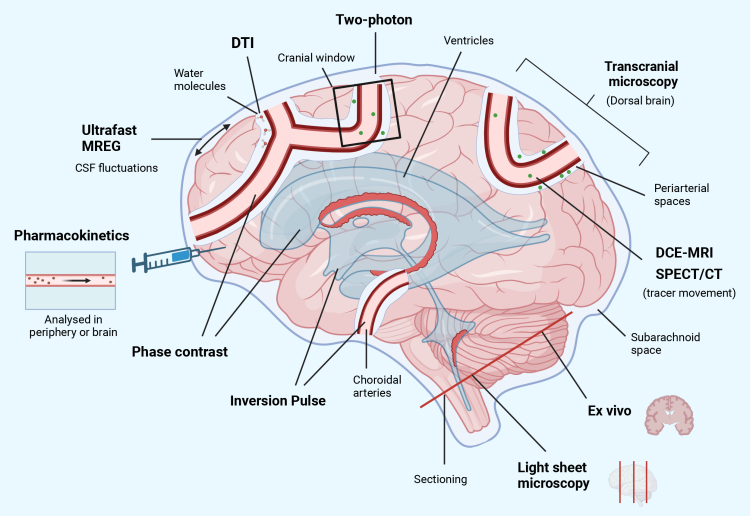

Figure 2 | **Schematic representation of the methods used to study glymphatics.**
DCE-MRI: Dynamic contrast-enhanced magnetic resonance imaging; DTI: diffusion tensor imaging; MREG: magnetic resonance encephalography; SPECT/CT: single-photon emission computed tomography/computed tomography. Created with BioRender.com.

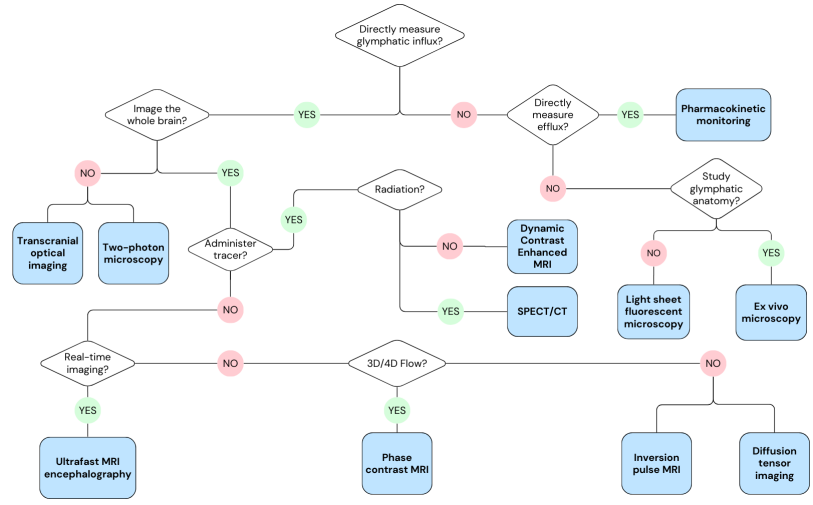

Figure 3 | **A proposed framework for selecting an appropriate method to study the glymphatic system.**
The choice of method depends on the specific research question being asked. MRI: Magnetic resonance imaging; SPECT/CT: single-photon emission computed tomography/computed tomography.

Small molecular weight (MW) tracers are typically transported quickly along the penetrating periarterial spaces into the cortical ISF after injection (Xie et al., 2013). In contrast, large MW tracers above approximately 40 kDa do not pass into the ISF, presumably due to the narrow gaps between astrocytic end-feet (Rennels et al., 1985; Iliff et al., 2012). The exact size threshold will depend on the properties of the molecule, such as charge or shape. Hence, large MW tracers can be used to label the perivascular spaces (Rennels et al., 1985; Ichimura et al., 1991; Iliff et al., 2012), but influx and clearance cannot be measured. It is important to note that tracer transport and distribution can vary significantly as a function of an experimental animal's sleep state. This can mask differences in results between large and small MW tracers. Such confounding factors will be highlighted later, in Section **Anesthesia and Confounding Factors**.

Whilst tracer analysis has been important in understanding glymphatic flow, many studies use the terms "water" and "fluid" interchangeably, which can be misleading (Salman et al., 2021a). It is important to distinguish that classical tracers measure paracellular fluid flow, whereas an $H_2{}^{17}O$ magnetic resonance imaging tracer tracks water movement (Alshuhri et al., 2021). $H_2{}^{17}O$ was shown to fully penetrate the rat rostral cortex within 10 minutes, whereas classical tracers did not penetrate even after 85 minutes. This is likely explained by the additional diffusion of $H_2{}^{17}O$ through AQP4, rather than classical tracers underestimating bulk ISF flow, as the authors concluded (Alshuhri et al., 2021). This suggests a need to establish more precise definitions in the glymphatic field.

