da Vinci's notebook

da Vinci's Notebook, vol. 8 (2016)

Seeing the Brain with CLARITY: Using a Hydrogel to Increase Three-dimensional Visibility of Post-mortem Neural Structures

Madeleine Kulak

First Year Student (B.Sc. Nanoscience)

College of Physical and Engineering Science, University of Guelph, CANADA

It is not entirely understood how the human brain works. It is clear, however, that the brain is the control centre of the nervous system, but how its structure relates to the way it functions is still unknown. This is due to its great complexity. The brain is an intricate network of over 85 billion specialized cells called neurons and about 100 trillion synapses which are spaces between neurons enabling neuron to neuron communication. A neuron has an average area of 50 μm^2 and the average synapse is about 20 nanometres (nm) long (Lee & Kim, 2014). The neural network can handle chemical and electrical information in such a way that allows memories to form, as well as maintaining physiological processes such as the heartbeat (Azevedo *et al.*, 2009).

If the brain is to be understood, there needs to be a way to integrate observations that happen at the intracellular, tissue and whole body levels (Silva, 2006). Furthermore, identifying how a cluster of neurons processes information requires registering trillions of bits of data associating form to function at the nanoscale, over time measured in nanoseconds (Lee & Kim, 2014). This is a major challenge for neuroscience because the quality of the data obtained depends on the sensitivity of the research tools used. Classical neuroscience relies on the microscopic observation of a few isolated neurons, which allows for very rough structural descriptions (Alivisatos *et al.*, 2013). To alleviate this challenge, researchers are adapting chemical and physical technologies that work at the nanoscale. One recently developed technique called CLARITY uses a nanomaterial to optimize the observation of neural networks and subcellular arrangements without damaging the brain tissue (Chung *et al.*, 2013).

CLARITY (Clear, Lipid-exchanged, Anatomically Rigid, Imaging-compatible, Tissue hYdrogel), is a tissue fixing technique that renders biological tissue transparent so as to easily observe large and bulky neural structures (Tyson & Hilton, 2015). Transparency is achieved by using a hydrogel, or a hydrophilic network of polymer chains made of acrylamide. The hydrogel forms through the bonding of acrylamide units (monomers) that are infused into post- mortem biological tissue. The network assembles across the neurons' cytosols and the extracellular spaces securing all hydrophilic structures such as proteins and nucleic acids within it (Zheng & Rinaman, 2015). The lipid constituents

are hydrophobic and remain loose in the mesh so they can be removed leaving cavities that improve visibility (Chung *et al.*, 2013).

Tissue clearing is useful because the brain has a high lipid content that encloses the water-based cytosols and extracellular spaces. The great limitation of the lipid-aqueous interface is that it scatters light and makes structures look cloudy, so no substantial depth of observation can be achieved (Chung & Deisseroth, 2013). CLARITY removes the lipid components and largely preserves the three-dimensional positions of other macromolecules (Chung *et al.*, 2013). The cavities in the hydrogel allow passage of other substances such as pigments to reveal neural paths across the brain that can be traced from the nanoscale to the macroscale (Shen, 2013; Silva, 2006). This integrated view of neural connections can help produce a map of the brain that relates structure to function and can increase understanding of how the brain changes as it grows, learns, or is afflicted by illness or injury (Alivisatos *et al.*, 2013).

