University of Colorado Anschutz Medical Campus

Optogenetics and Neural Engineering

Linear CLARITY Electrophoretic Tissue Clearing Chamber

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Introduction

Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging / Immunostaining / in situ-hybridization-compatible Tissue hYdrogel (CLARITY) as developed by Chung et al., 2013, is a method to affix a biological tissue sample in a hydrogel polymer and rapidly clear tissue of light scattering molecules. Large molecules such as proteins are mostly unaffected by the clearing process, therefore allowing for deep tissue imaging. CLARITY requires a Electrophoretic Tissue Clearing (ETC) chamber for holding the tissue as a Clearing Solution passes around the tissue and electricity applies force to the charged miscues, thereby speeding up the clearing process. Current chamber designs (http://forum.claritytechniques.org/) are suggested to either be bought as a unit from a supplier, 3D Printed, or fabricated through custom means. As reported in the CLARITY forum, these chambers are expensive, require expert tooling (3D Printers), frequently leak, are prone to variability, and can be difficult to expand. The methods suggested to secure the tissue within the electric field are variable and tissue-electrode contact has frequently been reported. Custom fabrication is prone to air leaks into the system and the electrode-solution interface may also introduce bubbles in the electric field. This can lead to uneven clearing or will over time build up and decrease the applied current.

Motivation

The Linear Clarity Electrophoretic Tissue Clearing (L-ETC, Figures 1-3) chamber, as developed by the Optogenetics and Neural Engineering Core of the University of Colorado School of Medicine, is an inexpensive and reliable CLARITY ETC chamber that can be easily assembled by a novice user. It provides several key advantages over current ETC chamber fabrication methods including secure tissue containment, more consistent and efficient tissue cooling, fast lipid removal, easier chamber fabrication, and very low initial costs. The L-ETC chamber aligns the Electric Field with Clearing Solution flow and is the main focus of this proposal. The L-ETC system as a whole (pump, refrigerator, and power supply) will also be discussed in terms of settings, but are considered general lab equipment and are therefore not considered in the cost. The cost discussed here also does not account for tissue nor the chemicals used. All chemicals used are of the same formulation as originally described, unless specified.

The L-ETC chamber is easily fabricated from ‘off the shelf’ parts, and are therefore subject to industry standards, thereby minimizing the risk of leaks. The tissue to be cleared is securely contained in a fine mesh cylindrical tissue strainer. The tissue strainer is fit into off the shelf plumbing components between platinum

mesh plate electrodes, creating the L-ETC. The detergent buffer clearing solution (as described in *Structural and molecular interrogation of intact biological systems,* Deisseroth 2013) is pumped through the L-ETC, forcing the solution over and through the tissue, expelling generated bubbles from around the tissue. A DC power supply applies an electrical field over the tissue and a refrigerator removes the heat from the solution generated at the platinum-solution interface. The L-ETC applies a uniform electric field, allowing for rapid tissue clearing. The temperature and pressure of the solution can be monitored at the inlet or outlet near the tissue. If necessary, the use of a simple refrigerator allows for stable, uniform cooling without the need for non-uniform ice baths or expensive cooling equipment.

The L-ETC system has shown success with mouse brains and spinal cords and rat lungs. Half mouse brains (n=20) have been successfully cleared in 2-3 days with the L-ETC and whole mouse brains (n=4) have also been cleared in 3-5 days. Smaller, 128 µm slices of mouse brain can be cleared in under a day (n=3). Rat lung slices (5 mm) were cleared in 2 days.

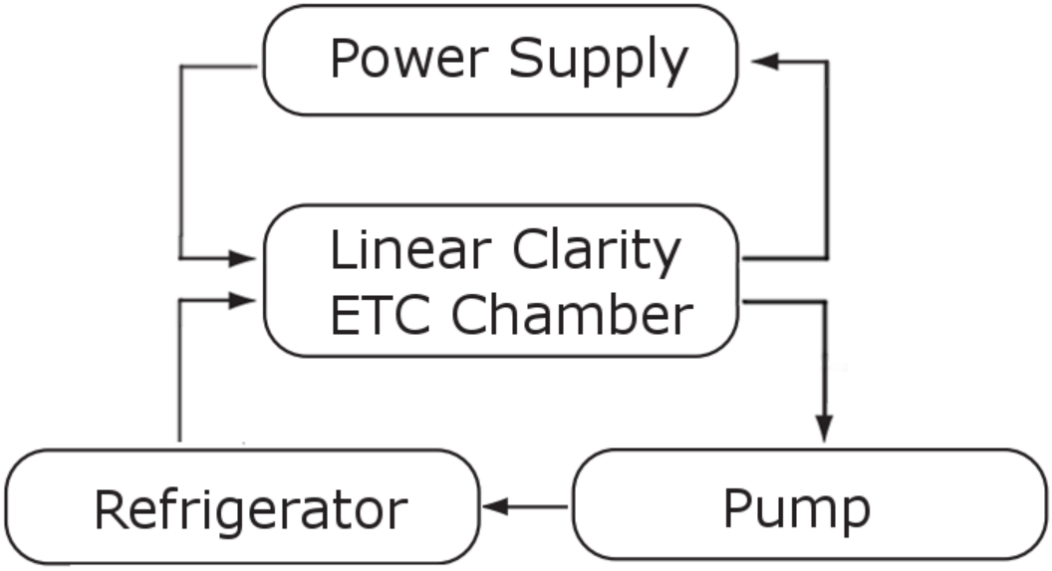

Figure 1: The CLARITY System consists of four main components. The L-ETC holds the tissue to be cleared between two platinum mesh electrodes and allows for linear flow of the clearing solution and electric current. As with other CLARITY Chamber systems, a DC power supply provides current over the tissue and a pump controls the flow of the solution. A dorm refridgerator provides sufficient heat removal, if necessary.

