Imaging tools to reverse engineer the brain

Kavli Lecture, Sept. 3rd, Oslo

By Winfried Denk
Max-Planck Institute for Medical Research, Heidelberg, Germany

Why are we getting better at finding things out? Part of the answer is that finding out how things work makes us better at finding the next thing out, and so on. We develop methods of observation and experimentation based on our understanding of the world. Understanding light, its wave nature and how materials interact with it, allowed the 19th century physicists and craftsmen to design microscope objectives that were in some sense of the word perfect: their resolution was no longer restricted by technical imperfections but was “diffraction-limited”, i.e. determined by an intrinsic property of the light, its wavelength. Similarly, knowledge of chemistry was used to synthesize dyes, which together with the microscope allowed the study of cells and tissues. The discovery of “cathode rays” (electrons), which have a much shorter wavelength (less than 4 picometers at 100 kV), ultimately allowed the construction of a microscope of vastly better resolution, still limited by diffraction as one should note. Other examples abound. One might even argue that it’s the weapons more than the warriors that drive progress in our understanding of the world.

Observing things is at the core of formulating questions and often a major contributor to the answers. Visible light, with its quantum energy falling in between that of vibrations and electronic excitations and thus often only lightly absorbed by matter, was, of course, the only way to “look” at things before the advent of film and of electronic detectors. Those detectors now not only provide access to wavelengths below the violet and above the deep red, but also are so exquisitely sensitive that signals comprising only a few photons can be recorded with confidence. Fluorescence, light generated (rather the merely reflected or scattered) by matter as it is exposed to light, can thus be detected even when it’s too weak to be seen by the naked eye. This has opened the door to extended microscopic observation and quantitative recording of cellular processes, greatly aided by fluorescent indicators for cellular messengers. Indicators, originally compounds synthesized in chemists’ laboratories (Tsien 1989) are being replaced by indicator proteins (Miyawaki, Llopis et al. 1997) made by cells themselves from genetic templates introduced by any one of a number of clever genetic tricks. In particular calcium, universally coupling electrical and biochemical signaling, has become, for better or worse, the functional microscopist’s generic activity signal.

What works very well for cells in isolation, however, can fail miserably when applied to real tissue extending in 3 dimensions. Even with the best objectives, blurred light from out-of-focus areas superimposes itself onto the highly resolved image from the focal plane, thereby obscuring it. Another
one of the physicists’ inventions came to the rescue: the laser. It enabled confocal microscopy (Amos, White et al. 1987; Amos and White 2003) and the use of non-linear optics (Gannaway and Sheppard 1978), which can match the confocal microscope’s optical sectioning ability while avoiding some of its drawbacks (Denk, Strickler et al. 1990).

Physicists, more precisely one physicist, Maria Göppert-Meyer (Fig. 1), realized early on (Goeppert-Mayer 1931) that multi-photon excitation should be possible. But why would one want to coax, at great effort and expense, two or more photons to do the work of one readily available photon? For those interested in the energy-level structure of molecules the answer was that 2-photon excitation drives different electronic transitions, its “selection rules” are different. This spectroscopist’s delight is a mere nuisance for the microscopist who instead makes use of the highly super-linear intensity dependence of multi-photon excitation to create the ideal scanning microscope. No incoming and outgoing cones of light encumber the small region of excitation, confined to sub-wavelength dimensions (Fig. 2), as it is trained onto a point of interest in the sample and then, a measurement of the fluorescence taken and recorded, moved onto the next point, and so on. This point-by-point scanning process sounds tedious (and is quite un-fashionably un-parallel) but can, in fact, be fast and fully automated. Importantly, it creates an image even when the signal to be measured cannot be focused. Scattered fluorescence, whole-cell current (Denk 1994) or, in the case of the electron microscope, secondary electrons, can all be recorded as the primary beam samples the target at high resolution.

Nervous tissue, the central nervous system in particular, is very different from other tissues in that most neurons have hundreds, some more than hundred-thousand cellular neighbors, most of which they touch in a meaningful way, by forming synaptic connections with them. It is this network of synaptic connections that gives the brain its computational abilities and shapes its activity; imaging activity in, and the structure of, the intact nervous tissue is probably important. As it turns out, the 2-photon microscope is well suited to look at activity inside intact tissue (Denk and Svoboda 1997). It works well with calcium indicators, both chemically synthesized ones and genetically encoded ones, uses infrared light, which is scattered less than visible light and, because the detection need not be spatially resolved, can use scattered fluorescence (Fig. 3), which greatly improves the detection efficiency compared to a confocal microscope (as much a 10000 fold at a depth of 500 microns into the brain).
The retina is unique among nervous tissues in that it scatters only little. This is, presumably, because the light forming the image on the array of photoreceptors has to traverse several layers of neuropil and cell bodies that are responsible for the first stages of image processing in the nervous system. Here the advantage of two-photon microscopy is that when imaging, say, the inner plexiform layer (IPL, Fig 4A) the outer segments of the photoreceptors are far enough away so that only little two-photon excitation of the visual pigment occurs (Euler, Hausselt et al. 2009).

