Supplementary Document: ITP

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Motivating example: A simple ITP experiment with typical LE/TE. In this experiment, we will visualize the ITP peak by introducing fluorescein in the TE (semi-infinite injection).

V1 Buffer preparation

1.0 Basic intro

- Here is our JOVE video which introduces ITP (physics and protocols).
- Here is our ITP review to be published in Chemical Reviews (background and physics).

1.1 Choice of buffers

Basic idea: We choose LE and TE co-ions (same charge as sample) to be buffer titrants. We then make the counterion the buffering weak electrolyte species (**Fig. 1**). When you do this, the pH of the TE and LE zones are about the same and governed by pH in the LE. Further, you ensure TE is buffered even if the TE ion is a relatively strong electrolyte.

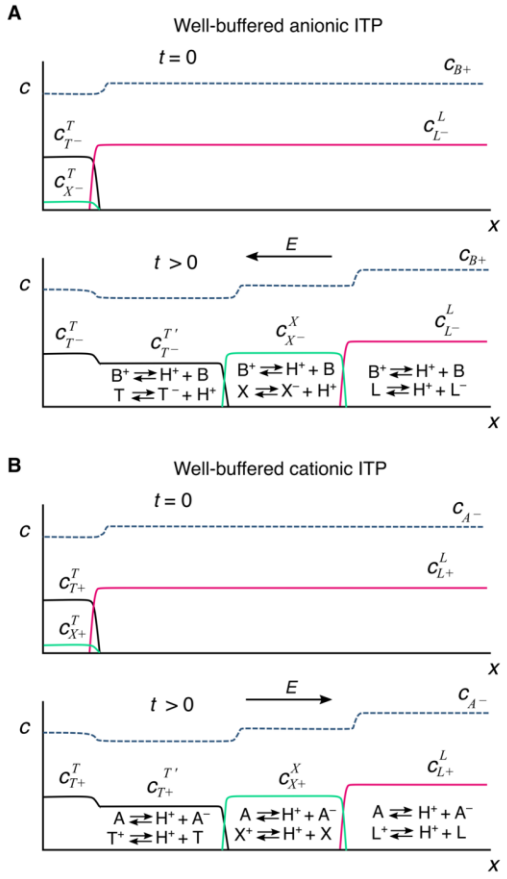

Fig. 1 Concentration versus position for well-buffered anionic (**A**) and cationic (**B**) ITP. Please see our CAFES tool (including the tutorial found at the bottom of the page) to assist buffer design.

1.2 Buffer preparation

1.2.1 Prepare the stock solutions

- Find Tris (Group A), HEPES (G), and PVP (G) powders in the Flammables Cabinet #1 in 218.
- Find 12 M liquid HCl in the "Acid" cabinet below the fume hood in 218.
- Find serological pipettes and filler in the overhead cabinets above the AB thermocycler in 218.
- Find 50 mL falcon tubes in the overhead cabinets left of the motorized microscope in 218A.
- Use the Acculab weighing scale near the PDMS workstation which has weighing boats and spatulas on the shelf above.
- Use deionized (DI) water from the sink (right faucet in any sink in 218/218A).
- Refer to video for weighing scale and serological pipette demonstrations.
- Prepare and label stock solutions as shown in the video and tabulated below.

Tris Stock (1 M)			
	Weight (g/mol)	***Mass (g)***	***Volume (mL)***
Tris (powder)	121.14	4.8	-
DI water	-	-	to 40

HEPES Stock (1 M)			
	Weight (g/mol)	***Mass (g)***	***Volume (mL)***
HEPES (powder)	238.3012	9.5	-
DI water	-	-	to 40

PVP Stock (2 % W/V)			
	Weight (g/mol)	***Mass (g)***	***Volume (mL)***
PVP (powder)	1300000	1	-
DI water	-	-	49

HCl Stock (1 M)		
	Concentration (M)	***Volume (mL)***
HCl (liquid)	12.178	3.3
DI water	-	36.7