The other major criticism of cisterna magna tracer injection is its potential to increase intracranial pressure (ICP) (Vinje et al., 2020), which may artificially drive tracer influx. However, in most injection protocols, the tracer is infused into the brain only for a short period of 5–10 minutes (Ramos et al., 2019). During the rest of the experiment, the pump is switched off. Therefore, tracer influx first occurs after ICP has reverted to its baseline, and changes in ICP due to tracer injection do not directly affect influx (Mestre et al., 2020b). Additionally, physiological changes such as Valsalva manoeuvres or head tilt cause increases in ICP greater than the 1–4 mmHg rise during tracer injection (Papadopoulos et al., 2004; Guild et al., 2015; Mestre et al., 2020b). A recent study used a dual-syringe system to control for changes in CSF volume and ICP (Raghunandan et al., 2021), but the results were equivalent to those of traditional tracer studies.

Following intraparenchymal injection, the total volume and rate of tracer entry could have a greater effect on ICP and tracer influx (Mestre et al., 2020a), as the neuropil is presumed to have a lower compliance relative to the cisterna magna (although this has not been directly measured in the literature). Hence intraparenchymal injections are typically used to study routes of glymphatic efflux rather than influx (Cserr et al., 1977, 1981).

Ex Vivo Techniques

Ex vivo techniques first involve tracer administration into the CSF or brain parenchyma of experimental animals, followed by analysis of tracer distribution in brain slices over time via fluorescent imaging (Cserr et al., 1981). This is particularly useful for studying efflux, as its measurement *in vivo* is challenging.

Table 1 | **Common tracers and their sizes**

Tracers	Large or small molecular weight tracers	Molecular weight (kDa)	Hydrodynamic radius (nm)	Methods	References
Magnetic resonance imaging contrast agents (gadolinium conjugates)	Small	0.6	≈ 2.6 (estimated)	Estimation relative to dextran 3	Ringstad et al., 2018; PubChem, 2024
Dextran	Small	3	2.33±0.38	Dynamic light scattering	Choi et al., 2010; Iliff et al., 2012
Amyloid-β	Small	4	0.9±0.1	Fluorescence correlation spectroscopy	Murphy and LeVine, 2010; Nag et al., 2011; Iliff et al., 2012
Dextran	Large	40	4.78	Capillary Viscometry	Armstrong et al., 2004; Iliff et al., 2013b
Horseradish peroxidase	Large	44	8	Dynamic light scattering	Rennels et al., 1985; Tan et al., 2016
Albumin	Large	67	3.51	Capillary viscometry	Ichimura et al., 1991; Armstrong et al., 2004; Mishra and Heath, 2021
Dextran	Large	70	10.2±1.4	Dynamic light scattering	Choi et al., 2010; Iliff et al., 2013b
Fluorescent microspheres	Large	≈ $2 \times 10^7 - 2 \times 10^{14}$ (estimated)	10–2000	Electron microscopy	ThermoFisher, 2010; Mestre et al., 2018a

Hydrodynamic radius refers to the size of a hypothetical sphere that diffuses at the same rate as the molecule in a fluid; kDa: kilodaltons.