There are three main steps in tissue preparation when using CLARITY, as shown in Figure 1. In the first step, a sample is submerged in a phosphate buffered saline solution (PBS) that contains formaldehyde and acrylamide. The system is maintained at 4 ˚C, which allows the acrylamide to be passively infused inside the cells. The formaldehyde acts as a crosslinker for the monomers that form the mesh (Chung *et al.*, 2013; Poguzhelskaya *et al.,* 2014). In the second step, the temperature is increased to 37 ˚C to facilitate molecular movement. The lipids are slowly flushed out of the hydrogel by

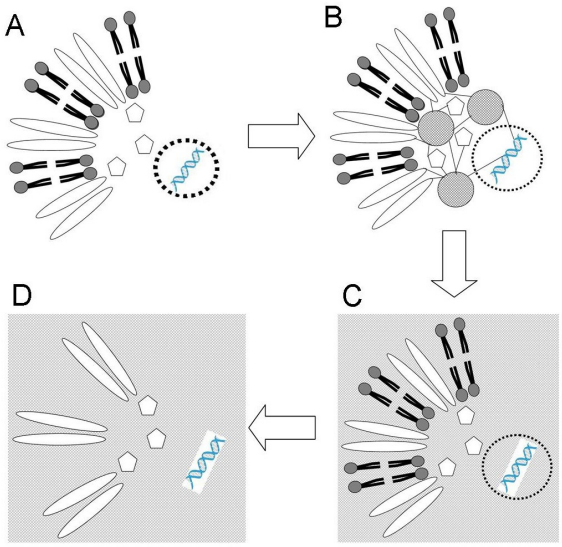

Figure 1. Simplified CLARITY steps.

A. Magnified portion of a neuron showing the cell membrane with phospholipids (black shapes) and proteins (white ovals), the cytosol containing hydrophilic components (white pentagons), and the nuclear membrane with phospholipids (dotted circle) encircling DNA.
B. Monomers are infused into the cell and crosslink gradually (shaded circles) attaching themselves to hydrophilic components.
C. A hydrogel fully formed across the cell and extracellular space.
D. Lipids are flushed out leaving in place only the hydrophilic components.

infusing the structure in an ionic detergent that is then subjected to electrophoresis (i.e., dispersion of particles using an electric field). In the third step the sample is washed in a new PBS to remove any residue (Chung & Deisseroth, 2013). The resulting structure is a hybrid between the hydrogel and the hydrophilic components (Alivisatos *et al.*, 2013). CLARITY can be used to prepare whole brains for observation as illustrated in Shen (2013).

The CLARITY hybrid is compatible with several types of microscopy such as fluorescence, confocal, and multiphoton, because visible-spectrum light can penetrate deep into the transparent multicellular matrix (Silva, 2006). Also, the

hydrogel protects most proteins from chemical damage with only a 4% loss (Zheng & Rinaman, 2015). This is an advantage to traditional tissue fixation using urea, which causes protein damage of about 41% (Chung et al., 2013). Preserving proteins and other structures has proven useful in research. For example, in an original experiment, Chung et al. (2013) clarified the entire brain of a three-month-old line-H genetic mouse. The hybrid brain was submersed in 85% glycerol and the neural structures were seen at a cellular scale under a confocal microscope. In a second experiment by the same authors, the brain of an eight year old autistic boy was observed at scales from 10 μm to 0.5 mm, using a high resolution SPIM (i.e., Selective Plane Illumination Microscopy) microscope.

The brain revealed multiple connections between the axons of separate neurons. This discovery was discussed by Chung and Deisseroth (2013) who suggested that the unusual brain structure might be related to autistic behaviour, because such multiple connections do not exist in normal brains.

Although CLARITY offers the potential to observe previously inaccessible information, the method has some disadvantages. For example, preparation of whole human brains can take up to ten weeks, and the electrophoresis process can destroy the hybrid if handled incorrectly. These issues prompted Poguzhelskaya and others (2014) to propose a modified procedure called CLARITY2. In this technique, the first CLARITY step was followed but after the hydrogel formed, the hybrid was cut into 1-1.5 mm thick slices instead of treating the brain intact. By handling a series of thinner samples, CLARITY2 simplified the total clearing time to two weeks, including electrophoresis. The clarified samples were observed under confocal microscopy and a light penetration of 250 μm up to 1.5 mm was achieved. However, the sliced samples treated with CLARITY2 required lengthy data integration using software to obtain a virtual, three dimensional image of a whole brain (Poguzhelskaya *et al.*, 2014). This highlights the fact that CLARITY is a very recent technique with great potential that needs further innovation to reduce sample destruction or long treatment periods when preserving intact brains (Chung & Deisseroth, 2013). Also, a concern with both techniques is that it is not yet established how long the tissue can be stored and preserved intact (Tyson & Hilton, 2015), as the first samples were processed in 2013.