1.2.2 Prepare the TE and LE
- Find pipette tips in the overhead cabinets to the right of the AB thermal cycler in 218.
- Find pH strips in the pH strips shelf in 218 (across the AB thermal cycler).
- Refer to video for demonstration.
- We recommend this step-by-step pipette tutorial.
- We also recommend you check out Table 3 of this article which recommends specific pipettes for specified volumes. As a rule of thumb, pipettes are most accurate closer to their upper (not lower) limit specified range. *E.g.*, a P20 pipette may be recommended for volumes between 2 and 20 µL, but it is most accurate near 20 µL.
- Prepare and label TE and LE solutions as shown in the video and tabulated below.
- TE and LE should respectively have pH of ** and **.
- (We recommend to prepare 10 mL volumes of TE and LE in 10 mL which is good for ~500 experiments assuming ~20 µL per experiment.)

TE			
	Stock concentration	***Final concentration***	***Volume (mL)***

Tris	1 M	100 mM	1
HEPES	1 M	50 mM	0.5
PVP	2%	0.5%	2.5
DI Water	-	-	6

LE			
	Stock concentration	***Final concentration***	***Volume (mL)***
Tris	1 M	200 mM	2
HCl	1 M	100 mM	1
PVP	2%	0.5%	2.5
DI Water	-	-	4.5

V2 Chip loading

2.1 Mounting the chip and the (optional) heater

- We recommend the use of a glass-ITO heater and controller from Cell MicroControls (mTCII, USA). (Heater discussed in detail in **V3**.)
- However, note that, if your experiment requires heating (*e.g.*, for CRISPR-ITP), then you must mount the heater now (before mounting the chip).
- Secure the desired chip above the stage (and heater) with clear tape.
- The heater thermocouple should be secured above the chip with clear tape.
- Ensure not to cover any channels with tape.

2.2 Presentation of the chips

2.1.1 Caliper chip (NS-12A)

- We obtain this chip at about $70/chip (see schematic) from Perkin-Elmer.
- We have previously purchased versions with or without the plastic caddy (see JOVE video for an example of the caddy).
- Diego strongly recommends the caddy-less version. Instead, plasma-bond PDMS reservoirs above the chip inlets. This allows the chip to make contact with the heater as well as reduces background auto-fluorescence (which comes from the plastic caddy).

