Ventricular Infusion and Nanoprobes Identify Cerebrospinal Fluid and Glymphatic Circulation in Human Nerves

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Background: Growing evidence suggests that cerebrospinal fluid circulates in human nerves. Several conditions encountered by the plastic surgeon may be related to dysregulation of this system, including nerve transection, stretch injuries, and peripheral neuropathy. The purpose of this study was to show how ventricular infusion and nanoprobes identify CSF and glymphatic circulation in neural sheaths of human nerves.

Methods: The technique of ventricular infusion using buffered saline was developed in 2017. The technique was used in a series of eight fresh cadavers before dissection of the median nerve, and combined with fluorescent imaging and nanoprobe injections in selected specimens.

Results: Eight cadaver specimens underwent ventricular infusion. There were six female and two male specimens, ages 46–97 (mean 76.6). Ventricular cannulation was performed successfully using coordinates 2 cm anterior to coronal suture and 2.5 cm lateral to sagittal suture. Depth of cannulation ranged from 44 to 56 mm (mean 49.7). Ventricular saline infusion complemented by nanoprobe injection suggests CSF flows in neural sheaths, including pia meninges, epineurial channels, perineurium, and myelin sheaths (neurolemma).

Conclusions: Ventricular infusion and nanoprobes identify CSF flow in neural sheaths of human nerves. CSF flow in nerves is an open circulatory system that occurs via channels, intracellular flow, and cell-to-cell transport associated with glial cells. Neural sheaths, including neurolemma, may participate in glucose and solute transport to axons. These techniques may be used in anatomic dissection and live animal models, and have been extended to the central nervous system to identify direct ventricle-to-pia meninges CSF pathways. *(Plast Reconstr Surg Glob Open 2022;10:e4126; doi: 10.1097/GOX.0000000000004126; Published online 17 February 2022.)*

INTRODUCTION

The purpose of this study was to show how ventricular infusion and nanoprobes facilitate the study of cerebrospinal fluid (CSF) and glymphatic circulation in the neural sheaths of human nerves. The concepts and techniques presented were developed between 2013 and 2017, and enable any interested researcher to investigate this emerging field.[1–4]

CSF circulation in paravascular channels, meninges, and neural sheaths is important to the plastic surgeon because it is associated with the blood-nerve and blood-brain barrier.[5–7] Damage to the blood-nerve barrier, perineurium, and myelin sheaths results in obligate Wallerian degeneration.[8–11] Injury to this circulation may therefore play a role in disorders commonly seen by the plastic surgeon such as nerve transection, stretch injuries, and peripheral neuropathy.[12–15]

Our understanding of the anatomy of CSF circulation in human nerves and brain is basic and limited at this time. Cruickshank and Mascagni identified parts of the CSF circulation of the human brain in 1786 and 1787 respectively, but mischaracterized them as part of the lymphatic circulation.[16,17] In 2012, Iliff et al used fluorescent dyes to show that CSF enters and is cleared from the interstitium of the brain in mice by perivenous and periarterial

CSF channels.[18] Because paravascular CSF channels are formed by the end processes of glial cells, Iliff referred to CSF flow in the murine cortex as the "glymphatic system" (glial + lymphatic). In 2015, Loveau et al identified dural lymphatic vessels in mice that drain CSF to deep cervical lymph nodes.[19]

Pessa et al identified epineurial channels and perineurium in human peripheral nerves as part of the neural CSF circulation in 2017.[1] The technique of ventricular infusion followed the hypothesis that CSF circulates through neural sheaths and meninges. The diameter of the probes used to further define this system (<10 nm) was dictated by the biology and structure of human nerve sheaths. Neural sheaths and meninges express a wide range of solute carrying proteins (SLC) involved in the active transport of glucose, lactate, pyruvate, and ketones.[20–22] Probes with a diameter closest to that of a glucose molecule (1.5 nm) were chosen. Examples are presented to show how these techniques may benefit future research in this field. Clinical implications of this work are discussed.

MATERIALS AND METHODS

Lateral ventricular infusion was performed on eight fresh cadaver specimens within 72 hours of death. The right cranium was used in all specimens for ventricular access. A posteriorly-based scalp flap was elevated after which a craniotomy was performed using the Stryker autopsy saw (Stryker Model 810, Kalamazoo, Mich.). The landmarks for access to the lateral ventricle are as follows: 2 cm anterior to coronal suture (x-axis); 2.5 cm lateral to the sagittal suture (y-axis); and 5–6 cm caudal (z-axis). Anterior-posterior positioning should be approximately 12 cm posterior to nasion. Sagittal position should correspond to the mid-pupillary plane (Kocher's Line).