Table 2 | **A summary of the main techniques used to study glymphatics**

Technique	Application	Advantages	Disadvantages	References
Ex vivo imaging	Can study influx or efflux by analyzing tracer distribution in brain slices	- Enables study of efflux - Can be used to study glymphatic anatomy	- Many animals required for statistical power - Artifacts in post-mortem processing	Rennels et al., 1985
Light sheet fluorescent microscopy	Uses a thin (nm- μM) sheet of light to excite only fluorophores in a certain volume	- Whole brain can be imaged - High processing speed - Greater resolution than magnetic resonance imaging (MRI) - Reduced photodamage and stress on samples	- Fixation may cause the collapse of perivascular spaces - Increased storage and computational power required	Bèchet et al., 2020
Two-photon imaging	Cerebrospinal fluid (CSF) tracer flow can be measured in superficial vessels	- High spatial resolution (μm) - High temporal resolution (ms) - Multiple fluorophores for color	- Invasive - Narrow field of view and ~250 μm depth - Motion artifacts	Ichimura et al., 1991; Iliff et al., 2012
Transcranial optical imaging	Fluorescent tracers injected into CSF and imaged through an intact skull with a wide field of view	- Can image a larger surface area on the dorsal side	- Limited depth - Cannot image ventral brain	Plog et al., 2018
Dynamic contrast-enhanced MRI (DCE-MRI)	CSF influx and efflux using contrast entry and elimination	- Whole-brain imaging - 3D image - Dynamic CSF flow information over time	- Low spatial resolution - Not in real-time - Adverse effects due to gadolinium-based contrast agent toxicity - Motion artifacts	Iliff et al., 2013a; Ringstad et al., 2017
Diffusion tensor imaging (DTI)	Study of water movement in perivascular spaces	- No contrast agent needed	- Only measures passive diffusion of water rather than CSF flow - Low spatial resolution - Only one region measured a time - Motion artifacts	Taoka et al., 2017; Harrison et al., 2018
Inversion pulse MRI	Study of CSF or blood flow	- No contrast needed - Real-time imaging - Can visualize detailed structures	- Time-spatial labeling inversion pulse findings may contrast with conventional physiology e.g. CSF backflows to lateral ventricles - Arterial spin labeling (ASL) can only measure in proximity to the choroid plexus - ASL also has a low signal-to-noise ratio	Posse et al., 2013; Yamada et al., 2014; Evans et al., 2020; Petitclerc et al., 2021
Phase contrast MRI	Velocity of blood or CSF flow	- No contrast needed - Real-time imaging - Gives 3D/4D information	- Difficult to study CSF within the brain parenchyma - High scan times due to low flow velocities - Limited quantification approaches - Motion artifacts	Battal et al., 2011; Dreha-Kulaczewski et al., 2017
Ultrafast MRI encephalography	Monitoring of CSF pulsations	- Real-time imaging - Non-invasive	- Limited spatial solution - Limited quantification approaches	Kiviniemi et al., 2016
Single-photon emission computed tomography/ computed tomography (SPECT/CT)	Can study influx and efflux	- Quantitative imaging of the whole central nervous system - Structural and functional information	- Ionizing radiation - Low spatial resolution (CT provides resolution only for hard tissue)	Lilius et al., 2022; Sigurdsson et al., 2023; Kroesbergen et al., 2024
Pharmacokinetic techniques	Uses modeling and diffusion of fluorescent tracers	- *In vivo* - Can directly study efflux	- Can be invasive - Kinetic modeling may not reflect clearance *in vivo*	Plà et al., 2022; Miao et al., 2024

Light sheet microscopy is a recent alternative to traditional *ex vivo* imaging. It relies on first administering a fluorophore, fixation of the brain with transcardial perfusion of paraformaldehyde, and finally clearing via the iDISCO protocol (Bèchet et al., 2020). By illuminating many slices with a sheet of light that excites fluorophores within a particular depth, a 3D image of the tracer's distribution can be built. Although the microscopes are expensive, once set up, this technique is cost-effective and fast- requiring only 25 minutes to image the entire brain, compared to the 2 hours needed for traditional *ex vivo* slicing and microscopy (Bèchet et al., 2020).

Interpretation of data derived from *ex vivo* techniques has been inconsistent. One issue is that perivascular spaces are absent from histological sections. This is believed to be due to the collapse of vessels upon death, which causes a tenfold decrease in the cross-sectional area of perivascular spaces (Mestre et al., 2018a).

Euthanasia also causes a pathological influx of CSF into the perivascular spaces (Ma et al., 2019). Therefore, CSF tracers are displaced into the surrounding smooth muscle layer and basal lamina as an artifact. This could be why several teams have concluded from their data that arterial pulsatility cannot drive advection (Asgari et al., 2016; Kedarasetti et al., 2020), in contrast to an opposing consensus that it does (Mestre et al., 2018a; Yokoyama et al., 2021). Hence, there has been a shift towards *in vivo* imaging techniques to resolve such outstanding questions.

Nonetheless, *ex vivo* techniques have proven invaluable for studying one niche of glymphatic anatomy. Dural lymphatics are important for immune surveillance, and their discovery can be attributed to the immunostaining of a whole-mount preparation of murine meninges (Louveau et al., 2015). The authors stained for T cells (CD3e) and endothelial cells (CD31). Unusually, T cells were aligned linearly next to the endothelial cells, which were later identified to be lymphatic endothelial cells (Louveau et al., 2015). Additionally, *ex vivo* immunofluorescence and electron microscopy led to the recent discovery of arachnoid cuff exit points (Smyth et al., 2024) – directly connected regions of dura and parenchyma due to discontinuities in bridging veins (**Figure 1**). However, it remains to be confirmed by other groups whether these structures are reproducible.

In Vivo Techniques

Fluorescent imaging

The earliest *in vivo* glymphatic imaging technique was two-photon microscopy (Cserr et al., 1981; Ichimura et al., 1991). CSF flow, for the first time, could be quantified in the live brain at a high spatial resolution of up to 1–2 μm, using fluorescent tracers. The general principles have been reviewed in detail elsewhere (Xu et al., 2024), but to summarize the procedure, the animal has a craniectomy, and the superficial cortex is imaged through a closed glass coverslip (Iliff et al., 2012). However, there are two notable issues with this method.