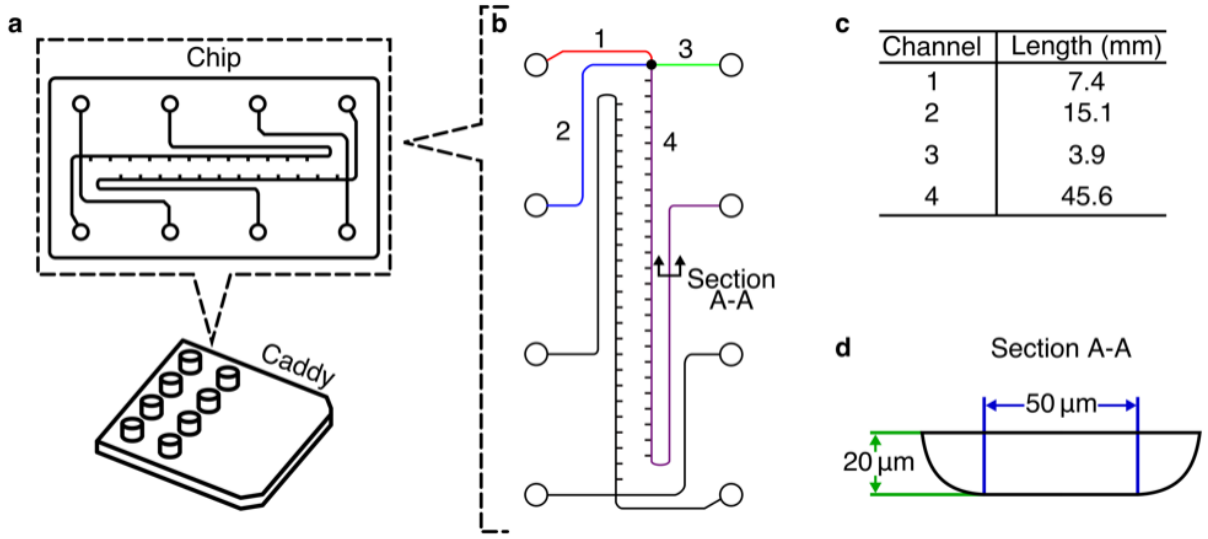

Fig. 2 (From the SI of Ramachandran *et al.*, 2020.) Schematic of NS-12A chip recommended for ITP experiments.

2.1.2 Custom chip
- We recommend ProtoLabs for 3D-printing plastic molds and to cast these molds with PDMS.
- We recommend plasma-bonding the PDMS channels onto glass for better optical access.
- Our group has tutorials for printing custom molds and fabricating chips.

2.3 Chip preparation
2.3.1 Chip washing
- To wash the chip, we will rinse the channels and reservoirs using a series of reagents.
- To wet the channels, load a reagent into reservoirs A, B, and D for the NS-12A chip (or just reservoir B for the custom chip). Then, seal a vacuum at reservoir C for the NS-12A chip (or reservoir A for the custom chip) for 15 s. Release the vacuum and allow the chip to sit for 1 min.
- Dry the channels by again sealing a vacuum at reservoir C for the NS-12A chip (or reservoir A for the custom chip). While the vacuum is sealed, use needle-tipped vacuum tube to dry the remaining reservoirs. It is crucial the chip is fully dry. Do this for about 30 s.
- The series of reagents to be used are listed below.

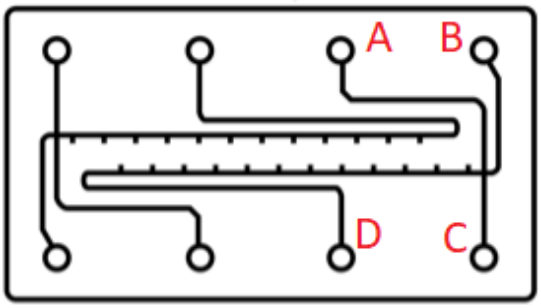

Fig. 3 Schematic of NS-12A chip with labels on each reservoir.

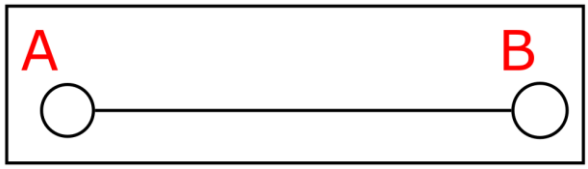

Fig. 4 Schematic of custom chip with labels on each reservoir.

Washing protocol	
Step number	***Reagent***
1	Triton-X (1%)
2	DI Water
3	DI Water
4	NaOH (1 M)
5	DI Water
6	DI Water

2.3.2 Loading of TE and LE into the chip

- For preliminary experiments, we recommend diluting the TE with fluorescein to allow visualization of the ITP peak.

Dye-containing TE (Fluorescein + TE)			
	Stock concentration	***Final concentration***	***Volume (µL)***
TE	1x	0.9x	90
Fluorescein	1 µM	0.1 µM	10

- For the NS-12A chip, load 20 µL of the LE into reservoirs A and D and load 20 µL of the dye-containing TE into reservoir B. Seal a vacuum at reservoir C for 15s and then use the needle-tipped vacuum tube to dry reservoir C. Finally, load 20 µL of the LE into reservoir C to complete the chip loading process.