The dura was punctured with a 16 gauge angiocatheter or a stylus using the Seldinger technique. The angiocatheter was advanced toward the ipsilateral medial canthus with gentle aspiration until placement within the lateral ventricle was confirmed. A bolus of 100 cm³ normal saline was infused over 5 minutes, after which the ventriculostomy catheter was connected to a syringe pump for continuous infusion at 100 cm³ per hour (SP 100I; World Precision Instruments, Sarasota, Fla.).

Dissection of central and peripheral nerves was then performed using standard surgical guidelines. The median nerve was used in this study because of its association with the common compression neuropathy, carpal tunnel syndrome. The nerve was dissected at the pronator canal, and just proximal to the antecubital fossa. Fluorescent injections were performed with quantum dots (Sigma Aldrich, USA) with a mean diameter of 6–10 nm. Nanomolecular gold and silver injections were performed with nanoparticles with diameters of 1.9 and 15 nm (Aurovist, Nanoprobes Inc., Yaphank, N.Y.). Nanogold was mixed 40 mg per 5 cm³ phosphate buffered saline for ventricular injection.

Outflow CSF pathways were excluded by ligation including spinal cord below T1, contralateral brachial plexus, and bilateral terminal CSF drainage. Right

Takeaways

Question: Does cerebrospinal fluid circulate in nerves?

Findings: Ventricular saline infusion, complemented by nanoprobes having a diameter of less than 10 nm, identifies CSF flow in the pia meninges, epineurium, perineurium, and myelin sheaths of nerves. CSF circulation in nerves is an open circulatory system that likely provides a medium for the transport of energy and solutes.

Meaning: Dysregulation of the CSF circulation in nerves may play a role in clinical problems facing the plastic surgeon including nerve transection, stretch injuries, and peripheral neuropathy.

cerebral ventricular injection was followed by continuous saline infusion. Right upper median nerve was biopsied after 30 minutes.

All dissections were performed at UTSW Willed Body Program. Anatomic dissection is exempt from IRB approval at this institution. All ethical guidelines were followed, and no personal data were collected except for gender and age. Cryosectioning was performed on tissues after paraformaldehyde fixation for 24 hours using the Leica CM 1850 cryostat to obtain 2–4 µm specimens. Histology specimens after Aurovist injection were treated with LEM Gold Enhance (Nanoprobes Inc., Yaphank, N.Y.) for visualization with light microscopy. Two-step indirect immunohistochemistry was done using routine protocols and reagents as previously described.[1–3] Primary antibodies tested include against GLUT-1; MCT-1; Phalloidin-488; GFAP; vimentin-cy3; Claudin1/3; and connexin (Sigma Aldrich, USA). All imaging was performed at UTSW (Leica SP5 microscope, Leica Microsystems, Jena, Germany).

RESULTS

Ventriculostomy

Eight (N = 8) fresh cadaver specimens underwent cerebral ventriculostomy and normal saline infusion within 72 hours of death. There were six female and two male specimens with an age range of 46–97 years (mean 76.6 years). Ventricular insertion was successful in all specimens using the coordinates provided: 2 cm anterior to coronal suture, and 2.5 cm lateral to sagittal suture. Depth of successful cannulation varied from 44 to 56 mm (mean 49 mm).

Gross Anatomy

The median nerve was dissected in all eight cadaver specimens after ventriculostomy and NS infusion at two locations: midforearm proximal to the pronator canal and just proximal to the antecubital fossa. Before ventricular infusion, epineurial channels can be identified but are flattened and indistinct (Fig. 1A). Epineural channels expand and are easily identified after ventricular NS infusion (Fig. 1B). Without ventricular infusion, epineurial channels exhibit no distinct pattern (Fig. 2A). After ventricular infusion, epineurial channels travel between individual nerve fascicles (Fig. 2B).

Fluorescent Imaging

Cerebral ventricular injection with a fluorescent nanoprobe (diameter: 6–10 nm) was performed in two cadaver specimens. Figure 3A shows flattened and indistinct epineurial channels before fluorescent imaging. With fluorescent imaging, epineurial channels are clearly defined and distended (Fig. 3B). Drainage from the epineurial CSF channels to adjacent lymphatic vessels is noted (Fig. 3B). With further dissection, perineurium was likewise noted to fluoresce.

