



Approaches for investigating plasmodesmata and effective communication

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Abstract

Plasmodesmata (PD) are integral plant cell wall components that provide routes for intercellular communication, signaling, and resource sharing. They are therefore essential for plant growth and survival. Much effort has been put forth to understand how PD are generated and their structure is refined for function and to determine how they regulate intercellular trafficking. This review provides an overview of some of the approaches that have been used to study PD structure and function, highlighting those that may be more widely adopted to address questions of PD cell biology and function. Extending our focus on the importance of communication, we address how effective communication strategies can increase diversity and accessibility in the research laboratory, focusing on challenges faced by our deaf/hard-of-hearing colleagues, and highlight successful approaches to including them in the research laboratory.

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Current Opinion in Plant Biology 2021, 64:102143

This review comes from a themed issue on **Cell Biology 2021**

Edited by **Siobhan Braybrook** and **David Mendoza-Cózatl**

For a complete overview see the [Issue](#)

Available online 23 November 2021

<https://doi.org/10.1016/j.pbi.2021.102143>

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Keywords

Plasmodesmata, Communication, Intercellular trafficking, Imaging, Fluorescence microscopy, Transmission electron microscopy, Scanning electron microscopy, Deaf, Hard-of-hearing.

Introduction

Plants require trafficking of metabolites and signaling molecules between cells and systemically to coordinate plant development and mount coordinated responses to abiotic and biotic stresses. With a few exceptions, almost all the cells of a plant are connected to its neighbors by plasmodesmata (PD), and the plant can therefore be

thought of as a symplast. PD are structurally complex nanopores that provide continuity of the plasma membranes and cytosols of connected cells. In many cases, the endoplasmic reticulum is also continuous between cells as a specialized structure, the desmotubule, which resides in the center of the pore. The major conduit for cell-to-cell trafficking is likely the cytoplasmic sleeve, the space between the desmotubule and the plasma membrane [1]. A major function of PD is the movement of sugars between plant cells and loading sugars into the phloem of source tissues for translocation to sink tissues. In addition to sugars, PD also traffic water, ions, small solutes, and macromolecules including proteins and RNAs. Recent work has further highlighted PD in systemic signaling, particularly the distribution of reactive oxygen species in response to local high light stress and demonstrated the role of PD-localized proteins in regulating this process [2**]. Viruses have evolved to take advantage of PD for their spread throughout the plant [3*]. Understanding how PD regulate the movement of signaling molecules is crucial to unraveling plant responses to stress and the environment.

PD are diverse and can be roughly grouped into three different morphological forms: simple, twinned, and branched or complex [4]. Transmission electron microscopy (TEM) studies of PD in *Nicotiana* sp. and other plants have shown that PD are usually 30–50 nm in diameter. Simple PD consist of a single pore, and they likely form during cytokinesis when strands of endoplasmic reticulum (ER) become trapped in the new cell wall forming between daughter cells, thereby representing primary PD [4,5]. Secondary PD are added to cell walls in the absence of cytokinesis, and they often form near existing PD giving rise to twinned [6] and branched PD [7]. Simple PD can be modified into complex PD as during the sink to source transition in leaves [8] or in maturation of phloem cells [9]. Other classes of PD have recently been described, suggesting that PD are even more structurally diverse. Type I (apparently lacking a cytoplasmic sleeve) and type II (having a cytoplasmic sleeve) PD were identified in *Arabidopsis* roots by TEM tomography [10]. There are also funnel-shaped PD that function in phloem loading [11].

A current challenge in plasmodesmal cell biology is correlating PD structure with function. For example, complex PD have been reported to traffic less than

simple PD [8], but mutants with increased trafficking have more complex PD than do their wild-type counterparts [12]. To decipher PD structure–function relationships, attempts have been made to measure PD permeability (trafficking capacity) and structure while causing minimal perturbations to tissues under examination. Here, we review a variety of approaches to studying PD and highlight which are emerging as promising approaches for investigating this fundamental aspect of plant biology. In parallel with our interest in plant intercellular communication, we also discuss our experiences with honing strategies for effective communicating with our deaf and hard-of-hearing (D/hh) colleagues in the environment of a research laboratory. Adoption of the successful approaches we present could facilitate the inclusion of D/hh persons in the scientific endeavor, given that they are often excluded from participation in the scientific community.

