Mounting of sections and Zipcoding process:

(Note this protocol describes conditions found to work for lymph node sections and PyMT tumor sections using an anti-mCD45-oligo conjugate. Other tissue types and antibody-oligo reagents may need their own optimization.)

1. Following from previous protocol, tissue sections in agar discs should be incubating in blocking buffer (RPMI+10ug/mL ssDNA and 0.5% BSA) for at least 30min @ 4C. I use a 24 well plate, enough area per well to accommodate like 5 4mm discs.

2. Either drain the blocking buffer using a pipette or carefully transfer the discs to a new well.

- The Ab-anchor needs to be hybridized to the caged overhang strand beforehand. Assuming you have followed the protocol earlier on reagent prep, use 5 uL of the conjugate + 5uL of 4uM NPOM caged S2 strand (**S2-4NPOM**) in the table. Incubate @ 42C for 10 min to ensure hybridization, then let cool to RT. Ready to use.
- Dilute this 10uL into 250uL in blocking buffer. Add any other fluorescently conjugated antibodies you are interested in and/or FcBlock, then add onto the discs and incubate 1.5 hrs @ 4C

3. Now wash the sections by pipetting out the staining solution, and pipetting in 1 mL of RPMI+0.5% BSA and HEPES and waiting 5min. Repeat 3x.

4. We can proceed to mounting the samples. For manipulating these fragile discs, I recommend a mini spatula created from melting a flat piece of plastic cut from an Eppendorf to the tip of a glass Pasteur pipette. It's a little ad hoc, but works well for scooping the sections out without 'folding' or tearing.

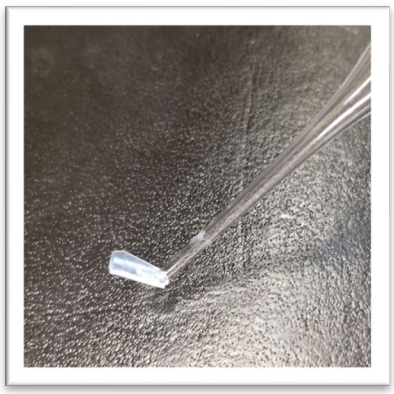


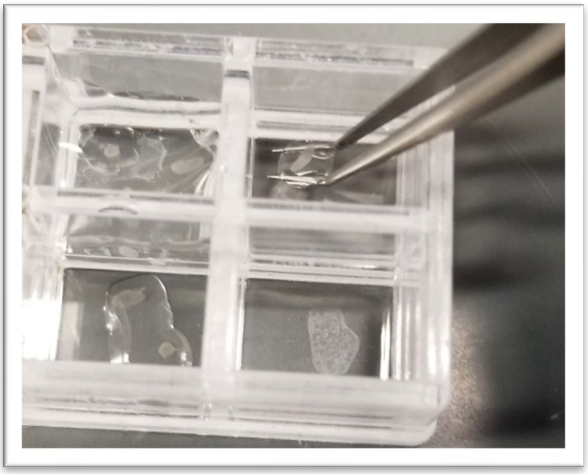
Fig. 1: homemade mini spatula for fishing out sections. Use a thin walled Eppendorf and carve out a slightly curved section into a fingernail shaped piece. Then heat up a Pasteur pipette, flatten the end to a 120 degree tilt, and then press into the plastic bit and hold until cool and bonded.

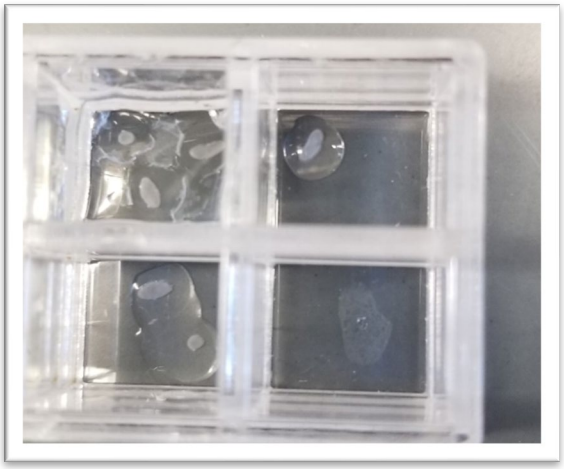
5. For the actual imaging and addition of reagents, I currently prefer a Lab-Tek 8 well (#155409) or an ibidi (#80821 u-Slide 8 well uncoated). The size of well is dependent on your slice sizes of course.

6. Fish your section out with spatula and lay flat in the well. Use a Kimwipe to carefully wick away excess liquid from the edges of the disc. Should look like Figure 2. It's important to remove as much excess liquid as possible for the Vetbond to properly adhere to the agarose disc.