Histology

Histology was studied in samples of median nerve harvested from three specimens. Median nerve was harvested from a 54-year-old male specimen before and after ventricular infusion. The perineurium shows tightly opposed concentric layers before ventricular infusion (Fig. 4A, arrows). After ventricular infusion, the perineurial layer is expanded and potential channels are noted (Fig. 4B, arrows). Ventricular infusion can be combined with immunohistochemistry to identify protein expression.
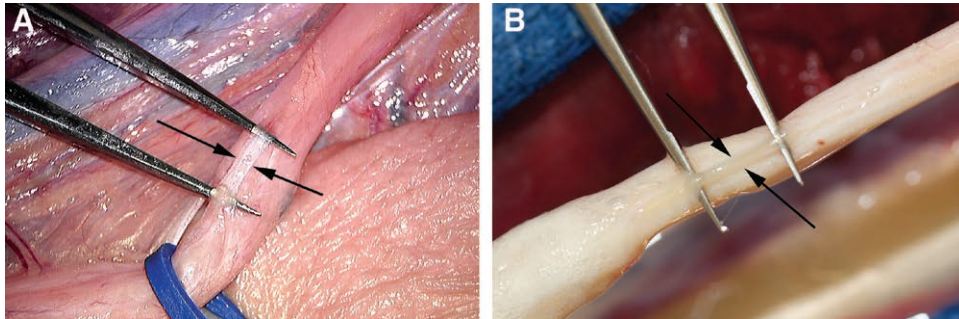
Fig. 1. A, Median nerve is dissected under the operating microscope in an 80-year-old male cadaver specimen. Before ventricular NS infusion, epineurial channels appear flattened and indistinct (arrows). B, Epineurial channels (arrows) become dilated and are easily dissected from the median nerve after ventricular infusion of NS.
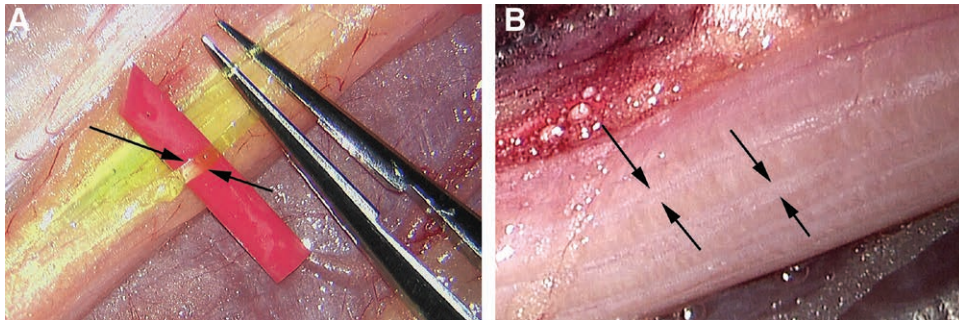
Fig. 2. A, Even with the operating microscope, epineurial channels (arrows) on upper median nerve are flattened before ventricular NS injection. A 93-year-old female specimen. B, After ventricular NS infusion, a regular pattern of epineurial channels (arrows) is observed on the median nerve that alternate between individual fascicles. A 46-year-old female specimen.
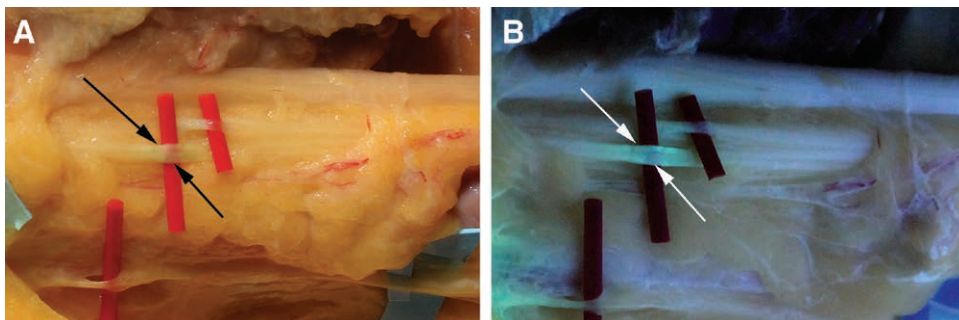
Fig. 3. A, Proximal median nerve has been identified in a 54-year-old male specimen. Epineurial channels can be identified but are flattened and indistinct (arrows). B, After ventricular injection of a nanomolecular fluorescent quantum dot, epineurial channels are distended and fluoresce (arrows). Drainage to adjacent subcutaneous lymphatic vessels is noted. Microsurgical dissection noted fluorescence of the perineurium and endoneurium/myelin sheaths. A 54-year-old male specimen.