Measuring PD trafficking

Fluorescent dyes were the earliest probes deployed for measuring PD permeability. Small dyes like 5(6)-carboxyfluorescein diacetate (CFDA) can be microinjected into the cytoplasm of cells, and the diffusion of the dye beyond the injection site is monitored by fluorescence microscopy (Figure 1). CFDA is a good indicator of symplastic transport because it is membrane impermeant, and only on entering the cytoplasm, it is cleaved by esterases to liberate the fluorescent CF molecule. The challenge with microinjection is the skill required for delivery into the cytoplasm while avoiding the vacuole which is the bulk of the cell volume in a mature plant cell. CFDA can also be used to measure systemic trafficking via the phloem [13], and it has been widely used for this purpose. Dyes closely related to endogenous plant molecules have been adopted for measuring phloem transport [14,15]. In the future, these may supplant CFDA as a symplastic tracer. A modern application for CFDA is the drop-and-see (DANS) assay developed by Jung-Youn Lee's group [16,17]. Here, a small measured amount of CFDA is applied to the adaxial leaf surface, and the radius of resulting fluorescence, presumably measuring intercellular trafficking, is measured on the abaxial surface. DANS has been used to characterize mutants [18] and measure PD trafficking capacity [2,19]. Although DANS has the advantage of being noninvasive, it has not yet been widely adopted, probably because the assay does not produce data with cellular-level resolution as the area of dye movement can be hundreds of square microns [17]. Other fluorescent molecules that have been used to assess PD permeability include caged fluorescein [20], 8-hydroxypyrene 1,3,6 trisulfonic acid [21–23], Lucifer yellow CH [9,24], and fluorescent dextrans of various molecular weights.

Fluorescent proteins have also been widely adopted as probes for measuring PD trafficking. Introduction of GFP-expressing constructs into plant leaves via *Agrobacterium* has been popular [25] although bombardment with plasmids for GFP expression is also possible [26]. The soluble cytoplasmic protein (GFP) moves between cells via PD by simple diffusion [27], and GFP movement is used to investigate PD trafficking in response to abiotic and biotic factors and changes in gene expression [28–30**]. GFP can also be stably expressed from tissue-specific promoters. The *AtSUC2* promoter is often used for expression of GFP in companion cells within the phloem ([31] and references therein), which permits GFP to traffic extensively through PD during unloading in the phloem. Tandem copies of GFP (2XGFP or 3XGFP) may also be used as probes although they do not traffic as extensively as GFP itself [22]. Thus, higher molecular weights of tandem copies of GFP are often used to test PD trafficking capacity. Differences in PD trafficking have traditionally expressed in terms of the PD size exclusion limit; however, we feel that this term is inappropriate because limits, clear cutoffs in the ability of PD to traffic molecules of a given size, are rarely observed. In addition, a size exclusion limit implies that the trafficking capacity of all PD at a given interface is the same, but this cannot yet be empirically demonstrated. DRONPA and photoactivatable GFP are GFP-derived proteins that can yield measurements of high spatial and temporal resolution [32]. DRONPA can be switched on and off with different wavelengths of light, allowing its movement to be observed in real time. The analysis of data used to assess PD permeability often varies widely between researchers and would benefit from more uniform standards for collection and statistical approaches, as recently proposed [33*].