Figure 2: Top: The L-ETC System. The power supply sits above the pump, chamber, and refridgerator. The wires from the power supply hang down, with the aligator clips at different heights. Middle: The L-ETC can be suspended at an angle to allow bubbles to rise up with exiting Clearing Solution. Bottom left: a simple refridgerator can house the solution reservoir and extract heat if necessary. Bottom right: a peristolic pump is more than adaquate to provide the recommended 1.2 L/min flow rate.

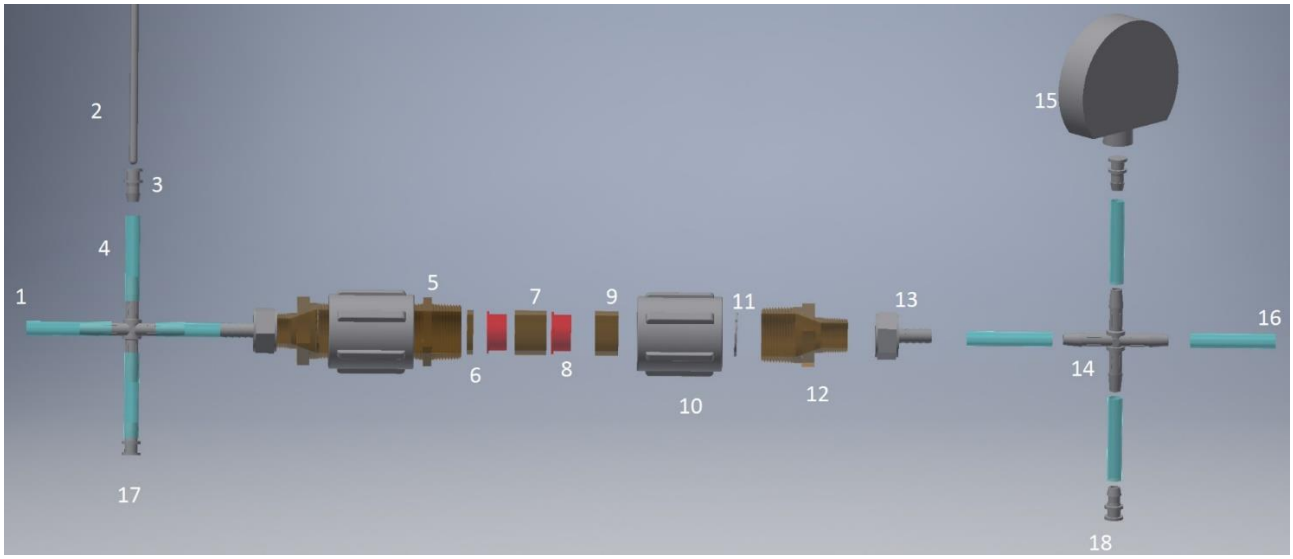

Figure 3: The Linear Electrophoretic Tissue Chamber (L-ETC). Assembly exploded on the right of Item 5 for ease of viewing, duplicate parts not numbered. The Clearing Solution flows from left to right (from 1-16). Current can flow either direction. All parts except for 6, 7, 8, 9, and 11 are 3D models downloaded from McMaster Carr (mcmaster.com). Wire and Clearing Solution not shown.

Item	Description	PN	Supplier	Qty nee	Qty in package	Price per Package	Total $	Total Cost Per
1	Solution Inlet	-	-	-	-	-	-	-
2	Thermometer	6182K51	McMaster	1	1	17.91	17.91	17.91
3	3/8" ID Barbed Tube Plu	2974K401	McMaster	4	10	8	8	3.2
4	3/8" ID Clear PVC Tube	5231K355	McMaster	12"	300	11.25	11.25	-
5	1 1/4" Hex Threaded Nip	46825K51	McMaster	1	1	1.94	1.94	1.94
6	1" PVC Pipe (spacers)	48925K93	McMaster	.178"	60"	5.27	5.27	0.02
7	1" PVC Pipe (spacers)	48925K93	McMaster	.681"	-	-	-	0.06
8	Sample Holder	21008-949	VWR	2	50	72.3	72.3	2.892
9	1" PVC Pipe (spacers)	48925K93	McMaster	.927"	-	-	-	0.08
10	1 1/4" Coupling	46885k185	McMaster	2	1	6.12	12.24	12.24
11	Platinum 2" x 3" with 4"	PLATINUM	The Amateur	1/5	1	25	25	5
12	1 1/4" to 3/4" Reducer H	46885K305	McMaster	2	1	2.91	5.82	5.82
13	3/8" Tube to 3/4" Femal	5372K222	McMaster	2	5	7.89	7.89	3.156
14	3/8" Cross Connectors	5463K97	McMaster	2	5	8.51	8.51	3.404
15	Pressure Gage	Not Nec	-	-	-	-	-	-
16	Solution Outlet	-	-	-	-	-	-	-
17	Positive Wire (NP)	Lab	-	-	-	-	-	-
18	Negative Wire (NP)	Lab	-	-	-	-	-	-
19	High Strength Sealant (N	7475A67	McMaster	1	1	9.41	9.41	9.41
20	Dorm Refridgerator (NP	Lab	-	-	-	-	-	-
21	Pump (NP) (~.4 L/min)	Lab	-	-	-	-	-	-
22	Power Supply (NP) (28V	Lab	-	-	-	-	-	-
23	TaegaSeal PTFE Tape	4591K12	McMaster	1	1	2.31	2.31	2.31
	Totals						176.13	65.13