- For the custom chip, load 40 µL of LE into reservoir B. Seal a vacuum at reservoir A and then use the needle-tipped vacuum tube to dry reservoir A. Finally, load 40 µL of dye-containing TE into reservoir A to complete the chip loading process.

2.2.3 Connecting the electrodes

- Connect platinum electrodes to the chip reservoirs B and D for the NS12-A chip (or reservoirs A and B for the custom chip) and tape the electrodes on the stage.
- NOTE: the electrodes should make contact with the liquid in the reservoirs.
- We use platinum electrodes previously purchased from uGems.

V3 Current controlling and heating

3.1 Keithley operation

- The Keithley 2410 1100V SourceMeter (Keithley Instruments, USA) is a so-called source meter which can be used to both prescribe and measure electrical conditions. For example, we can set a small current and then measure the required voltage. Check each Keithley's maximum current and voltage limits before use. For ITP, we prefer to use the Keithley in constant current operation. In constant current mode, the ITP velocity (e.g. the velocity of the LE co-ion) is constant. In semi-infinite injection mode, constant current mode results in constant rate of accumulation of sample into ITP zone.
- We here will describe the knowledge needed to use the Keithley for ITP experiments. You should refer to the Keithley manual for further details.

3.1.1 Preparing the Keithley for an ITP experiment
- The Keithley is connected to a PC via RS-232 and RS-232-to-USB converter (there are multiple of these converters in the lab).
- Note to which COM port is the Keithley connected.
- Connect the cables which lead to the platinum wires to the "INPUT/OUTPUT" (rightmost) ports on the Keithley.
- Turn on the Keithley by pressing the button on the bottom left. Allow the device to initiate for a few seconds before beginning PC control.
- The following settings must be specified for the Keithley to work with the MATLAB script.

Keithley settings	
Baud rate	19200
Data bits	8
Parity	none
Terminator	<CR>

3.1.2 Operating the Keithley via MATLAB
- Our lab has written a straightforward MATLAB code which applies a constant current (varies voltage) to the Keithley output. It also outputs live plot of current and voltage versus time.
- The code is located in the "keithley_control" folder found in the Desktop of 211 PC (else contact Neelanjan Akuli at neel2021@stanford.edu). The filename is "CurrentMonitoringMatlab_GPIB_COM_I_v7_Diego.m".
- Please refer to the code (which is heavily documented) to understand its function.
- Most importantly:
    - Update the COM port (variable name "Keithleys") that is specified in the code.
    - Update the current (variable name "Current") to the desired current. E.g., 4 µA for an NS-12A.
    - Simply initiate the script from the top to apply the constant current. Exit the script by closing the live plot.
- IMPORTANTLY: Once you start the code, the Keithley will apply a potential across the electrodes. Ensure these electrodes are properly placed in the reservoirs and do not make contact with persons or conductive surfaces.
- If you start the script and immediately see the voltage climb to > 1000 V, then one of your electrodes is likely disconnected.

3.2 ITO heater operation

- We use a commercially available heater and controller from Cell MicroControls (mTCII, USA) that is a piece of glass coated with an indium tin oxide (ITO) layer. ITO acts as the conductive (high resistance) element for heating and both the glass and ITO layers are optically clear so we can image the setup from above or below.
- After mounting the heater and thermocouple (see **V2 Chip loading**) connect the heater and thermocouple to the proprietary controller cable (a single gray cable shown in **Fig. 6**). Connect the gray cable to one of controller channels (there are two). Also connect the controller to a power supply (this will automatically turn on the controller).
- The top number on the display is the measured temperature on Ch1. The bottom number on the display is the measured temperature on Ch2. An "open" indicates that no thermocouple is connected to the relevant channel.
- To set the desired temperature: Press the ▲ button. Press either ▲ (for Ch1) or ▼ (for Ch2). Then use either button again to set the desired temperature. Press the ↵ button when done.
- To turn on temperature control: Press the ↵ button. Press either ▲ (for Ch1) or ▼ (for Ch2).
- To turn off temperature control: Press the ↵ button. Press either ▲ (for Ch1) or ▼ (for Ch2).