First, two-photon imaging is invasive. The glymphatic system is fragile and even the slightest manipulation can disrupt the pressure gradients that drive CSF flow. For example, intracranial pressure drops following craniectomy (Plog et al., 2019). Even without penetration of the dura, craniectomy alone can impair tracer influx via reduced arterial pulsation (Plog et al., 2019). It was also shown that influx fell by 40% when a glass pipette was inserted into the parenchyma (Mestre et al., 2018b). Nonetheless, many imaging protocols require such invasive methods, although some trauma can be alleviated by leaving the needle in place during experiments (Ramos et al., 2019).

Second, two-photon microscopy is limited to measuring depths of up to 250 μm below the surface of the cortex (Iliff et al., 2012; Xie et al., 2013), which is only suitable for studying surface level glymphatic flow, such as meningeal lymphatics (Louveau et al., 2015). This limitation also applies to more recent fluorescent imaging techniques, such as transcranial optical imaging, used to visualize the dorsal cortex (Plog et al., 2018). Additionally, the imaging field of view is small, ranging from 0.8 to 20 mm^2 (Cramer et al., 2021). Although experiments are performed on a limited region of the cortex, results are often generalized to the whole brain, which could be misleading. For example, one foundation of the glymphatic hypothesis is that there is a 60% decrease in the brain extracellular space in awake versus sleeping mice (Xie et al., 2013). In this experiment, extracellular space was measured via a cranial window. This idea was challenged by a later study (Gakuba et al., 2018), which used diffusion weighted imaging and apparent diffusion coefficient as a proxy for extracellular space, and found no significant differences in awake and anesthetized mice (Gakuba et al., 2018). Furthermore, another group measured apparent diffusion coefficient in human volunteers with magnetic resonance imaging (MRI) and reported no global changes in apparent diffusion coefficient (Demiral et al., 2019). However, there were regional changes, such as in the cerebellum and left temporal pole. This highlights that a small cranial window is insufficient to study such complex global glymphatic changes, and thus whole-brain imaging techniques are required.

Contrast-enhanced magnetic resonance imaging

Glymphatic transport in the whole brain was first captured using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) (Iliff et al., 2013a). A paramagnetic contrast agent, typically a gadolinium-based compound (GBCA), is injected either intrathecally, intracisternally, or intravenously preceding the scan (Iliff et al., 2013a, Naganawa et al., 2017, Ringstad et al., 2018). The distribution of the tracer over time can be used to quantify influx, or indirectly study efflux using computational approaches (Iliff et al., 2013a).

DCE-MRI is used to study pathologies, such as aging, traumatic brain injury, dementia, and stroke (Taoka and Naganawa, 2021). DCE-MRI also enabled the study of human glymphatics (Eide and Ringstad, 2015; Ringstad et al., 2017). Although administration of high-dose GBCA can cause encephalopathy, leading to death, low-dose GBCA is typically used to diagnose conditions of CSF leakage (Arlt et al., 2007; Eide and Ringstad, 2015). Ringstad et al. (2017) conducted the pioneering study and concluded there was a difference in CSF flow between idiopathic normal pressure hydrocephalus patients and controls. In controls, the GBCA distributed in a pattern consistent with the rodent brain, but the CSF signal amplification was much slower – perhaps due to differences in brain size, metabolic rate, vasculature, and AQP4 polarization (Iliff et al., 2013a; Eidsvaag et al., 2017). GBCA distribution also revealed that the final destination of glymphatic drainage is the cervical lymph nodes (Eide et al., 2018).

Contrast-free magnetic resonance imaging

There are several MRI-based approaches to study glymphatics, which do not involve tracer administration due to its invasiveness, inability to image in real time, and potential toxicity. Here, we focus on diffusion tensor imaging, phase contrast MRI, inversion pulse MRI, and ultrafast MRI encephalography as they are the most common techniques used.

Diffusion tensor imaging (DTI) can be used to analyze CSF movement. One variation of DTI is diffusion tensor image analysis along the perivascular space (DTI-ALPS), which measures the diffusivity of water molecules in perivascular spaces at the level of the lateral ventricles (Taoka et al., 2017). However, it is unclear whether the signal truly reflects perivascular flow in the glymphatic system. This is because cerebral waste clearance includes both diffusion and advection, whereas DTI-ALPS only measures passive diffusion of water as it uses a high b value (~1000 s/mm^2) (Kaur et al., 2024). In DTI, the b value quantifies the diffusion weighting, with a high value reflecting the increased sensitivity of the image to slower water movement. Furthermore, generalizing the results of DTI-ALPS to the whole brain could be misleading as DTI-ALPS primarily measures diffusivity in deep cerebral white matter, where perivascular spaces are considerably richer and denser in the cortex relative to subcortical regions (MacGregor Sharp et al., 2020). Additionally, perivascular spaces constitute only around 1% of white matter, so it is unlikely that the DTI-ALPS index can distinguish perivascular water diffusivity from other sources of directional water movement, such as along fiber tracts (Ringstad, 2024). DTI-ALPS is also constrained by the imaging plane as diffusivity measurements are limited to the x, y, and z axes, and by head movement, which may introduce imaging artifacts, although a method to retrospectively correct for the head angle has been proposed (Tatekawa et al., 2023).