Table 1: L-ETC parts list. Item numbers consistent throughout paper. Some parts are not pictured in Figure 1 (labeled NP in description). Component of the system beyond the chamber are considered lab equipment and are not specified or considered in the price (example: pump, refrigerator, power supply, wrench).

Basic L-ETC System Components

The Linear Clarity ETC Chamber system can be fabricated from 'off-the-shelf' industry standard parts (Table 1). For ease, most of these parts can be found on McMaster.com. Most parts are from the plumbing industry and therefore subject to the standards of UTS overseen by ASME/ANSI. Such standardization results in parts that can easily and reliably be assembled and disassembled with minimal risk for leaking when assembled correctly. This standardization allows for uniformity across chambers (and labs). Furthermore, standardization also allows for part replacement and chamber expansion. All plumbing parts may be assembled by hand or with assistance from a large wrench. The T-Barbed connections to the 3/8" tube may be frequently and reliably connected and disconnected simply by pushing/pull on the components, allowing for easy access to the tissue.

The Clearing Solution (as described in CLARITY Protocol, Chung, et. al, 2013) flows linearly from the inlet (Item 1), through a series of tubes and connectors that can hold up to two whole mouse brains (Item 8), sandwiched between two platinum mesh plates (Item 11), out the outlet (Item 16), through a pump (Item 20, not pictured), and is cooled in the Clearing Solution reservoir. Current flows through electrically insulated wire from a DC power supply. The wires enter the ETC chamber (Item 17, wire not pictured) and pass through the solution until it reaches the platinum plate cathode (Item 11). The current passes through the solution and tissue(s) to the anode platinum plate, connected to a wire that will exit out the system (Item 18, not pictured). Auxiliary environmental monitoring devices, such as a Thermometer (Item 2) and a pressure gauge (Item 15) can quickly and easily be mounted at the solution inlet or outlet, but are not considered necessary.

One tissue sample holder (Item 8, WVR PN 21008-949) can securely constrain a whole mouse brain, securing the tissue in a safe chamber away from electrodes, yet allowing for tissue expansion. The outer dimensions of the sample holder dictated the size of subsequent parts. If larger tissue samples or whole body clearing is required, larger tissue holders may be chosen, resulting in a larger chamber. The parts that hold the tissue do not allow for light penetration and thereby minimize photo bleaching.

Assembly of the L-ETC

The tissue strainers are of cup shape. If the pump is of good consistent pressure, the tissue should remain held against the base of the cup by the flow of fluid. However, it is possible that tissue could move against the direction of fluid flow, and therefore it is advisable to completely secure the tissue. The tissue in the second, downstream tissue strainer in contained by the base of the first strainer. In order to fully contain tissue in the first, upstream tissue strainer, the first spacer (Item 6) can be fitted with cell strainer mesh. Simply take a cell strainer and cut off the base. Then place the base in the washer and affix the two with silicone (Item 19).

Figure 4: The tissue strainer comes with a tab (right) that can be cut (middle) for holding tissue (Item 8). The top of the tissue strainers can be cut off and fixed into a spacer to ensure complete security of the tissue (Item 6).The size of the tissue strainers dictates the size of all other components of the L-ETC.

As shown, the Threaded Nipple (Item 5) holds two tissue samples in series. It is possible to add additional tissues in series by attaching another Threaded Nipple with more Couplers (Item 10), thereby creating a line of Couplers in series. However, it is preferable to expand the system by adding an additional chamber in parallel (see L-ETC Expansion below).

Figure 5: Right: the tissue strainers fit (Item 8) into the spacers (Item 6, 7, and 9). When stacked, they are the same height as the Threaded Nipple (Item 5) that houses them. Left: The components are shown in exploded view for consideration. As shown, Clearing Solution would flow from right to left.

Theoretically, the Coupling, Reducer, and Tube to Pipe Connector (Items 10, 12, and 13 respectively), could be one part. These three parts serve to connect the Threaded Nipple to the tubing size suited to the pump. For the given tissue strainer size, a 1 ¼" Threaded Nipple was chosen and the pump of the lab was well suited for 3/8" ID tubing. If a more standard size Threaded Nipple was chosen (example: 1"), then Items 10, 12, and 13 can be combined as one part (example: McMaster Item 5372K136, ¾" Tube ID x 1" Male Pipe size).