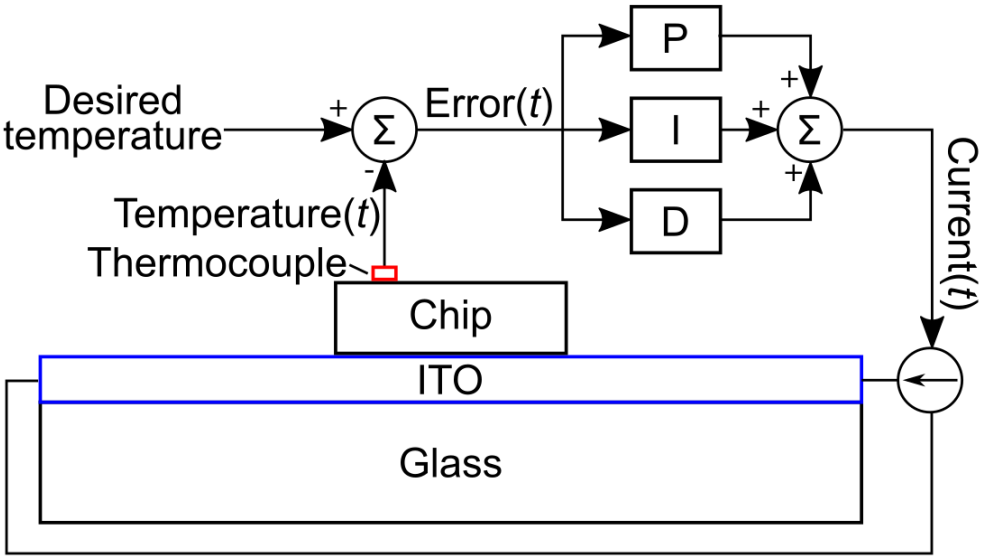

Fig. 5 Schematic of heater and controller with feedback signal. The heater is only necessary for ITP experiments which require controlled temperatures above room temperature (*e.g.*, ITP-CRISPR experiments).