Inversion pulse MRI magnetizes fluid (such as CSF or blood) for use as an endogenous tracer. One variation is time-spatial labeling inversion pulse (Yamada, 2014), which uses CSF as the tracer. This method has elicited interesting results which contrast with conventional physiology, such as the finding that CSF backflows from the third ventricle to the lateral ventricles (Yamada, 2014). Another variation is to magnetize arterial blood as a tracer, termed arterial spin labeling (ASL) (Evans et al., 2020). ASL has been used to study the blood-CSF barrier and CSF production from blood water. However, the signal is also observed around the cortex, pointing towards a blood-CSF exchange site in the subarachnoid, possibly related to arachnoid cuff exit points or AQP1 in pial vasculature (Petitclerc et al., 2021; Li et al., 2023). A significant limitation of ASL is that CSF analysis is predominantly constrained to proximal regions of the choroid plexus as it is difficult to separate the signal from large draining veins, although it is possible to image the subarachnoid space surrounding the cortex when using a modified MRI sequence (Petitclerc et al., 2021). ASL also has a low signal-to-noise ratio, and a larger region of interest is required for analysis (Joseph, 2021). Alternatively, 3D T2-Fluid Attenuated Inversion Recovery with a T2 inversion recovery pulse is a third variation of inversion pulse MRI, which nullifies the CSF signal before the image is taken, allowing visualization of detailed structures such as dural lymphatics and drainage along nerves. However, this method may not fully suppress CSF near bone or air, resulting in artifacts near the skull base (Oshio et al., 2021; Albayram et al., 2022).

Phase contrast MRI can measure the velocity of CSF or blood. Four-dimensional phase-contrast

imaging enables the assessment of vascular morphology hemodynamics (Ha et al., 2022). For example, four-dimensional phase-contrast imaging allows the study of intracranial arterial pulsatility, which has been shown to correlate with glymphatic neuroimaging markers (Xie et al., 2024). However, using four-dimensional phase-contrast imaging to image the CSF flow itself is more difficult. This is due to slower velocities and a long T1 longitudinal relaxation time, leading to longer scan times and potential motion artifacts (Rivera-Rivera et al., 2024). Furthermore, using phase contrast to study CSF flows within the brain is still technically challenging. Hence, imaging is limited to the subarachnoid spaces and ventricles. Although one phantom study attempted to measure creeping intraparenchymal CSF flows, this was not validated *in vivo* (Magdoom et al., 2019).

Finally, ultrafast magnetic resonance encephalography detects CSF pulsations within 100 milliseconds of each other. It uses ultra-fast gradient echo pulses and has enabled the detection of cardiac and respiratory pulsations, which affect CSF flow (Kiviniemi et al., 2016). However, ultrafast magnetic resonance encephalography is limited by its spatial resolution, with a voxel size of 3 × 3 × 3 mm^3. Additionally, it depends on blood oxygen level dependent signals, which are an indirect measure of neural activity and fluid dynamics rather than directly measuring CSF itself. It also generates large volumes of data, requiring advanced computational methods for analysis.

Single-photon emission computed tomography/ computed tomography

Single-photon emission computed tomography (SPECT) imaging is one of the few alternatives to MRI for imaging glymphatic flow. Combining SPECT with computed tomography (CT) allows for tracer detection in 3D with a higher sensitivity and specificity than MRI (Sigurdsson et al., 2023). Nevertheless, its low spatial resolution of ~0.9 mm results in difficulty resolving small compartments. Therefore, it is challenging to accurately track the distribution of the tracer between blood, CSF, and ISF within the brain. Instead, the authors used indirect methods, such as analyzing a region of interest in the heart to estimate the tracer levels in the systemic circulation (Sigurdsson et al., 2023).

Pharmacokinetic techniques

We have thus far focused on imaging techniques, but one group has developed a novel method that can directly measure efflux *in vivo* using pharmacokinetics (Plá et al., 2022). This method avoids the need for *ex vivo* tissue, which can introduce post-mortem artifacts and distort efflux predictions (Mestre et al., 2018a; Ma et al., 2019). Furthermore, this approach reduces the number of animals required, since real-time efflux can be assessed in individual mice.

Evans blue (DB53), a blood-brain-barrier impermeable dye, is first injected into an experimental animal's striatum (Plá et al., 2022). The pharmacokinetics of DB53's efflux is determined by continuous measurement of signal in the femoral vein. DB53 can be detected with a high signal-to-noise ratio *in vivo* in blood vessels and is retained in the vasculature for up to 3 days due to its high affinity for albumin (Wolman et al., 1981).