After ventricular infusion, the median nerve was harvested from a 76-year-old female specimen and tested for F-actin in Figure 5A (green fluorescence). The perineurium (arrows) has been re-expanded by ventricular infusion. On higher magnification, potential CSF channels in the perineurium (arrows) can be identified (Fig. 5B).

DISCUSSION

This work suggests that neural sheaths and meninges participate in CSF circulation and solute transport in central and peripheral nerves. Interest in this field began in 2013 when epineurial CSF channels were first observed during the release of an entrapped median nerve.[1] The author initially mischaracterized CSF channels as part of the lymphatic system, an error that others have made throughout history.[16,17,23–27] Ventricular infusion and nanoprobe injection were developed when this error was corrected in 2017.[1–4,28–31]

CSF flow in nerves may be described as an open circulatory system that provides a fluid medium for the transport of energy molecules and solutes. In humans, CSF circulates from cerebral ventricles to pia meninges of brain and spinal cord, and from there to perineurium, endoneurium, and to the neurolemma of myelin sheaths. CSF traveling in neural sheaths can drain to peripheral lymphatic vessels, or may recirculate directly into the thoracic duct (Fig. 6).[3,4]

CSF may flow in neural sheaths via three mechanisms, which include circulation in channels,[1] intracellular flow (cytoplasmic),[2] and cell-to-cell transport (gap junctions)[3] associated with glial cells (Fig. 7A–C).[18,32–34] An important distinction exists between the glymphatic system described in the mouse cortex and CSF flow in neural sheaths.[18] Paravascular channels (glymphatic system) are described as a "sink" or an outflow circulation that may help clear solutes such as amyloid B. In contrast, CSF flow to pia meninges, perineurium, and myelin sheaths in nerves is more likely an inflow circulation that provides a fluid medium for energy and solute transport to axons.

Ventricular infusion with buffered saline by itself answers two important questions. First, microsurgeons frequently observe clear fluid weeping from the proximal end of transected nerves during nerve repair. Ventricular infusion suggests that this clear fluid is CSF. Ventricular infusion also answers the question why routine nerve biopsies fail to identify CSF pathways. Nerve biopsy transects CSF pathways, allowing them to drain and decompress. Epineurial, perineurial, and myelin sheath CSF pathways flatten, and are easily overlooked on routine inspection. Ventricular infusion restores the anatomy to a more accurate in vivo appearance.

After it was found that cerebral ventricular infusion of saline produced flow in nerves, the next step was to choose (the size of) a suitable probe. Human nerve sheaths express
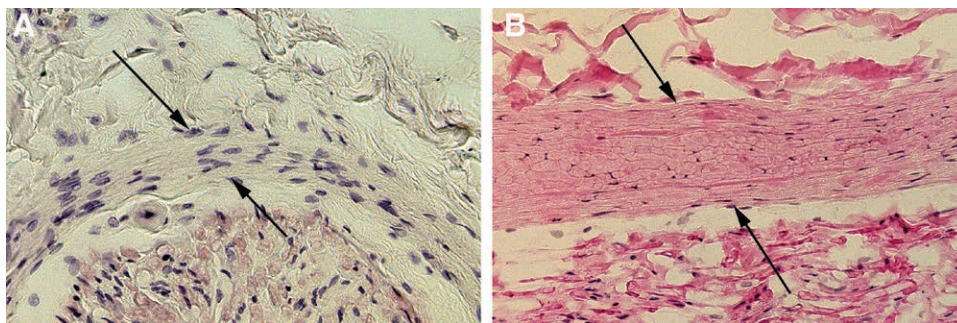
Fig. 4. A, Median nerve histology before ventricular infusion shows perineurium with tightly opposed concentric layers (arrows). An 87-year-old female specimen. B, Same nerve harvested after ventricular infusion shows the perineurium has been expanded (arrows).
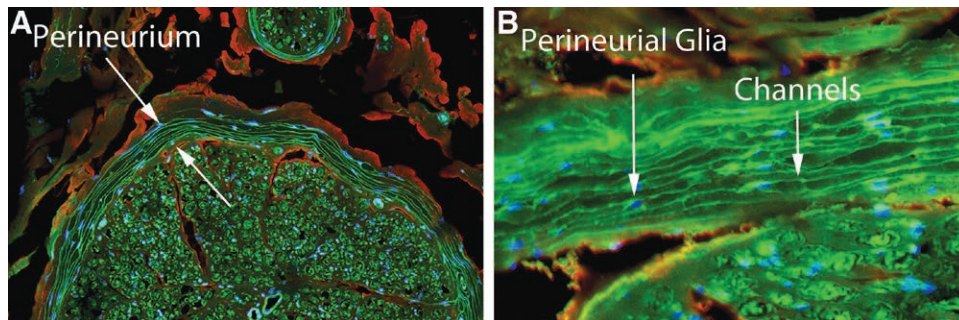