Measuring callose

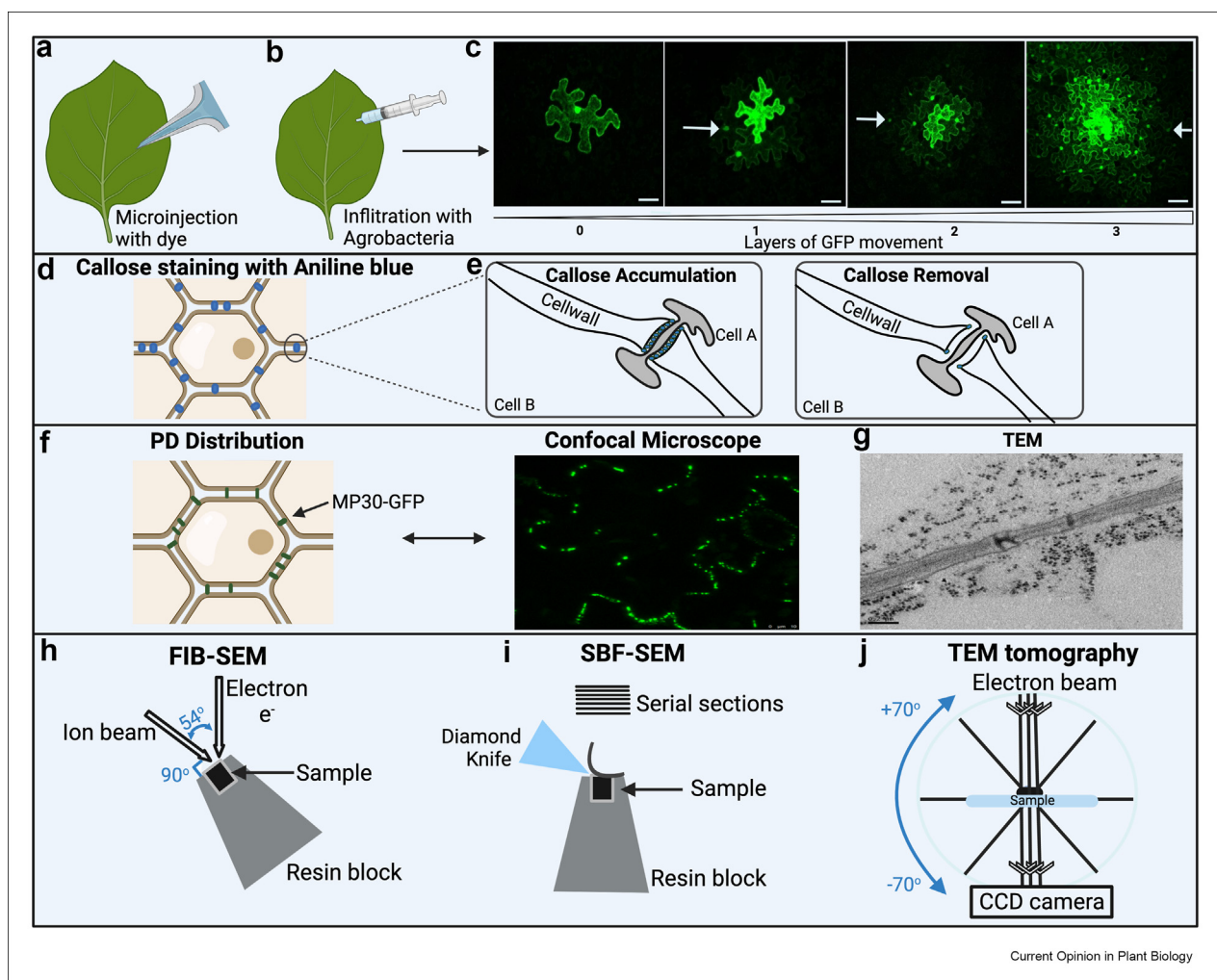
The regulated accumulation of callose, a β -1,3-glucan polysaccharide, at PD controls intercellular trafficking (reviewed in [34–36]). As high callose levels at PD correlate with lower intercellular trafficking, the level of callose in a tissue has been used to determine the status of PD. Staining with the pigment aniline blue is the most commonly used method for measuring callose levels [37] (Figure 1). It is relatively easy to fix tissues and then apply aniline blue, although care must be taken during tissue isolation to avoid inducing damage and subsequent callose deposition at those sites [37]. Another advantage of aniline blue is that it can be used to examine a relatively large amount of tissue. Measuring callose by Immuno-electron microscopy (immunoEM) using anticalllose antibodies gives greatly improved resolution over aniline blue staining, allowing the visualization of callose at certain positions along PD and the distance from PD to be ascertained [38,39]. Despite these advantages, this approach is not very

commonly used, perhaps because of the time, cost of reagents or perceived difficulty, and expertise needed for TEM. Despite its widespread use as a proxy for PD permeability, the correlation of callose with PD trafficking is not straightforward, and recent data suggest it is more complicated than previously assumed [40]. In addition, although it is easy to observe callose accumulation, it is harder to measure reductions in callose levels that may be associated with increased permeability. Data on callose levels should be interpreted with caution, and assumptions about PD trafficking should be limited to actual measurement of trafficking.