Functional (calcium) signals can thus be recorded during visual stimulation (Denk and Detwiler 1999).

Thomas Euler, who joined my lab in 2000, just after I had moved from Bell Laboratories to the Max-Planck Institute in Heidelberg, used two-photon microscopy to explore calcium signaling in starburst amacrine cells (SACs) and found that their dendrites act as independent computational units and respond preferentially to stimuli that move away (centrifugally) from the soma (Euler, Detwiler et al. 2002). These direction-selective signals are generated by a mechanism that is intrinsic to the dendrite, which we know because the signals persist even when inhibitory synaptic transmission is blocked.

What resolution do we need when we want to discern the structure of the entire network rather than the activity in individual cells or neurites? Reconstruction of the network requires the tracing of all the “wires” (neurites) and the identification of all synaptic contacts. Neural wires can be as thin as 50 nm in caliber and a resolution well in excess of that is needed to follow them. This rules out diffraction-limited light microscopy, but not, in theory, super-resolution optical imaging (Hell 2007). Electron microscopy certainly has sufficient resolution; Sidney Brenner and his colleagues exploited this when they set out to reconstruct the “mind of a worm” (White, Southgate et al. 1986). The resulting first cellular connectome took more than 10 person-years to complete, most of them spent analyzing rather than acquiring the data. Given that there are only a little more than 300 neurons in C-elegans, what is the hope of reconstructing even a fraction of the mammalian nervous system?
About a decade ago we set out to automate three-dimensional electron microscopic imaging, which essentially consists of cutting heavy metal-stained and plastic-embedded tissue into a series of slices, each a few tens of nanometers thick, and imaging them one after another. Rather than observing the cut slices in a transmission electron microscope, we decided to image the face of the tissue block, using a scanning electron microscope. This is done each time before a slice is cut by an ultra-microtome integrated into the microscope chamber (Denk and Horstmann 2004). As it turns out, this had been tried a few decades ago (Leighton 1981), but the microscope and data handling technology at the time weren’t ready for a serious application to circuit analysis. The original motivation for serial block-face electron microscopy (SBEM) was complete automation of image acquisition. Several additional advantages emerged. For one, the slice to be cut is aligned perfectly with the following one, simply because both are still part of a solid block of plastic rather than flimsy slivers. Also, the removed material is discarded and thus does not need to remain intact, which allows the slices to be substantially thinner (Fig. 6) than the 40 nm thickness that can be reliably cut during the production of a serial-section ribbon. In fact, since the removed material is no longer needed, other methods of removal can be exploited. In particular, the use of a focused beam of gallium ions has become popular in recent years (Knott, Marchman et al. 2008). While limited in field of view, FIB-SBEM allows cutting at increments of well below 10 nm while imaging with higher electron doses and thus achieving higher signal-to-noise ratios than are possible with diamond knife-based SBEM.

The contrast in the SEM is generated by detecting either (low-energy) secondary (SE) or (higher-energy) backscattered electrons (BSE). The number of electrons scattered backwards depends strongly on the atomic composition, because heavier nuclei scatter electrons much more strongly. In the transmission electron microscope this leads to a loss of signal. In the SEM, the signal is enhanced. The images look, however, rather similar if the SEM contrast is inverted, which is how we display them (Fig. 7). Block-face imaging requires the use of low electron energies (<3 kV) to distinguish the information from the slice to be removed (Hennig and Denk 2007) from deeper layers. While the generation of SEs is per se not dependent on the nuclear charge, BSEs are efficient generators of secondary electrons (SE2s), thereby imparting on them their material-contrast. Benjamin Titze in my lab has developed an automatic coating system that covers the surface after each cut with a film of palladium, conducting but still thin enough to only slightly weaken the BSE contrast from the underlying block. This solves the charging problem encountered when imaging insulating specimens in the SEM, while providing a better signal-to-noise ratio than low-vacuum imaging for both BSE and SE imaging.