Assembly: Platinum Plates

The platinum plates from The Amateur Chemist (Item 11) can replace expensive platinum wire frequently used in other ETCs. These are plates typically used to apply coatings to jewelry. They are porous, allowing for fluid to flow around and through the plates, cooling the interface and providing a more uniform electric field. These plates come with a stem that can be removed with a grinder or simply cut with metal shears. The plates can then be cut into near-circles of diameter 1.275" with metal shears. This allows for several platinum electrodes to be made from one plate. Drill an appropriate size hole in the top of two Tube Plugs (Item 3) for the wire to pass through. Feed the wire through the Plug, tube, cross connector, tube, tube to pipe connection, and the reducer (Items 18, 1, 14, 1, 13, and 12). It has been reported on the CLARITY Forum that the clearing solution can quickly degrade any exposed copper, so it is important to insulate the copper wire from the solution, silicone recommended (Item 19). The copper wire can be directly connected to the platinum plate and then all exposed copper insulated. Alternatively, the copper wire can connect to platinum wire, which can then be connected to the platinum plate (pictured).

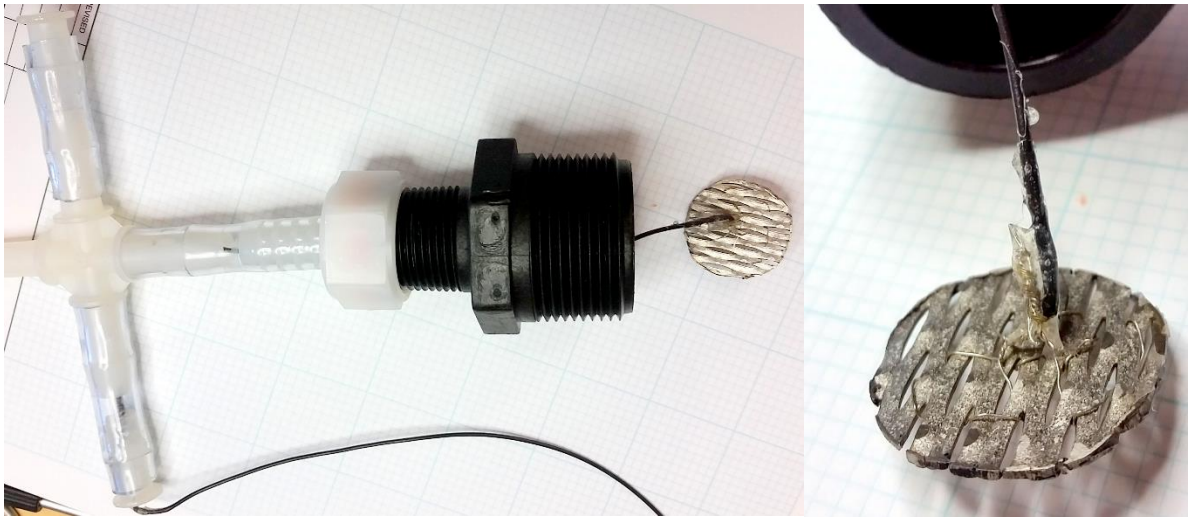

Figure 6: Right: Wire enters the L-ETC at the bottom through a silicon sealed T-Barb connector (Item 18) and leads to the platinum plate (Item 11). Once the wire is connected to the platinum plate, the plate can be pushed into the Reducer (Item 12) and fixed with silicone. Left: copper quickly degrades in the Clearing Solution. Encapsulate any exposed copper with silicone. As shown, the copper wire connects to platinum wire which connects to the platinum plate. It is possible to directly connect the copper to the platinum plate, but it should be encapsulated with silicone.

Assembly: Spacers

For the given tissue holder size, the spacers used to separate the tissue holders (Items 6, 7, and 9) can be made from one PVC tube (McMaster 48925K93). Note that tolerance mismatch between these parts may require some sanding to allow the spacers to fit into the Threaded Nipple (Item 5, McMaster 46825K51). The Threaded Nipple holds the spacers, tissue holders, and tissue snugly in the middle of the electric field.

Assembly: Sealing

Place the platinum electrode (Item 11) as far back in the Reducer (Item 12) as possible. Fix the electrode to the reducer with a small amount of the silicone around the edge of the platinum electrode. Then take up any slack in the wire through the plug. Seal the plug by applying the sealant to both the inside and outside of the plug. Other auxiliary connections (example: thermometers or pressure gauges) can be made similarly (drill through a plug, insert the device, and seal with the sealant). Connect the wires to the power supply (Item 22, Not Pictured) with easily removable connection (ex: Alligator clips).

Figure 7: Wire, thermometers, and pressure gauges can be connected to the L-ETC by drilling a hole in a T-Barb connector (Item 18) and sealing both the inside and outside with silicon.

The barbed tube fittings are removable (twist and pull). This allows for auxiliary components (thermometer) to be moved around to other locations. This is also handy for gaining access to the tissue: simply unplug one end of the L-ETC from the pump (ex: the tube/barb connection shown at Item 1). Disconnect the wire from the power supply near Item 17. Hold the chamber with the inlet up, such that drips flow down into the system. Because the inlet is now disconnected, it may rotate freely. Twist the couplings (Item 10) apart by hand, keeping the outlet stationary. Then remove the tissue within the Hex Nipple (Item 5) with a wrench.