Determining PD structure and measuring distribution of PD

Viral movement proteins (MPs) tagged with fluorescent proteins have emerged as excellent probes for PD [3]. Tobacco mosaic virus MP30, although preferring complex PD in some tissues [41,42], can label all types of PD [4], and it has been widely adopted as a PD marker (Figure 1). MP17 from the Potato leafroll virus specifically labels complex PD [4,43], and it can therefore be used as a marker for these types of PD. These proteins can be stably expressed in transgenic plants and used in this way to observe PD; however, they often have effects

Figure 1



Experimental approaches used to study PD and intercellular trafficking. (a) Fluorescent dyes and proteins can be introduced into leaf cells by microinjection. (b) Fluorescently labeled proteins can be transiently expressed in leaf cells by agroinoculation. (c) Fluorescent probes are subsequently imaged by microscopy, and a pattern of cells containing the probes is usually observed when PD permeability allows probe trafficking, with larger areas representing more PD permeability and trafficking. Images show layers of cells into which GFP has moved, with no movement and movement to 3 layers being depicted. Arrows point to nuclei of neighboring cells into which GFP has trafficked. (d) Aniline blue can stain callose at PD (blue dots). (e) Representation of the distribution of aniline blue around PD, with aniline blue accumulating along the entire length of a pore. (f) PD distribution as revealed by MP30-GFP accumulating in PD at the cell periphery in a confocal micrograph. (g) Thin-section TEM is used to observe complex and simple PD in a cell wall. (h) FIB-SEM combines ion beam milling with an electron beam in one setup. (i) For SBF-SEM, a microtome is mounted inside the microscope to produce serial images of the newly cut surface. (j) TEM tomography involves the capture of images as a sample is rotated through a tilt series. This was created with BioRender.com.

on plant development and metabolism, thereby complicating studies [43–46]. We suggest that it is more prudent to adopt transient expression by bombardment or *Agrobacterium* to express these markers [29], as this avoids the need to generate transgenic plants that would have modifications in PD but yet develop fairly normally. In addition to viral MPs, the plant proteins PLASMODESMATA LOCATED PROTEIN1 and PD CALLOSE BINDING PROTEIN1 can also be fluorescently labeled for use as PD markers. An exemplary use of these PD markers to study PD cell biology is the development of a high-throughput imaging platform and accompanying algorithm, PDQUANT, for identifying cells and counting PD [7]. This approach was used to document several features related to the development of complex PD that would otherwise have been recalcitrant to discovery. Unfortunately, such approaches have not seen widespread adoption. There remains a need for (semi-) automated imaging and analysis approaches for rapid, reliable, and unbiased data collection and analysis, and we are certain that such approaches will advance the quality of information about PD distributions.

TEM has a long history as the go-to method for studying PD structure and morphology and measuring PD distribution. Despite its popularity, in thin-section TEM, only a single slice of around 60–70 nm thickness, of a three-dimensional (3D) structure, is visible. This produces a snapshot of PD, and usually only a few (or portions thereof) PD in a wall are captured (Figure 1). The details of PD morphology are therefore often obscured, and only time- and labor-intensive scoring of many sections and PD can give a somewhat reliable estimate of PD form and distribution in a tissue [12]. Nonetheless, thin sections can be used in interesting ways, for example, when combined with freeze fracture and fluorescence in a new technique dubbed 3-D immunolocalization confocal microscopy [47]. This technique has been used to measure PD density of PD at the mesophyll–bundle sheath interface in C4 grasses grown under different light regimes and made evident that PD size and distribution are dynamic in response to growth conditions [48*].

Detailed analysis of the ultrastructure of the PD nanopores requires use of 3D approaches, typically TEM tomography (Box 1 and Figure 1). The resulting 3D models produced allow higher magnification and better resolution of structural components, particularly in the *z* axis [49]. Ideally, samples are prepared for imaging by high pressure freezing and subsequent freeze substitution [50], the gold standard for preparing plant samples for electron microscopy [51]. Recently, TEM tomography has been used to reveal new forms and structural variants of PD. Type I and type II PD were discovered by TEM tomography [10], and this technique was also used to identify PD defects

in the *phloem unloading modulator (plm)* mutant [52]. TEM tomography has also been used to examine the interaction of viruses with PD during infection [53]. Given the power of this technique, TEM tomography should be the gold standard for determining PD (ultra)structure.