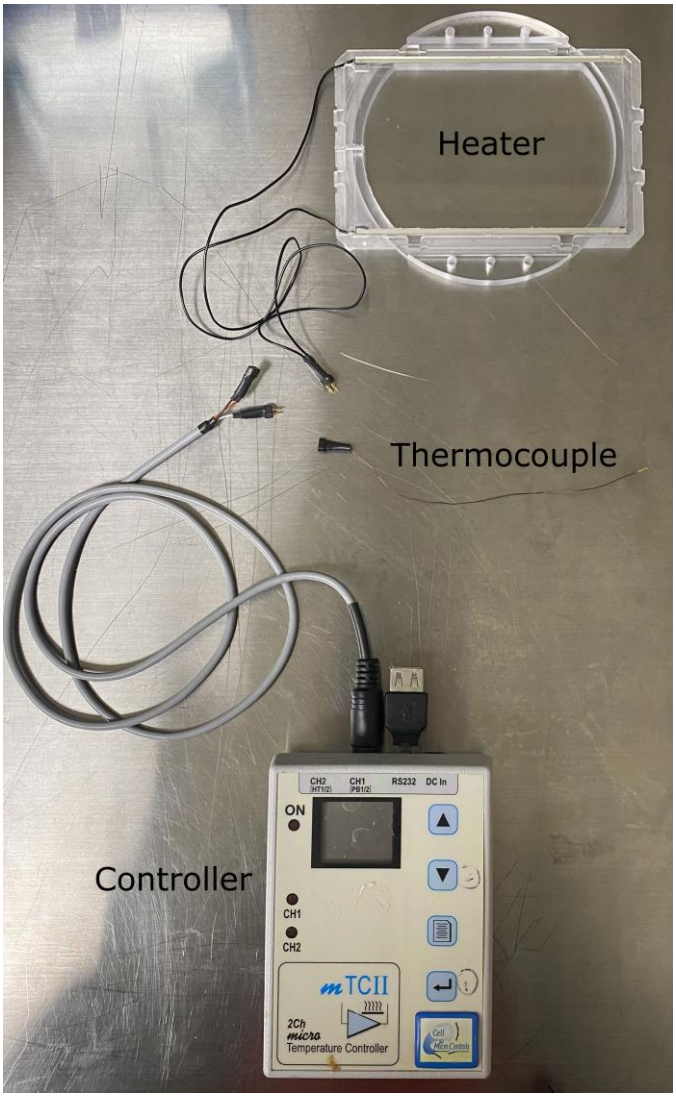

Fig. 6 Heater and controller with gray cable.

V4 Imaging

4.1 Setup the microscope

- This section covers brief microscopy basics necessary for the ITP experiment. We refer the reader to the MicrocopyU tutorials on Numerical Aperture, Depth of Field and Depth of Focus, Resolution, and Introduction to Fluorescence Microscopy.
- We here will use a 10×/0.4 objective (Nikon, Japan), 0.50× demagnification lens, and blue-green filter cube (**).
- The demagnification can be used to increase fluorescence signal and increase field of view with negligible loss of resolution. It is often best to demagnify right up until you observe vignetting of the image on the camera (i.e. the image from the microscope starts to become smaller than the camera sensor).

4.2 Connect the LED/camera to the microscope

- High power LEDs (Solis-3C, Thorlabs, USA) are strongly recommended for ITP experiments.
- Mount the LED to the back of the microscope using a Thorlabs Nikon/Olympus adapter.
- Connect the LED to the LED driver (Thorlabs, USA).
- Turn on the driver and set to "Brightness Mode" with full power.
- Tip to use mercury bulbs/lasers: turn them on *before* turning on the CCD, because they briefly send out very strong EMF signals which damage CCDs.
- We here use an ORCA-Flash4.0 v2 sCMOS camera (Hamamatsu, Japan).
- The camera should be connected to its proprietary power source and to the PC by USB 3.0 cable.
- Turn on the camera by flipping the on/off switch in the back. After a couple of seconds, the PC should make a noise indicating it has detected the camera.

4.3 Use the software to find and focus on the chip

- We recommend to use the software (rather than eyepiece) to find the chip channels more easily.
- Open the Hamamatsu proprietary software HCImageLive. (Although free, this software is not available online and you must obtain it from a Hamamatsu representative. Diego has previously contacted hcsupport@hamamatsu.com and provided the camera serial number.)
- Now begin the process of finding the chip.
    - Turn the microscope Z knob until the objective is as far as possible from the chip.
    - Align the LED illumination (from the objective) to the chip by physically examining the microscope stage (do not use the camera software at this step). Try to align the light emanating from the objective with a channel on the chip.
    - Turn the Z knob so that the objective slowly approaches the chip. Alternate between looking at the HCImageLive output (you should see a channel come into focus) and physically looking at the stage (to avoid crashing the lens).
    - If you cannot focus onto any channel, try moving the stage in X or Y slightly. Repeat the last two steps.

4.4 Use the software to acquire data

- Refer to the HCImageLive manual.
- Click on the "Capture" tab on the left.
    - Input a file name and location (cannot be stored on an external drive or with "." in the filename)
    - Specify an exposure time.
    - Under the "Binning and SubArray" subtab, you can choose a binning or region of interest (ROI)
    - Click "Capture" to take an image.
- Images are saved in .TIFF file format and we recommend the FIJI to view images.

V5 Finishing an experiment

- Once an ITP experiment is done, dry the chip (see **2.2 Chip preparation**). Perform the first three washing steps (i.e., Triton-X (5%) -> Water -> Water).
- Turn off the heater, camera, LED driver in no particular order.