Parameters and Considerations

Rapid success (2 days; half mouse brain cleared) has been observed with the following parameters. Considerations are also given.

Use: Cooling

"Most proteins from mammals have a temperature optimum for their biological function at around 37°C. Temperatures above 43°C will denature most mammalian proteins more or less quickly. At 55°C, complete denaturation takes place within one or two hours, at 95°C only a couple of minutes." (http://research.uni-leipzig.de/uspdu/docs/Protein%20guide_Storage_Working.pdf; Universität Leipzig).

The application of electricity can cause heating at the electrode-solution interface. Excessive heating can damage the tissue to be cleared, including protein degradation. Monitor the amount of heat that is generated by monitoring the temperature at the inlet and outlet of the chamber. Should the temperature be at or near that which could cause protein degradation, two inexpensive heat removal methods are recommended. We found that heat removal was not necessary for our experiments. Protein degradation can be observed in tissue that appears with a yellow tint.

Solution Reservoir: the amount of Clearing Solution used is dependent on the amount of solution that will fill the tubing, chamber, and pump. One liter should be adequate. Clearing solution returning to the reservoir can introduce bubbles. So it is recommended that the returning solution tube end above the reservoir and the tube for the solution leaving the reservoir be at the bottom of the reservoir, far away from any introduced bubbles.

Method 1: Ice bath. The solution reservoir can simply sit in ice, changed periodically. This method is very inexpensive. However, the SDS in the Clearing Solution can precipitate out at low temperatures. Because an ice bath can have local extreme temperature gradients, SDS can precipitate and allow solids to clog the filters.

Method 2: Dorm refrigeration. Caution: Freon is a toxic chemical frequently used in refrigerant systems and can be under pressure. It is frequently used without any additive orders or colors, and is therefore generally undetectable with the senses. Inhalation can lead to death. This method should only be considered after great care is taken to ensure that the Freon lines are not disturbed. Research online for you particular refrigerator model is recommended; many instructional videos exist of people retrofitting refrigerators for 'kegerator' applications.

A simple and inexpensive old refrigerator can be retrofitted to pull heat from a CLARITY system. The solution reservoir can be placed in the refrigerator, with two holes in the side to allow for tubing to bring the solution in and out of the refrigerator. Care must be taken when drilling into a refrigerator to ensure that you do not puncture the Freon tubing when your HR Manager is walking their kid around on Take Your Kid to Work Day (true story). It is therefore advised that you start by using a razor to cut away the plastic *on the inside* of the refrigerator. This will allow you to either see the Freon tubing or you can use a probe to feel around the insulation of the refrigerator wall and ensure that there is no lines or wires. Then drill from the inside outward. If

an appropriate drill size is chosen, the tube will fit snugly and no sealant is required. Connect the solution outlet (Item 16) to the inlet of the pump. The outlet of the pump should flow into the refrigerator.

Bubbles

Bubbles can get stuck in the chamber, altering the flow of solution and electricity. Bubbles can be introduced into the system at the electrode-solution interface or more likely at incomplete sealing of components. Components that are not to be frequently separated (see Assembly: Barbed Tube Fittings and Tissue Access; example Items 12 and 13) can be better sealed with the application of silicon (Item 19). Connections that will frequently be separated can be better sealed with a high quality plumbers tape (Item 23). TaegaSeal PTFE Tape is made to MIL-T-27730A, and is therefore chemically inert and 99% pure. Bubbles can further be expelled from the L-ETC by placing the chamber on a slight incline, with the outlet above the inlet of the chamber.

Pump

The pump chosen for this set up is a peristaltic pump, though success has been reported with inexpensive pumps frequently used in aquatic tanks (reference?). Increasing the number of stages on a pump will ensure more uniform flow and pressure. A check valve was tested to ensure unidirectional flow, but created unacceptable pressure peaks and is therefore not recommended. Success was observed when pump has a flowrate of around 0.4L/min. A higher flow rate of 1.2 L/min was tested, but no benefit was observed (n=4). A slower flow rate was not tested; keep in mind that slower flow will result in higher heat buildup.

Electrical

For safety considerations it is advisable to place the power supply above anything that can leak liquid. Because the wires connecting the power supply need to be frequently connected/disconnected (alligator clips), it is likely that there will be exposed electrical contact. Shorts in these hanging wires can be minimized by cutting the positive terminal to a different length than the ground. Success was observed when 30 VDC was applied, resulting in 0.02-0.05A (dependent on flow, bubbles, and tissue). This was the maximum output for the given power supply. Higher output could clear faster (or risk damaging the tissue), but stochastic, AC may be a more promising avenue of research.

Originally, it was chosen to have the conventional current flow opposite the direction of fluid flow (anode placed downstream). It was thought that the electrical forces should flow with the stream of solution. Because the SDS micelle with extracted lipids are negative, they would be pulled to the anode with the flow of solution. However, the anode and cathode were switched (n=4), and no noticeable difference was observed.

Clearing Solution

It was hypothesized that more rapid clearing could be achieved by increasing the SDS concentration, while maintaining the same level of buffers. Therefore, an 8% SDS clearing solution was used to clear four half brains. With the same applied voltage (30 VDC), the current doubled (reaching 0.09 A). However, the same time was required to clear the tissues and there appeared to be more bubbles, so this avenue of investigation was abandoned.