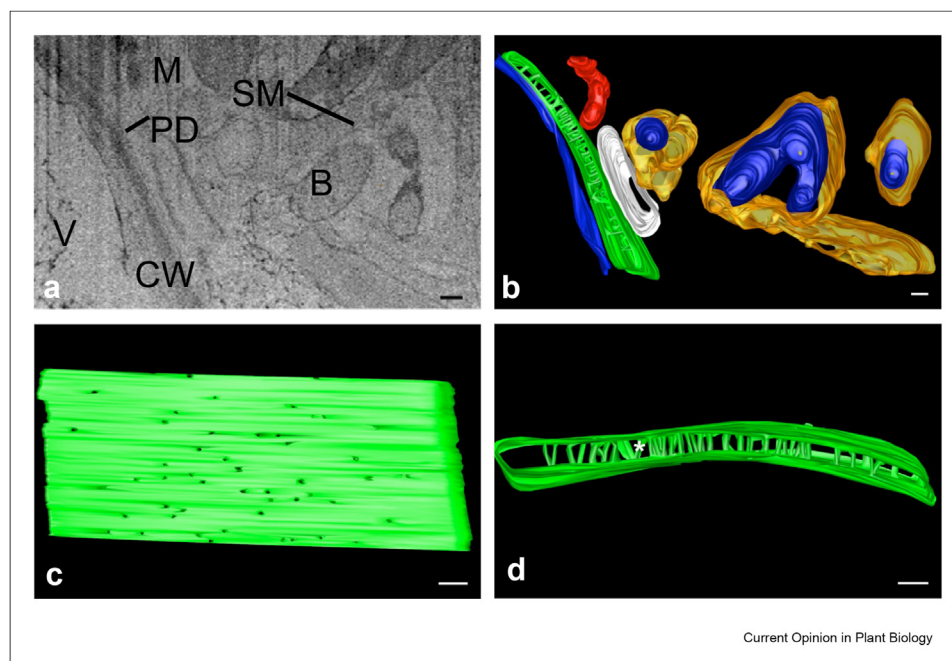
Scanning EM (SEM) is typically used to generate images of sample surfaces and does not reveal internal structures; however, combining serial SEM images can be used to produce 3D projections of an object, allowing 3D analysis of larger sample volumes and thicker specimens than TEM tomography. Two approaches for serial SEM-based imaging are serial block face–SEM (SBF–SEM) and focused ion beam–SEM (FIB–SEM), and both have been adopted to analyze PD structure and distribution. Importantly, the same samples that are prepared for thin-section TEM and TEM tomography can be used for 3D SEM [54]. SBF–SEM was used to reveal defective sieve pore formation in the root phloem of the *choline transporter1 (cher1/clt1)* mutant [55] and to discover funnel PD in the phloem-pole pericycle in the *Arabidopsis* roots [11]. A pipeline for adopting SBF–SEM for imaging PD distribution and structure has recently become available [56**], and we expect that this will lead to wider adoption of the 3D imaging technique for PD analyses. FIB–SEM analysis of *Nicotiana benthamiana* leaves generated a 3D projection of approximately 10 μ m of the cell wall and the subtending chloroplasts [1]. The presence of pit fields of PD was revealed in this 3D projection, and even with images collected every 20 nm, there was sufficient detail to determine whether individual PD were simple or complex [54]. Any plant tissues can be imaged by FIB–SEM, as demonstrated by examination of PD in the nitrogen-fixing nodules on the roots of a legume (Figure 2).

In summary, there exists a comprehensive suite of resources that can be used to decipher how PD form and function. There are several mutants with defective PD in which these techniques could be applied to examine PD cell biology [57]. Future improvements in these techniques, especially increased temporal and spatial resolution and ease of sample preparation in high-end imaging, will no doubt enhance this research area further. We expect that electron microscopy–based approaches will soon regain their prominence in PD studies as these approaches are unparalleled in their ability to resolve the PD nanopores.