Fig. 5. A, Immunohistochemistry has been performed for F-actin (green fluorescence) on a sample of median nerve after ventricular infusion. Perineurium has been expanded (arrows). 200×. B, Perineurial glia cells, the nuclei of which have been stained with DAPI, appear to form perineurial CSF channels, much like what is observed in the glymphatic system and in myelin sheaths. 800×.

solute carrier proteins (SLC) involved in the active transport of glucose (GLUT-1/SLC2A1) and lactate (MCT-1/SLC16A2). Fluorescent and metallic probes with a diameter closest to that of a glucose molecule were chosen.

The size of probes used to study CSF pathways, especially when introduced by injection into the cerebral ventricle, is a critical factor. Historical studies failed to identify CSF flow to peripheral nerves simply because the dyes used were too large to travel through CSF pathways.[35–38] Although suitable for vascular and lymphatic studies, methylene blue, India ink and even submicron fluorescent dyes are unable to enter many of the CSF pathways in nerves and brain.[39] In addition, large molecules fail to identify cell-to-cell transport of CSF and solutes (eg, glucose and monocarboxylates) that may occur through gap junctions known to exist between adjacent Schwann and other glial cells. When these size constraints are followed, CSF flow in neural sheaths and meninges may be studied using a variety of nanoprobes, including fluorescent, metallic, and functionalized-energy transport molecules (Table 1).[40,41]