Cleaning

After each use, thoroughly pump water through the system. Over time, particles may build up on the platinum electrodes. This may degrade the electrical interface, decreasing the current for a given voltage. To clean these off, remove the tissue from the system, and run the system with reverse polarity.

Chamber Expansion

The Linear Clarity System is designed as shown for serialized clearing of two separate tissues (it is recommended that their relative positions be altered daily). More ETC Chambers could be added in series, but this type of expansion would expand the electric field, resulting in uneven clearing(?). Therefore, it is recommended that additional chambers be added in parallel, with separate power supplies.

If the pump can push enough volume for adequate flow through two or more chambers, separate the chambers with wye connectors (see McMaster PN 5463K725). Measure the output of each chamber. Because the fluid resistance through each will likely be different, it is likely that the rate of fluid through each will be different. You can ensure even flow through each chamber for this with Clamp-Style Pinch Valve for Tubing (McMaster PN 53345K39).

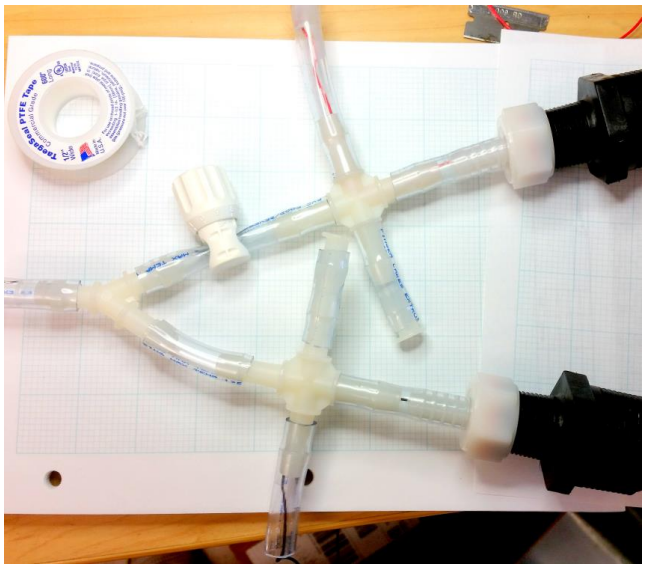

Figure 8: The L-ETC is expandable, but it is recommended that the system expand in parallel, such that the Clearing Solution flows through multiple chambers, each with their own power supply. A wye connector will separate the fluid and a Clamp-Style Pinch Valve can regulate the flows to ensure even flow between chambers.

Acidity and Filters

Monitor the pH of the solution daily and replace when the Clearing Solution has a pH of below 7.5-8; typically daily. It has been suggested that a carbon filter be placed within the solution flow to remove small particles that can detach from the tissue or chamber. Success has been reported with carbon filters frequently used in Aquarium set ups. The L-ETC set up has shown success without any filters. The screen mesh of the tissue strainers has catches large particles before they can become lodged in tissue.

Completion

What is determined as completely ‘clear’ is inherently subjective. The tissue needs to be as optically clear as is necessary for a clear image. Over clearing can remove too much protein and can cause tissue ‘yellowing.’ Do note that the tissue will continue to clear a bit after removal from the clearing chamber until it is well rinsed in PBS. Furthermore, some Index Matching Solutions will also clear the tissue. It is therefore recommended that tissue be removed from the chamber prior to being completely clear.

BCA Protein Analysis

Coming soon.