The importance of communication for a diverse scientific workforce

In our laboratory, our interest in communication extends beyond that mediated by PD. Similar to how diversity enriches an ecosystem with species capable of using different resources and thriving in different

Figure 2



Focused ion beam–scanning electron microscopy (FIB–SEM) reveals nodule ultrastructure and PD. (a) An SEM image of a region of a nitrogen-fixing nodule formed on *Medicago truncatula* roots by *Sinorhizobium meliloti* showing an infected cell and a companion uninfected cell. The image is a single slice of a series of 104 images. (b) Reconstruction of a FIB–SEM tomogram of a *Medicago truncatula* nodule. A total of 104 serial SEM images were combined to generate the 3D model. (c) Many PD span the cell wall shared by the infected and uninfected cell. (d) 3D reconstruction also allows PD structure to be determined; a branched plasmodesma is marked with a white asterisk. Vacuole (V, blue); cell wall (CW, green); mitochondria (M, red); bacteroid (B, dark blue); symbiosome membrane (SM, orange); scale bar = 500 nm.

microenvironments, science also benefits from using the full range of perspectives present in our population. D/hh individuals are part of the diverse views that can strengthen our science community. One of the biggest challenges encountered when training deaf scientists is communication, and careful, effective communication is a major benefit for everyone. Effective communication is foundational to innovation.

Ineffective communication, on the other hand, can significantly hinder scientific discovery. For example, traditionally, scientific methods, protocols, and research articles are written in an abstract, overly complicated style. Excessive jargon, acronyms, and using the passive voice are common practices that can obfuscate even relatively simple concepts. Scientists enter the laboratory with diverse skills, abilities, resources, and perspectives, and many potentially talented scientists may find this style of writing impenetrable. Effective communication is also critical when considering scientists with different abilities, for example, D/hh scientists. Therefore, we advocate that any consideration of scientific methods must also consider the diversity of scientists who will use the method and emphasize accessibility for the broadest

range of scientists. Toward that goal, here, we will discuss our experience accommodating D/hh scientists in research laboratories and, specifically, how we have adopted relatively simple structural and cultural changes to increase accessibility to the techniques mentioned previously.

One of the authors of this review is a deaf scientist, and our group frequently engages other early-career D/hh individuals in our research. Careers in science, technology, engineering and mathematics (STEM) provide a route for improved economic outcomes for the D/hh individuals, as D/hh individuals in STEM careers with bachelor's degrees earn twice more than those without degrees [58]. One of the first challenges faced by a D/hh scientist might be access to scientific seminars, laboratory meetings, and so on. People learn in diverse ways, and American Sign Language (ASL) is the preferred medium for communication for many D/hh persons (in the United States; other countries have their own sign languages). Thus, deaf people (those who use sign language) learn everything visually. Often their visual attention is divided between a speaker/instructor, an interpreter, and a visual aid such as a slide or video. One challenge with the deaf and science is the

absence of signs for many terms used in science. One striking example is the term ‘culture’. The ASL sign for culture incorporates the sociological and historical aspects of the noun, whereas in the laboratory, ‘culture’ is used to refer to growth under laboratory conditions. Another frequent challenge we have encountered is a vague hesitance to accommodate the use of ASL and ASL interpreters at scientific forums, a common incorrect assumption being that there are technological solutions that are equally effective and cheaper. Our experience is that closed captioning and even transcription by a human often fail to correctly caption scientific terms and contexts, resulting in nonsensical outputs like ‘the oxen levels in plants’! Happily, however, most colleagues are willing to be flexible and make necessary accommodations as appropriate once they are made aware of the challenges faced by D/hh scientists. In the same way we strive to develop and deploy effective means for understanding PD and intercellular trafficking in plants, we are endeavoring to develop effective approaches for communication with our D/hh colleagues in research because communication between scientists is as important as between plant cells.

Here are a few simple suggestions, based on our experience, that can lead to working successfully with D/hh scientists in the laboratory/research environment.

1. Unless the interpreter is a scientist (rare), always having an interpreter in the laboratory may provide access to information, but doing so will limit interactions between the D/hh scientist and laboratory members. For effective communication, we encourage laboratory members to learn how to directly work and communicate with their D/hh colleagues rather than relying on a full-time interpreter. When communicating directly with D/hh colleagues,
 - a. As a first approach, write! Do not be afraid to write or draw to help understanding.
 - b. Always face a deaf person and make and maintain eye contact while you are talking.
 - c. Try not to look away or cover your mouth as many D/hh people rely on lip reading to help them understand you.
 - d. Check your lighting and make sure your face is not in shadow and there are no strong lights or sunshine in the D/hh person’s eyes.
 - e. Keep your distance by standing 1–2 m away from the D/hh person. This is especially important for hearing-aid users, lip-readers, and signers.
 - f. Speak clearly, slowly, and steadily. Do not mumble, shout, or exaggerate—it distorts your lip patterns.
 - g. If there is more than one person in a conversation, take turns to talk. Do not talk at the same time or over each other.