Staining is central to almost any imaging project, as the runaway success of green fluorescent protein shows. Giving contrast to what one needs to see and nothing else can enable, or at least greatly
simplify, the analysis of the data acquired. Unfortunately, following the wires and identifying synapses imposes conflicting demands on the stain: in the first case only the plasma membrane should be visible while in the second case additional structures, such as vesicles and membrane thickenings, are needed to recognize synaptic contacts (Fig. 8). Because following the wires is, arguably, the more difficult task, with errors having more severe consequences (a single missed branch point can mean the loss of hundreds of synapses), we optimized plasma-membrane staining by introducing horseradish peroxidase before tissue fixation which then, with the help of hydrogen peroxide, causes the oxidative precipitation of (highly osmiohilic) diamin benzidine. Although vesicles and membrane thickenings are not visible in this case, the shape and size of a contact between neurites often allow one to infer the presence of a synaptic connection (Briggman, Helmstaedter et al. 2011).

Information on structure and on activity can augment each other, in particular when obtained from the same piece of tissue. One case in point are bistratified direction-selective retinal ganglion cells (DSGCs), which come in four flavours, morphologically indistinguishable but each sensitive to a different cardinal direction. Kevin Briggman imaged the calcium responses of all cells in a region of the ganglion-cell layer while visual stimuli moving in different directions were applied (Fig. 9). After that, he prepared this region for electron microscopy and imaged it over the course of about 6 weeks in the serial block-face microscope. Next (with the help of some undergraduate students and a software tool that we called KNOSSOS (Helmstaedter, Briggman et al. 2011) and that was coded by Jörgen Kornfeld and Fabian Svara), he traced 5 DSGCs of known preferred direction, starting from their somata. Also traced were a number of SACs. Then, Kevin analyzed the connections between the SACs and the DSGCs by inspecting about 10000 close encounters (< 1.5 microns) between their skeletons. Of those, fewer than 1000 were accompanied by a contact with the characteristic shape of a synapse (as verified using a conventionally stained data stack) at another 2500 locations cells merely touched each other. For the “synaptic” contacts but not for unspecific “touches” the preferred direction of the postsynaptic cell was strongly correlated with the angle of the presynaptic neurite, confirming that DSGC are connected to SACs such as to harvest their direction-selective dendritic signals (Briggman, Helmstaedter et al. 2011).
Moritz Helmsaedter took a different dataset (also acquired by Kevin Briggman) and, with the help of more than 100 undergraduate students, again using KOSSOS, obtained skeleton tracings of the neurites of all cells that had their somata in the inner-nuclear or ganglion-cell layers. Then he developed, in collaboration with Viren Jain and Srini Turaga (both then in Sebastian Seung’s lab at MIT), a neural network-based boundary classifier and used it to create an automatic volume segmentation (Fig. 10). The remaining false splits in this segmentation were healed by collecting all segments overlapping each skeleton and then fusing them into complete volume reconstructions of all traced cells. These volume reconstructions now allowed Moritz to detect and quantify contacts between cells and construct a connectomic matrix (Fig. 11).

The rows and columns of this matrix are sorted by cell class (ganglion, amacrine, and bipolar) and within those classes by type. An attempt to classify all bipolar cells into the classes known from the work of Heinz Wässle and colleagues (Ghosh, Bujan et al. 2004) was largely successful but a group of cells stubbornly refused to fit into any of those categories (but was similar to a cell type described for the macaque (Joo, Peterson et al. 2011) and may correspond to one described in mouse (Badea and Nathans 2004)). These orphan cells (Fig. 12) have a wide axonal field, are rare and sharply stratified. They appear to be connected mainly to one amacrine-cell type. Among bipolar-cells, type 5, unlike the other types, shows no clear mosaic, instead there is massive overlap of axonal fields. This indicates (Ghosh, Bujan et al. 2004) that there are multiple, morphologically indistinguishable sub populations. The contact patterns between type-5 bipolar and ganglion cells are highly variable. About one third of the cells are, for example, strongly connected to the “W3-local edge detector”, about one third only weakly, if at all. When selecting only the latter cells, a reasonable mosaic emerges (Fig. 13).