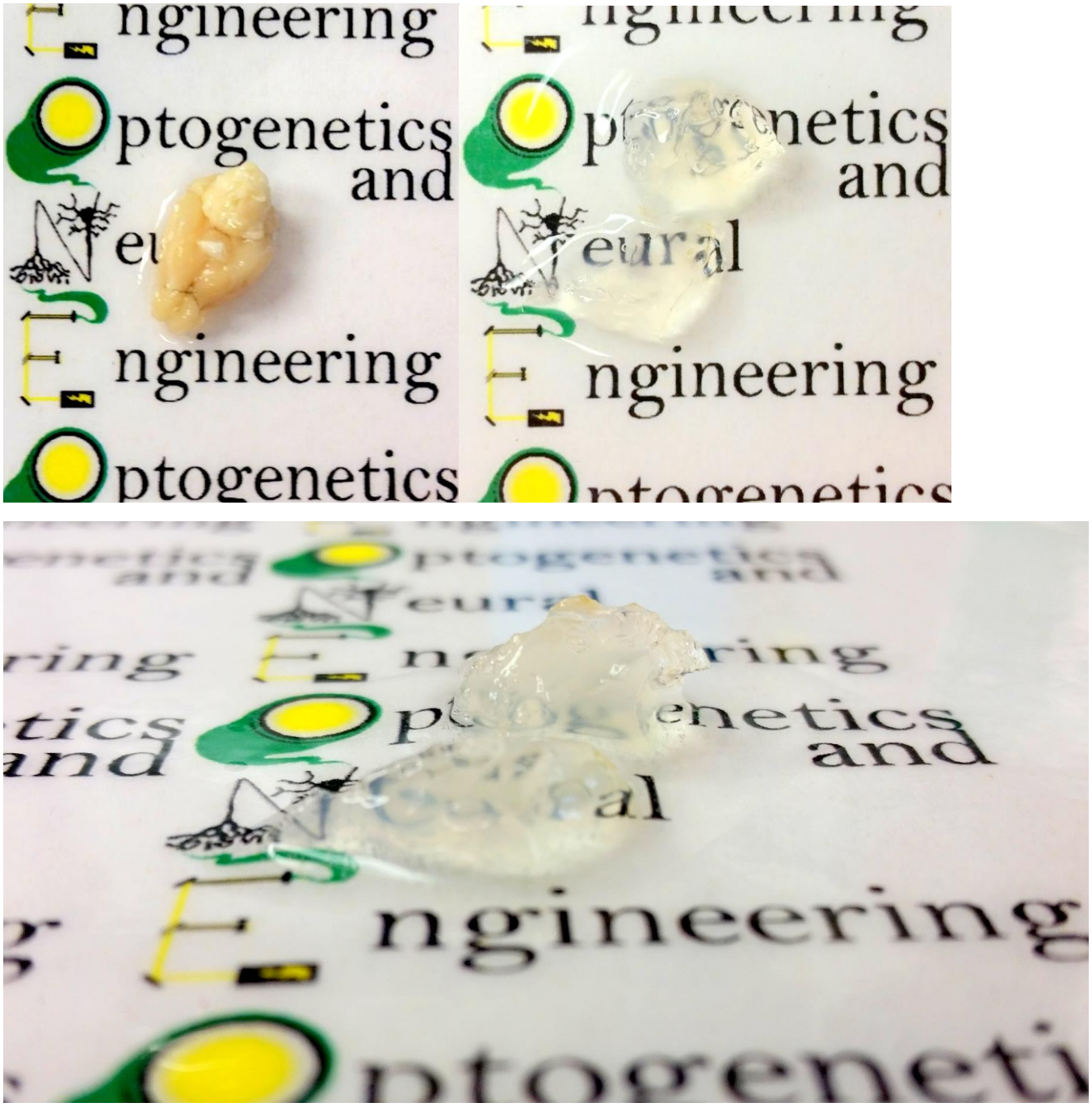

Figure 9: Top left: a whole mouse brain is shown prior to clearing. Top right: two 5 mm thick slices of mouse brain is shown on the bottom after clearing for 2 days in the L-ETC system. Bottom: The same slices are shown at a different angle.

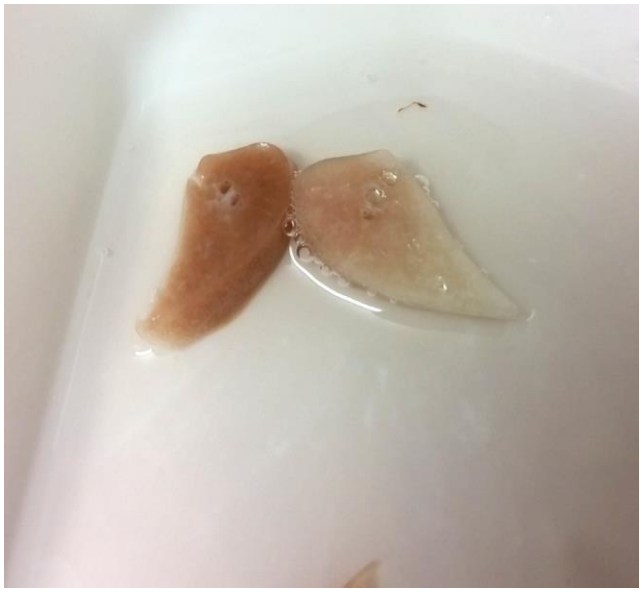

Figure 10: The same rat lung (5 mm sections) is shown uncleared (left) and cleared with the L-ETC system (right) in 2 days.