- h. Repeat and rephrase as necessary. We strongly recommend to rephrase, and if all that fails, write it down (see a.).
2. Provide laboratory ‘warning’ signals such as timers that vibrate and/or have flashing lights and mirrors to promote awareness when working on the bench or hood. For example, when our D/hh colleagues are intently working at the microscope, turning the room lights on and off quickly is an effective way to get their attention.
3. Give preferential seating and/or set up a work environment that avoid visual barriers, for example, replace cubicles with open desks. This will allow D/hh scientists to feel included among their hearing laboratory colleagues. Isolation is the number one reason why aspiring D/hh scientists abandon their research careers.
4. When displaying any sort of complex structure, movie, or animation for the first time during a lecture, do not speak. After allowing time for complete observation, repeat the movie/animation with narration. A good example from our laboratory is when we are analyzing a rendering of a PD tomogram, we all watch in silence before we begin discussion, to ensure that our D/hh colleagues can appreciate all structural details before their attention is returned to the ASL interpreter or the captioning of the discussion.
5. Write detailed protocols for complex laboratory procedures, including all the ‘obvious’ details for using laboratory equipment (see 1.a.). This approach benefits not only our D/hh colleagues but also undergraduates and other inexperienced or new workers.
6. Ensure that trained ASL interpreters are available for training new D/hh laboratory members who depend on ASL, for example, for safety training during onboarding or when a new instrument like a confocal microscope is being introduced.

Our work on PD has benefitted from the keen observations of our D/hh colleagues, and we are hopeful that the D/hh and others with more diverse backgrounds will one day be fully integrated into the research community.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors apologize to their colleagues whose excellent work they could not cite because of article length. They are greatly appreciative to Drs. Jacob Brunkard, Rebecca Bart, and Brad Binder for their thoughtful suggestions and members of the Burch-Smith laboratory for helpful feedback. They thank Dr. Brandon Reagan for allowing them to use his work in Figure 2. This work was supported by the NSF through a Graduate Research Fellowship to AFS and grant to TBS (MCB 1846245).

Box 1.**TEM tomography**

TEM tomography reconstructs the internal three-dimensional structure of an object by taking serial-tilting projection images by TEM and back projection of these images [49]. Typically, 100–150 nm-thick sections of resin-embedded fixed samples are examined at 100–120 kV. Images are collected from -70° through $+70^\circ$ of tilt (commonly -65° to $+65^\circ$), and an image is collected at every degree of tilt or as desired with a CCD camera. For maximal resolution, the sample is rotated 90° , and a second tilt series of images is collected to produce a dual-axis tomogram. Images are computationally recombined using software like IMOD [59] to produce 3D models.

One limitation to TEM tomography for studying large cellular volumes is sample thickness, which is restricted to around 150–200 nm on standard TEM operating at voltages below 300 kV.

FIB-SEM

Focused ion beam milling combined with SEM (FIB–SEM) uses a beam of ions (usually gallium) to slice away successive layers of a sample, with the SEM collecting an orthogonal image of each layer uncovered. Samples can be milled in steps as small as 5 nm, and the resulting images can be used to generate three-dimensional images of biological cells and tissues with excellent z-axis resolution [60]. Although FIB–SEM produces similar images to TEM, FIB–SEM allows serial reconstructions of larger volumes of tissues to be generated quickly and automatically. One major advantage of FIB–SEM is that the thickness of a sample that can be analyzed is only limited by the time one is willing to spend collecting images.

SBF-SEM

Serial block face–scanning electron microscopy (SBF–SEM) uses a diamond knife mounted in the machine to repeatedly cut the sample section by section, generating serial sections that are then imaged by scanning electron microscope. The images are then computationally recombined to enable high-resolution 3D imaging of biological samples which have been fixed, stained, and embedded in resin. It is also amenable to other materials that can be sectioned using an ultramicrotome. SBF–SEM can provide nanometer-level ultrastructural data over relatively large fields of view and up to several microns in x, y, and z axes.

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- ** of outstanding interest

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