DB53's pharmacokinetics revealed new insights. First, by comparing the efflux times of free DB53 and DB53 bound to bovine serum albumin, efflux was shown to be size-dependent, contradicting previous literature (Cserr et al., 1977; Cserr et al., 1981). However, as only two compounds were tested, additional studies are necessary to determine whether the size limitation of clearance is linear or has a threshold. Furthermore, there appear to be at least two efflux paths (Plá et al., 2022): one with fast kinetics ($t_{½}$ = 50 minutes) and another with slower kinetics ($t_{½}$ = 240 minutes). Investigation of these efflux routes was beyond the scope of the study, but it is tempting to consider these paths representing advection and diffusion (Koundal et al., 2020).

A similar pharmacokinetic strategy has also recently been used by Miao et al. (2024) to develop novel insights into glymphatic clearance during sleep. The authors injected 4 kDa FITC-dextran into murine caudate putamen (rather than in the periphery, as was done by Plá et al. 2022), and recorded the time taken for it to be detected in the frontal cortex as a readout of clearance. Unusually, clearance was lower in sleep relative to awake and anesthetized mice (Miao et al., 2024), challenging previous literature (Xie et al., 2013; Jiang-Xie et al., 2024). However, it is possible that the craniotomy surgery on the mice resulted in the collapse of the fragile pressure gradients needed to drive glymphatic flow, although the authors delayed FITC administration by a week after the procedure, and argue this is sufficient for recovery. Additionally, one group highlighted that different tracer volumes were administered to the awake, anesthetized, and sleeping mice groups, which may account for these findings (Kroesbergen et al., 2024). Despite the ongoing controversy generated by this study, we highlight the potential for this technique to measure efflux or glymphatic flow in other domains, such as neurodegeneration, which may yield interesting results.

Anesthesia and Confounding Factors

We have already discussed how the choice of certain techniques has led to a debate in the glymphatic literature. For example, researchers used two-photon imaging to study changes in extracellular spaces during sleep, although imaging was limited to a small cranial window that may not reflect global brain changes (Xie et al., 2013). Similarly, *ex vivo* data has been used to investigate whether arterial pulsations can propel CSF in perivascular spaces, despite induced postmortem artifacts (Asgari et al., 2016). More recently, the craniotomy performed to measure the clearance in the murine frontal cortex may have been too invasive to study glymphatics (Miao et al., 2024).

Another factor that has led to debate is confounding variables in protocols. For example, it was previously unclear whether the AQP4 channel was necessary for CSF movement from the periarterial space to the parenchyma. In 2012, it was first shown that APQ4 knockout mice had deficient solute transport and clearance capacity relative to controls (Iliff et al., 2012). However, Smith et al. (2017) reported no difference in their APQ4 knockout mice and rats. A subsequent meta-analysis, with data from five independent groups, refuted the negative findings (Mestre et al., 2018b). The methodology of Smith et al. was not presented in sufficient detail to allow independent replication, with key details missing such as the injection approach and rate, choice of anesthesia, and age of the animals. It is likely that differences in experimental design led to a differing conclusion. Here, we focus on the confound of anesthesia, given its extensive study.

Glymphatic imaging typically requires anesthesia for invasive procedures such as cannulation (Xie et al., 2013; Ramos et al., 2019), but anesthesia-dependent effects have been reported (Benveniste et al., 2017; Gakuba et al., 2018). The 2012 AQP4 study used ketamine/xylazine (Iliff et al., 2012), whereas Smith et al. (2017) used Avertin. Later experiments compared CSF flow under different anesthesia protocols and found that influx was highest under ketamine/xylazine, followed by isoflurane and dexmedetomidine, isoflurane and pentobarbital, and finally Avertin (Hablitz et al., 2019). In the recent article that demonstrated glymphatic flow increased during anesthesia, the authors used 20 mg/kg xylazine (Miao et al., 2024), whereas a 2013 study used 10 mg/kg xylazine (Xie et al., 2013), which could be one factor underlying the difference in results. Such anesthesia-dependent effects on CSF flow may be explained by their mechanisms of action. For example, noradrenaline is a key regulator of glymphatic transport (Xie et al., 2013), and xylazine's action on the brain as an α2 adrenergic receptor agonist may cause increased influx (Kitano et al., 2019). Glymphatics methodologies must therefore be stringently controlled for such anesthesia effects. However, a "gold-standard" anesthesia regimen has not yet been established, and further work is needed to fully understand the underlying molecular basis of anesthesia-dependent effects.