Myelin is considered a neural sheath, and ventricular injections in humans suggest that the neurolemma of myelin sheaths may participate in CSF circulation and glucose transport. CSF flow in neurolemma is confirmed when a nanomolecular gold probe is injected into median nerve via a peripheral lymphatic: the probe travels to the neurolemma of myelin sheaths surrounding individual axons (Fig. 8A, B). Gold nanoprobe is noted in the cytoplasm of Schwann cells (Fig. 8B).
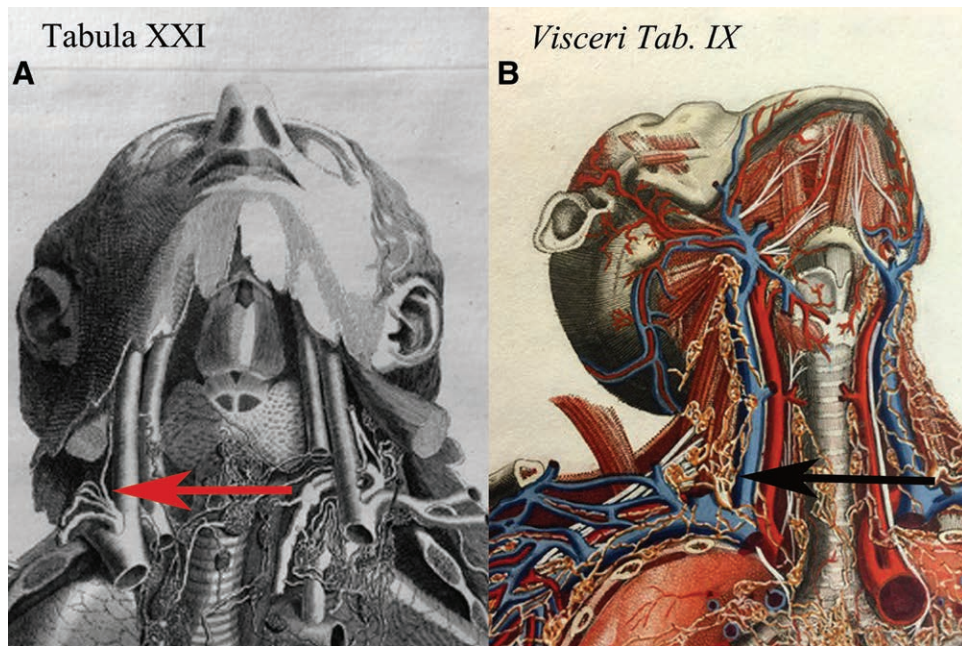
Fig. 6. Mascagni identified the terminal CSF drainage of human brain and nerves in 1787. A, The terminal cerebral drainage traveling along the posterior internal jugular vein, with epineurial CSF vessels traveling from nerve roots to the thoracic duct (red arrow). B, The terminal cerebral CSF drainage of brain as a plexus of vessels (black arrow). Reproduced with permission from Countway Medical Library.
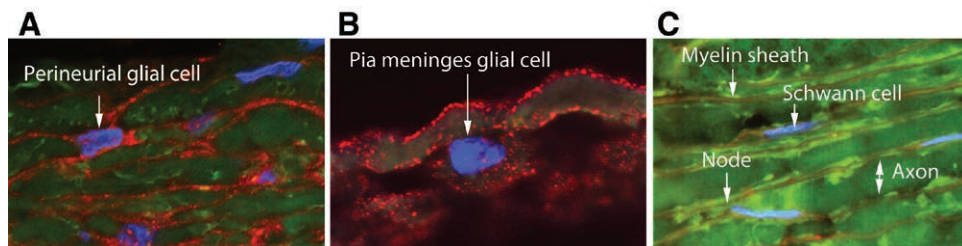
Fig. 7. A, Glia cells contribute to the structure of many CSF and glymphatic pathways throughout the central and peripheral nervous systems. Perineurial glia cells (arrows) express the glucose transporter-1 protein. 3000×. B, Glia cells are found in the outer surface of pia meninges in human spinal cord, and participate in CSF circulation as an analogue of perineurium. 3000×. C, Ventricular infusion suggests the neurolemma of myelin sheaths may contribute to CSF flow and solute transport. After infusion, human optic nerve shows dilated neurolemma (arrow, myelin). Schwann cells are a peripheral glial cell that forms these sheaths, and are similar to oligodendrocytes found in the central nervous system. A node of Ranvier (Node) is seen. 3000×.

Table 1. Techniques and Materials Used to Identify and Study CSF Circulation in Human Nerves and Brain

Access to CSF Circulation	Pros and Cons of Approach
1. Ventricular	+Applies to brain and nerves +Identifies circulation in meninges and neural sheaths +Identifies spinal canal to pia meninges CSF pathways (cord) +Identifies direct ventricle-to-pia meninges CSF pathways (via glial cells) +Identifies CSF flow via myelin sheaths
2. Subarachnoid	+Applies to brain and nerves −Does not identify spinal canal to pia meninges CSF pathways −Does not identify direct ventricle-to-pia meninges CSF pathways
3. Communicating lymphatics	+Identifies CSF flow to epineurium, perineurium, and neurolemma +Best way to confirm epineurial-to-lymphatic communications −Technically challenging; requires specialized techniques
4. Direct CSF channel injection	+Peripheral access via epineurial channels in nerves +Central nervous system access via falx and sagittal sinus channels +Retrograde access via terminal CSF drainage in carotid sheath −Technically more difficult than ventricular injection
Injectable probes	
1. Buffered saline (NS, PBS)	+Increases diameter of meninges and neural sheaths +Improves histological evaluation −Best results require simultaneous immunohistochemistry
2. Fluorescent probes	+Can be used during anatomic dissection −Size limited to nanomolecules (<10 nm) −May wash out during tissue processing
3. Molecular probes	+Can be aggregated for light microscopy +Tend to remain in tissues during processing −Size limited to nanomolecules (<10 nm) −Aggregation technique distorts neural sheath histology
4. Functionalized energy molecules	+Include glucose, lactate, pyruvate, ketones +May identify specific parts of CSF and glymphatic system +Can be linked to any probe to study active energy transport

A variety of peripheral neuropathies are related to myelin sheath and Schwann cell dysfunction.[26–42] Traumatic nerve stretch injuries are associated with disruption of the myelin sheath.[14,15] Demyelinating diseases such as multiple sclerosis affect cranial nerves (eg, optic, trigeminal), which are easily studied using these techniques.[42,43] Both genetic (eg, Marie-Charcot-Tooth; Ehlers Danlos) and acquired (eg, Type-2 diabetes) neuropathies may affect Schwann cell function and gap junction proteins.[44–47] There appears to be a relationship between the clinical symptom of pain and myelin damage. It is interesting that perineural injection therapy with dextrose has been used to treat complex regional pain syndromes.[48]

