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Getting more CLARITY to gain the third dimension

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Until the invention of the microscope, classical anatomists only relied on their naked eyes for their studies. In this way, studies of tridimensional human anatomical structures were carefully conducted by the pioneering Padovan School in the fourteenth century: Vesalius (1514-1564), Realdus Colombus (1516-1559), Gabrielle Fallopio (1523-1562) and Fabrizio da Aquapendente (1537-1569). When tissue slices were first observed through a microscope, anatomists could obtain a much closer view of the structures, getting a detail that they had never imagined before, although at the price of renouncing the third dimension. Manual, first, and digital techniques of 3D reconstruction in recent years have allowed us to obtain tridimensional images from stacks of serial sections. However, full power 3D microscopy of large, complex organs seemed a very difficult task.

This aim seems to be closer according to a recent paper published in *Nature* by the team of Karl Deisseroth at the University of Stanford (Chung et al., 2013). This paper describes a novel technique called CLARITY by their authors. The technique uses the cationic detergent SDS (sodium dodecyl sulphate) to clear the tissue by washing out lipids. This procedure of clearing thick sections of tissues was already known. However, in previous applications, despite the formaldehyde fixation of the tissue, which forms covalent bonds between proteins and contributes to stabilize the tissular structure, many proteins were lost after SDS permeabilization. The novelty of CLARITY is the combination of formaldehyde fixation and SDS treatment with acrylamide infusion. In brief, the tissues are infused at 4ºC with formaldehyde, acrylamide and heat-activated initiators of acrylamide polymerization. Then, the temperature is raised to 37ºC, the acrylamide polymerizes, and the formaldehyde cross-links not only proteins and nucleic acids but also these biomolecules to the acrylamide monomers, first, and then to the polymer. The result is a stiff, nanoporous tissue-hydrogel hybrid that retains large molecules as proteins and nucleic acids allowing for lipid extraction with SDS. This procedure has shown that more than 90% of all proteins remain in the tissue after clarification.

The authors of the paper have demonstrated that CLARITY allows for the study of whole brains from adult mice, which became transparent and could be analyzed *in toto* with long working distance optical microscopy. Furthermore, the retention of the proteins and nucleic acids allowed the use of classical techniques of immunohistochemistry and in situ hybridization (ISH), whose results could be studied directly in three dimensions, with no need for time-consuming reconstruction from serial sections. The authors have also shown that CLARITY can be used on formalin-fixed human brain tissue obtained in autopsies. They were able to follow individual nerve fibres in fragments of 0.5 mm in thickness from the frontal lobe of an autistic patient, samples which had been stored for six years.

The possibilities of using CLARITY in neuroanatomy are breathtaking. The study of brain circuitry at the level of individual fibres, virtually impossible by reconstruction of serial sections, becomes a relatively easy task especially if the nerve fibres or the neurons are labelled by immunohistochemistry, ISH, or if transgenic animals are designed to express fluorescent proteins in subsets of neurons, as described below. Regarding immuno-

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histochemistry, Deisseroth and co-workers demonstrated that CLARITY allows for multiple rounds of immunohistochemical staining – i.e., some antigens can be detected, and then the antibodies can be washed out and successive rounds of antigen detection can be performed on the same polyacrylamide-embedded tissue without damaging the structures or the antigenicity of the tissue. With these techniques, brain circuitry and gene expression might be directly compared in brains from individuals with neurological disorders and healthy controls.

CLARITY could also allow for another amazing possibility: to generate a library of brains that can be stored, exchanged, studied and reused by different neurobiologists. Thus, it is conceivable that a "bank of brains" could be added in the next future to the current banks of antibodies, probes, cells or tumors.

Finally, another exciting possibility would derive from the convergence of the CLARITY technique with BRAINBOW (Fig. 1), a technique developed in 2007 by the group of Jeff W. Lichtman and Joshua R. Sanes in the Department of Neurobiology at Harvard Medical School (Livet et al., 2007; Smith, 2007). The technique was originally designed for the mouse, but it has been modified to be used in other animals, such as zebrafish and *Drosophila* (Hadjieconomou et al., 2011; Hampel et al., 2011; Pan et al., 2011; Boulina et al., 2013). In BRAINBOW mice, the neurons are genetically engineered to randomly express different amounts of red, yellow, orange and blue fluorescent proteins, by inserting in the genome multiple copies of a genetic construct that can be recombined in many ways to produce more or less amounts of these proteins. The combination of the colours provide neurons labeled with a specific hue. This allows us to map simultaneously the projections of more than 100 neurons that are emitting light with different wavelengths. The possibility to study BRAINBOW brains with CLARITY will probably increase greatly our knowledge about the "connectomics",that is, the study of the brain circuitry that aims to explain complex cerebral functions such as behaviour.

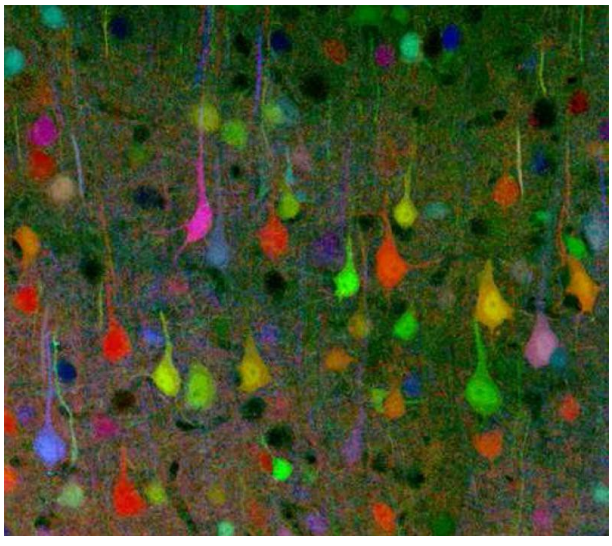

Fig. 1. A BRAINBOW example: mouse neurons labeled with fluorescent tags. Author: Stephen J. Smith. Source: Smith (2007). Reproduced from Wikimedia Commons.

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