One point of debate is whether anesthesia is a true reflection of sleep. Despite sleeping and anesthetized animals having similar changes in extracellular spaces and delta wave profiles (Xie et al., 2013), there are still unanswered questions. One study concluded that 4 hours of isoflurane treatment did not lead to normal sleep homeostasis in rats (Mashour et al., 2010). However, when 1-hour doses of isoflurane were used at a concentration that induced delta waves, it caused sleep homeostasis (Nelson et al., 2010). In this study, the delta waves recorded were skewed below 1.5 Hz, which is unusual, since this is typical of later sleep stages when sleep pressure is low (Britton et al., 2016). This may suggest that some anesthesia protocols are better at modeling the effects of natural sleep than others.

Finally, many alternative factors can affect glymphatic transport, which must be controlled. One example would be circadian effects. Whereas past studies have shown that tracer movement increased with sleep or anesthesia (Iliff et al., 2012; Xie et al., 2013), Gakuba et al. (2018) used near-infrared fluorescent imaging and DCE-MRI to demonstrate a reduction of CSF flow with anesthesia. Notably, this study scheduled imaging during the active crepuscular phase of mice, between 8–10 pm, rather than the inactive

day phase. Consequently, the timing of imaging must be controlled. Examples of other potential confounding variables include the degree of neural activity, arterial pulsatility, exercise, diet, sleep deprivation, body posture, and stress (Gędek et al., 2023; Jiang-Xie et al., 2024).

Future Methods

There have been several recent innovations that hold potential for studying glymphatics. We will first highlight some more established techniques before speculating on the future.

Miniature fluorescent microscopy

Advancements in two-photon and three-photon imaging have enabled *in vivo* measurements in freely moving mice (Zong et al., 2017; Zhao et al., 2023). Head-fixed mice are constrained physically and experience emotional stress, which may affect glymphatic flow (Wei et al., 2019). Instead, a miniature microscope, weighing only 2.15 g, can be fitted to the mouse's head (Zong et al., 2021). Although only neurons have been imaged in the sensorimotor and visual cortex (Zong et al., 2017; Zhao et al., 2023), the microscope permits a field of view of 420 × 420 × 180 μm^3, and a depth of up to 1.2 mm, which has the potential to measure glymphatic flow even in subcortical structures. For the first time, the glymphatic system can be studied at high resolution about behaviors such as feeding, fighting, and mating. However, there is almost no data in the literature, possibly due to the current lack of access to this technology.

Genetic manipulations

In addition, there is untapped potential in using genetics to study the glymphatic system. The ideal tracer should be an endogenously produced waste substance that is exported into the ISF. This would minimize invasive cannulation, which can disrupt fine pressure gradients (Mestre et al., 2018b). Amyloid-β and lactate are two such examples of waste molecules. Although there are no genetically encoded amyloid-β fluorescent sensors, many lactate sensors have been developed (Xu et al., 2016). Several studies have used viral vectors to deliver fluorescent probes into mice with high spatial resolution (Li et al., 2023; Nasu et al., 2023), and there has even been a successful extracellular lactate biosensor (Nasu et al., 2021). This has been tested in the visual cortex and muscle tissue of mice but is yet to be studied in perivascular spaces (Li et al., 2023; Nasu et al., 2023). If the probe works, the next step is to engineer a tissue-specific transgenic mouse line in which glymphatic efflux can be measured using lactate as a proxy because brain lactate is inversely correlated with glymphatic clearance (Lundgaard et al., 2017). Although MRI probes should be ideally used to avoid cranial windows, there are currently no useful genetically encoded MRI contrast agents for studying the glymphatic system (Dan et al., 2023).

Moreover, genetically encoded neurotransmitter sensors also have potential. An example would be the fluorescent noradrenaline sensors nLightG and nLightR (Kagiampaki et al., 2023). Using such tools can give new insights into which neurotransmitters are involved, especially since noradrenaline is hypothesized to mediate the effect of sleep and anesthesia on CSF flow (Xie et al., 2013; Benveniste et al., 2017).

In vitro microfluidic models

A physiologically realistic *in vitro* model of glymphatic transport is urgently needed. As discussed in the previous section, *in vivo* experiments are subject to variation depending on myriad variables including anaesthetic type, time of day, and animal age. There is a need for a reductionist model that can be used to examine variables systematically. Although non-invasive imaging techniques in humans are progressively increasing their resolution and signal-to-noise ratios (Feinberg et al., 2023), tools are required for ease of manipulation, drug discovery, and experimentation.