In general terms, data obtained with CLARITY or CLARITY2 can be combined with data from other technologies. For example, the brain of a live mouse can be implanted with electrodes to produce certain behaviour such as moving a leg (Alivisatos et al., 2013). The targeted section can be clarified post-mortem to identify the set of neurons involved in the behaviour and therefore delineate a cell map that can link form to function. This in turn could guide further research to record the interaction of neurons in real time.

Conclusion

The complex physiology of the brain involves events that affect millions of cells simultaneously. In addition, microscopic observations are difficult due to the high

lipid content of this organ. For these reasons, neuroscientists and nanoscientists are converging to develop better tools to record and integrate neurological data so a map of the brain can be drawn. CLARITY and CLARITY2 are new tissue preparation techniques that use an acrylamide-based hydrogel to secure neural structures in a three dimensional arrangement, as well as making tissue transparent and easy to observe. Overall, cleared brain samples provide a spatial view of neural networks that can help relate form to function. However, tissue clearing raises interesting questions, most notably regarding the length of time that the samples can be preserved. Further multidisciplinary studies are needed to compile neurological information that can help in understanding how the brain works.

References

Alivisatos, A. P., Andrews, A., Boyden, E., Chun, M., Church, G., Deisseroth, K., & Zhuang,X. (2013). Nanotools for Neuroscience and Brain Activity Mapping. *ACS Nano, 7*(3), 1850-1866.

Azevedo, F., Carvalho, L., Grinberg, L., Farfel, J., Ferreti, R. E., Leite, R. E., & Herculano-Houzel, S. (2009). Equal numbers of neural and nonneural cells make the human brain an isometrically scaled-up primate brain. *Journal of Comparative Neurology, 513*(5), 532-541.

Chung, K., & Deisseroth, K. (2013). CLARITY for Mapping the Nervous System. *Nature Methods, 10*(6), 508-513.

Chung, K., Wallace, J., Kim, S.-Y., Kalyanasundaram, S., Andalman, A., Davidson, T., & Deisseroth, K. (2013). Structural and Molecular Interrogation of Intact Biological Systems. *Nature, 497*(7449), 332-337.

Lee, J.-W., & Kim, M. M. (2014). *Nanotechnology-Neuroscience Convergence*. Handbook of Science and Technology Convergence. Cham: Springer International Publishing.

Poguzhelskaya, E., Artamonov, D., Bolshakova, A., Vlasova, O., & Bezprozvanny, I. (2014). Simplified Method to Perform CLARITY Imaging. *Molecular Neurodegeneration, 9*(1), 1- 5.

Shen, H. (2013, April 10). See-through brains clarify connections [video]. Retrieved from http://www.nature.com/news/see-through-brains-clarify-connections-1.12768

Silva, G. A. (2006). Neuroscience Nanotechnology: Progress, Opportunities and Challenges. *Nature Reviews Neuroscience, 7*(1), 65-74.

Tyson, A., & Hilton, S. A. (2015). Rapid, Simple and Inexpensive Production of Custom 3D Printed Equipment for Large-Volume Fluorescence Microscopy. *International Journal of Pharmaceutics*.

Zheng, H., & Rinaman, L. (2015). Simplified CLARITY for Visualizing Immunofluorescence Labeling in the Developing Rat Brain. *Brain Structure and Function*, 1-9.

da Vinci's Notebook (ISSN 1923-5720) is sponsored by the
College of Physical and Engineering Science, University of Guelph, Canada.