Recently, we got interested in how birds learn and produce mating songs, that is to say, my former Bell Labs colleague Michael Fee (MIT) and one of his former postdocs, Michael Long (NYU) both approached us with different questions about the wiring in the part of the brain that controls song production and learning. Producing a song requires the activation of muscles in a precisely timed way. Michael Fee and his colleagues discovered that cells in an area called HVC fire sparsely but in strict synchrony to the song (Hahnloser, Kozhevnikov et al. 2002).
How is this activity pattern generated? One possibility is that the cells form a synaptic chain (Fig. 14), each cell triggering the next with a certain delay caused by action-potential travel time, and the delays inherent in synaptic transmission and action potential firing. Such a chain would have a characteristic connection matrix, with most connections above the diagonal if the cells are sorted in time along both the rows and columns, as a presynaptic cell (row) will always connect to a postsynaptic cell that fires later. While such an ordering can be found using the connection pattern alone, an important question is whether the structural order corresponds to the ordering of the activity in time. The plan is for Mike Long’s lab to use 2-photon calcium imaging during singing to map the timing and then send the tissue to Jörgen Kornfeld (in my lab), who would then map the connectivity in the same piece of brain using the serial block-face microscope. Song learning requires synaptic plasticity in specific synapses. To test whether in “area X” the synapses made by sparsely firing, song-synchronized axons originating in HVC are of the right “morphological” type, i.e. terminate on spines, Jörgen Kornfeld (in collaboration with Michale Fee’s lab) is reconstructing axons that synapse onto the spines and dendritic shafts, respectively, of a “median spiny neuron” and finds distinct differences in the morphologies of the reconstructed axons.

Both in the retina and in the bird brain, we can address certain questions by looking at the local circuitry but, inevitably, the desire arises to follow at least a few selected processes beyond the edge of the dataset to see where they come from. This is related to the fact that, in the end, the brain has to decide which one course of action to take in light of all the evidence provided by present (and past) sensory experience. Sensory information needs to be distributed widely to different processing modules and the results of all that parallel processing has to be integrated. Thus our curiosity will not be satisfied until we can, at least in principle, follow the flow of information to anywhere in the brain.

How can we get a data set that will allow this? Cutting living or freshly fixed tissue into blocks small enough to be prepared with traditional techniques is likely to leave surface damage that will make it difficult, if not impossible, to trace especially small processes from one piece to the next. Shawn Mikula has, therefore, developed a procedure to stain the whole mouse brain (Fig. 15). During every incubation step, substances need to diffuse from the solution surrounding the sample to every point in the sample (and vice versa during each washing step). Diffusion, which transports a solute very quickly over small distances (1 micron is bridged in about a millisecond), slows down dramatically as distances increase (for 100 microns 10 seconds are needed and for 5 mm, 8 hours). Patience is required for large samples but, as Shawn found out, it pays off. By increasing the incubation steps to 48h hours and the washing steps to 8 hours each, it is possible to obtain whole-brain samples that are not only

![Figure 14. Neuron-chain model for the generation of sparse, well timed sequence](image1)

![Figure 15. Whole mouse brain stained and embedded for EM imaging.](image2)

![Figure 16. Block-face image of cross section through whole brain](image3)
stained uniformly (Fig. 16) but are also plasticized well throughout, as cutting test from various locations in the brain show. In this case we focused on making the myelin visible so we can follow all myelinated axons. This would not give us a cellular-level connectome but would provide a map of all major connections between areas (sometimes called a projectome). Following all myelinated axons should be possible at a resolution of 80 nanometers, which, at 40 MHz pixel rate, would allow the acquisition of data for a whole brain in about one year.

At such a rate it would take 50 years or more to acquire data at 20 nm resolution, which would be needed to trace every neurite and identify every synapse. One could farm the imaging out to a whole factory hall full of electron microscopes but for that one would have to cut and collect large slices or divide the brain into small blocks without loss at the interfaces. Alternatively one could employ a multi-beam SEM (Fig. 17), which uses many electron beams in parallel, resulting in an aggregate pixel rate well above one GHz, bringing down the total acquisition time to a year or so.

There remains the problem of what to do with a large room full of hard drives that contain basically grainy black and white images. (at about 400 microliters a mouse brain contains about 50 peta (10^15) voxels of the required size. Those data would fill about 12500 4 terabyte disks). At a rate of 10 h/mm it would take about a 100 million person hours to completely skeletonize one mouse brain. This seems like a lot of hours until one looks at how much time Americans alone spend playing video games (about 50 billion hours/year). Now we only have to make tracing neurons fun (addictive?). While getting computers to do the analysis has been much more difficult than I initially expected, having the data for a whole brain might provide a strong incentive for more computer scientists to try harder to find an algorithm that works.

Acknowledgements

I would like to thank all the colleagues and collaborators who have contributed to this work and also Julia Kuhl for her help with graphics and proof reading.

Literature cited