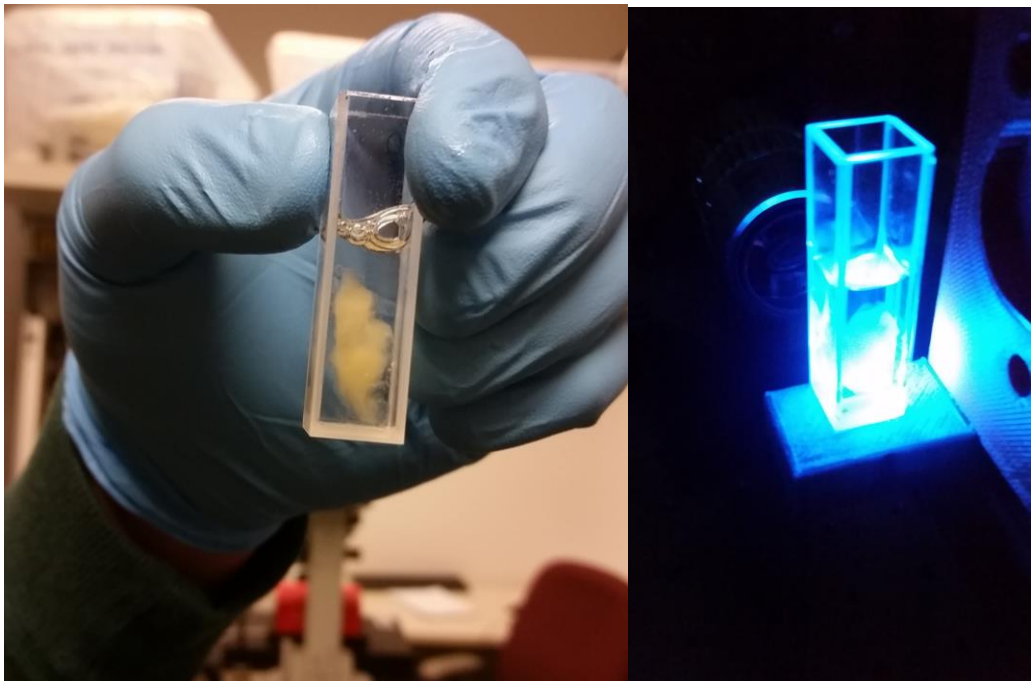

Figure 11: Left: a half mouse brain is placed into FocusClear for imaging. Right: a custom Light Sheet Microscope is used for deep tissue imaging.

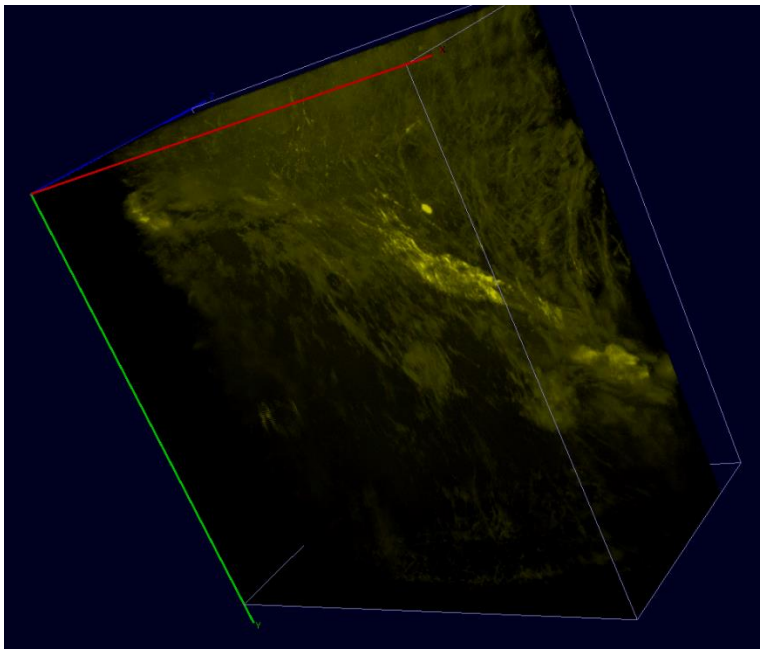

Figure 12: A 3D rendering of light sheet images of the cholinergic neurons of the olfactory system of a mouse. Mouse ChAT-tau-GFP line where a tau-GFP fusion protein is expressed downstream of the ChAT promoter.

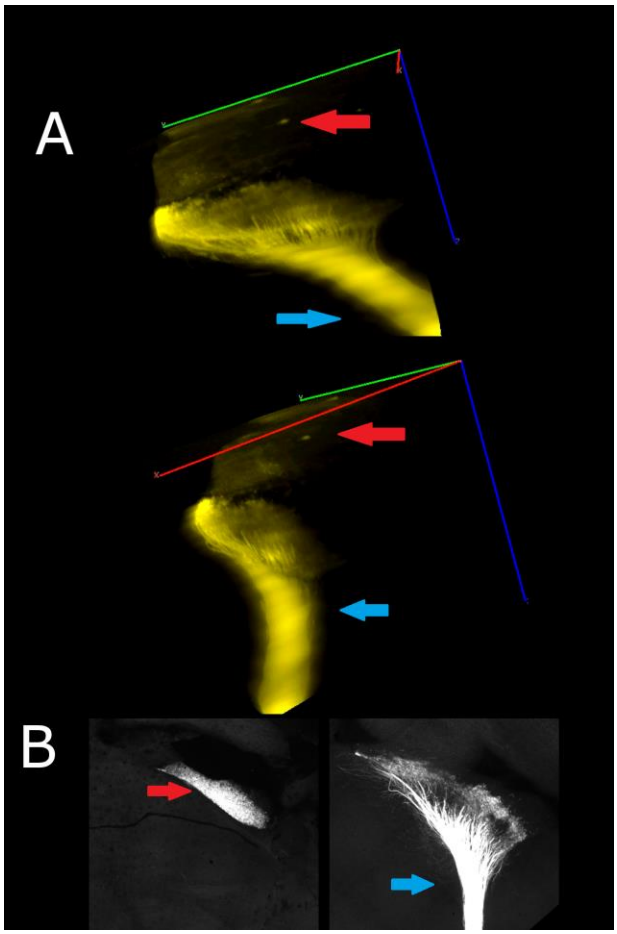

Figure 13 Panel A: Light-sheet microscopy data showing cholinergic fibers of the Medial Habenula (red arrow) coalescing into the dense fasciculus retroflexus (blue arrow). Panel B: Epifluorescence microscopy data showing the same anatomical features in a 150um slice cleared by PACT passive-clarity techniques. Even with

these relatively thick tissue sections it not possible to capture the full extent of the Medial Habenula nucleus with the fasciculus retroflexus projection.

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