In vitro microfluidic models are one promising approach as they have the potential to better emulate the brain microvessel environment including the unidirectionality of flow, lack of biomembranes, physiological levels of shear stress, and presence of the cylindrical geometry typical of blood vessels to facilitate the direct cell to cell interactions and mechanobiology of extracellular matrix (Salman et al., 2020, 2021b). The first step towards a microfluidic glymphatic model was published in 2022 (Soden et al., 2022). The device consisted of two parallel microchannels in a 3D astrocyte hydrogel, as shown in **Figure 4**. Human brain microvascular endothelial cells were seeded in the left microchannel, which has a diameter of 250 µm, to form an endothelial sheet. CSF drainage was quantified by (1) adding a tracer to the left channel; (2) letting it redistribute for a defined period; (3) measuring the volume of culture medium in the right channel; and (4) quantifying the level of fluorescence on both sides. Thus, tracer flow could be determined. The group tested the effects of lipopolysaccharides, amyloid-β, and AQP4 inhibition using the inhibitor TGN-020, which has been questioned as an AQP4 pore inhibitor, and they all impaired glymphatic drainage, corresponding to previous literature reports (Iliff et al., 2012; Mestre et al., 2018b; Manouchehrian et al., 2021; Salman et al., 2022). The group later tested the effects of amyloid oligomers on AQP4 polarization using an improved device that more accurately modeled advective ISF flow (Yslas et al., 2024). This was achieved by adding ten times more medium to the left reservoir relative to the right, creating a hydrostatic pressure gradient (Yslas et al., 2024).

Nevertheless, this "glymphatics-on-a-chip" model is very limited. The model does not clearly separate the three fluid compartments of the gliovascular unit: the vascular lumen containing blood, the perivascular space containing CSF, and the parenchyma. Hence, *in vivo* glymphatic drainage is not recapitulated. Specifically, there is no modeled gliovascular unit, a lack of penetrating arterioles, and no suitable flow system.

However, bioengineering holds the potential to resolve these issues. Regarding the lack of penetrating arterioles, groups have bioengineered functional, hierarchical vessel networks (Szklanny et al., 2021). Though most of the vessels produced via bioprinting are larger than 600 µm, vessels as small as 10 µm have been made by using laser-based approaches such as multiphoton lithography (Arakawa et al., 2017). This is particularly useful for studying glymphatics in capillaries, as the functional importance of their perivascular spaces is unexplored. This is because their perivascular spaces are much smaller relative to arterioles, and there are challenges in *in vivo* imaging resolution (Hablitz et al., 2021). A combination of these techniques has the potential to generate a physiologically realistic vascular glymphatic model. Secondly, to address the lack of pulsatile blood flow, which is needed for CSF movement, unilateral flow pumps have been developed (Offeddu et al., 2021). The next step is to incorporate these into microfluidic models.

Conclusion

Technique choice has led to much controversy in the glymphatic field, especially since there is no established "gold-standard." The use of tracers is the dominant measurement technique, and despite initial concerns of a supraphysiological rise in ICP, this was later shown not to be the case. The field faced early technological constraints, relying initially on *ex vivo* techniques that lacked dynamic flow information and suffered post-mortem artifacts. *In vivo*, two-photon imaging also proved unreliable with its narrow field of view and depth. Consequently, the current standards are non-invasive methods such as MRI and SPECT (summarized in **Table 2**), which offer predictive modeling capabilities despite challenges in resolution at the capillary and lymph vessel scale.

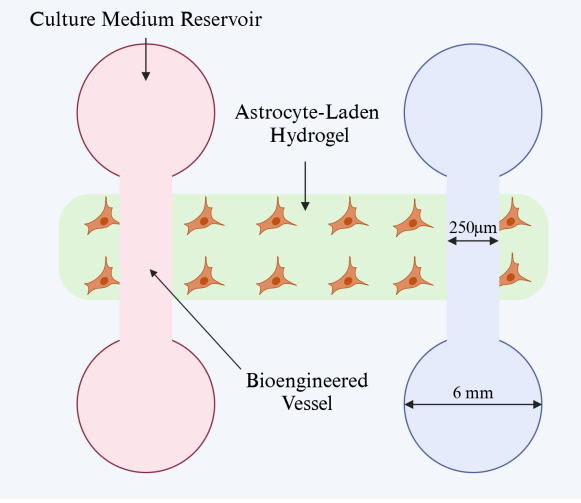

Figure 4 | **The first step towards a glymphatics microfluidic model.** Reprinted with permission from Soden et al. (2022). Created with BioRender.com.

However, even with the advent of 7T MRI, such imaging is still insufficient to study efflux and does not enable direct experimental manipulation of glymphatics. Novel techniques, like miniature fluorescent microscopy and "glymphatics-on-a-chip" models, hold promise to address these issues, but a more suitable *in vitro* glymphatic model is urgently needed. Furthermore, differing protocols have caused issues of reproducibility (e.g., with anesthesia) since the field was initially unaware that glymphatic flow could be affected by so many factors. Standardized protocols are now needed to assure reliable, reproducible results, and to enable the progression of this exciting field.

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Conflicts of interest: *PK, RMB, and MMS are founders and shareholders in Estuar Pharmaceuticals. LU has been offered vesting shares in Estuar Pharmaceuticals. KA declares no conflicts of interest.*

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