Disruption of the CSF circulation of nerves may be important in other problems that confront the plastic surgeon.[12–15] Nerve repair is immediately affected by this information. Precision of epineurial repair is improved by avoiding epineurial CSF channels that travel between individual nerve fascicles (see Fig. 2B). Because the perineurium (or pia meninges of central nerves) participates in CSF flow, iatrogenic damage to this system should be avoided.[13]

The concepts and techniques described facilitate the study of CSF and glymphatic circulation in nerves and enable any interested individual to participate in this research (Table 2). It is simple to adapt these concepts and techniques to live animal models. Specialized equipment
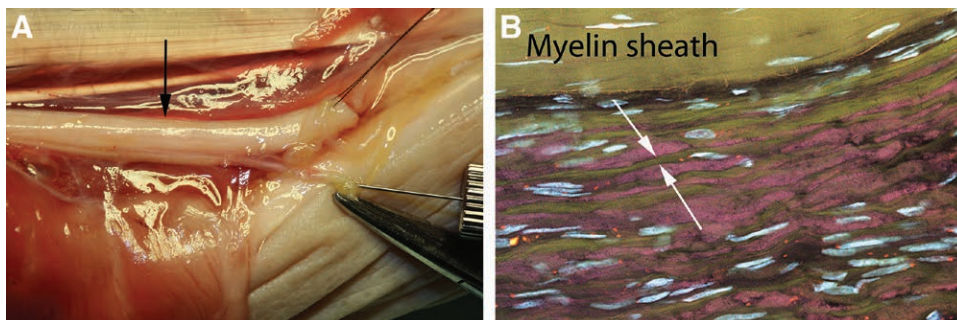
Fig. 8. A, CSF infusions may be performed retrograde from a draining lymphatic to the median nerve. A 15 nanometer gold probe was injected after infusion. B, The neurolemma of myelinated nerves (arrow) have been expanded by the infusion, and gold has been transported into the cytoplasm of Schwann cells.

Table 2. Basic Concepts Evolved from Human Anatomical Dissection, and Help Guide Techniques Used to Study the CSF and Glymphatic Circulations

Concept	Explanation	Technique
Ventricular injection excludes fewest potential CSF pathways	CSF flows from ventricle-to-pia meninges; to subarachnoid space; to spinal canal	Ventricular access
CSF channels readily drain and decompress	CSF pathways are collapsible; CSF drainage is redundant	Continuous ventricular infusion; in-situ fixation
CSF pathways exclude large molecules	CSF and glymphatic circulation is part of the blood-brain and blood-nerve barrier	Use nanoprobes
CSF circulation is continuous but *slow*	Volume replaced every 24–48 h; channels, intracellular and cell-to-cell transport	Study proximal nerves; stage nerve biopsy after ventricular injection (30 min–2 h)
Many (not all) CSF pathways are associated with glial cells	Plasma membrane related to structure of CSF channels	Use appropriate antibody (eg, Schwann cell—EAAT1)

is not necessary, and probes exist that can be aggregated and imaged using light microscopy (see Materials and Methods). Because CSF circulation is slow (ie, cell-to-cell transport), cranial nerves (eg, optic; trigeminal; vagus; and spinal accessory) are most easily studied (Table 2).

Surgical experience, anatomical knowledge, and microsurgical skills enable the plastic surgeon to make significant contributions to this emerging field. Both human anatomical dissection and animal models are important because key structural differences exist between the two. For example, the terminal CSF drainage of human nerves and brain can flow directly into the thoracic duct; in rodents, CSF recirculates to the deep cervical lymph nodes.[1–3,11] Structural differences are also noted in the development of specific neural layers, especially epineurium and perineurium. Although this study was limited to nerves, the techniques presented are applicable to study of the central nervous system as well. CSF injection of human brain with fluorescent nanoprobes suggests that pia meninges participate in CSF flow and glucose transport.[1] Perineurium and pia meninges are embryological analogues, so research in one tissue tends to reinforce findings in the other. Ventricular injection, especially when performed in conjunction with nanoprobes, is a simple technique that may benefit future research in this field.

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