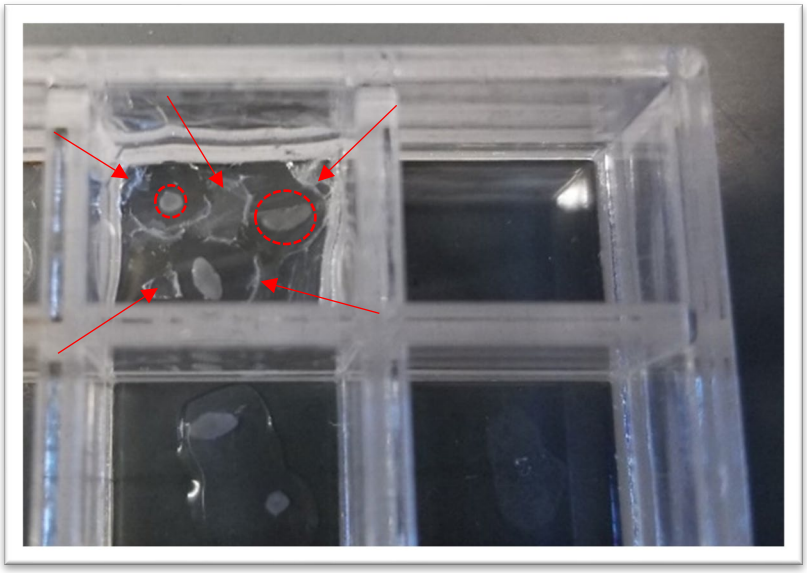
7. Now use a fine pipette tip, a p20 tip works well for this purpose. Dip into Vetbond (3M) and then dab at edge of the disc. The Vetbond should naturally wick towards the wet disc. Repeat for at least 4 evenly spaced 'dabs' to get a solid anchoring. Vetbond's adhesive action is activated by water so any excess liquid will cause the Vetbond to cure before interacting with the agarose itself. The benefit of the excess

agarose on the edges is now apparent: we can affix the section flat, without getting it into our precious sample.





8. Now flood the chamber with buffer, slowly pipetting onto the disc outwards to fully cure the Vetbond and submerse in liquid. You should see cured Vetbond (white, delineated by arrows) surrounding your agarose disc which surrounds your sample (dashed circle below). Ideally no Vetbond should get into the sample itself. You are now ready to image, photo-uncage etc.



9. At this stage, wash the discs with imaging media (RPMI-phenol red+ssDNA+0.5%BSA+HEPES+1mM EGTA to inhibit nuclease activity by depriving them of Ca2+) and leave in this media during imaging. RPMI was chosen as ideal for lymph node sections, can be adapted for other media formulations e.g. DMEM.

10. Proceed with imaging using your favorite method. In our original ZipSeq publication, we used simple wide-field epifluorescent imaging. Multiple fields of view were acquired and stitched together using

Metamorphs scan slide function. This is not ideal but that's what our mosaic DMD was controlled with. Later iterations will hopefully be performed on the more widely used MicroManager platform.

11. Define your regions of interest and illuminate the first one with 365 nm light to uncage. The exact time necessary for full uncaging needs to be empirically tested as every light source and illumination system is unique. For reference, our Mosaic DMD is illuminated with a collimated Mightex GCS-0365-48-A0510 365nm High Power LED used at half-power setting. Full uncaging is reached with a pulse of 250ms at this power level funneled through a Zeiss 20X objective (part # needed). It is critical that you choose an objective with good UV transmittance!

12. Once illuminated, remove media and add your Zipcode 1-containing imaging media with the Zipcode at 0.5 uM concentration. Let incubate at least 5-10 min at RT for hybridization.

13. Wash 3X times by gently pipetting in media and removing with a pipette. Let these washes sit for a minute before removing. Add media with blocking strand (S2 blocking strand in parts list) @ (0.1 uM) and let incubate a further 4 minutes. Remove and apply another 1 minute wash.

14. Repeat step 11-13 as needed to define your regions

15. Using forceps, carefully remove the tissue section from its agarose disc, or remove the whole agarose disc (no worries, the bits of agarose will be strained out prior to sorting or encapsulation) and transfer to a desired receptacle. Here is where you have to determine how you want to make your single cell suspension.

- Mechanical Dissociation only: This works for lymph node sections, used in the manuscript. Place on a 40um strainer and use a syringe plunger to grind against the filter, using FACS Buffer (PBS+2% FCS + 1mM EDTA) to wash cells through.
- Enzymatic Digestion: This option requires some more prior testing. Several enzyme mixes are 'dirty' preps that contain significant amount of nuclease activity. Obviously avoid adding DNAseI to the digest. This nuclease activity can be partially mitigated through addition of ssDNA to the digest which acts as a 'decoy' for our tags. Nevertheless, minimizing the amount of nuclease activity and time spent at 37C is crucial. In the paper, and in lab we find a mix of collagenase I and IV (Worthington Biosciences) is gentle to the tags while yielding good cell recovery from tumor. Meanwhile collagenase XI is quite harsh to DNA tags.
    a. Prepare your **digest mix 100 U/mL of Coll I and 400 U/mL of Coll IV in RPMI**
    b. Finely mince your section using a razor blade or scalpel, like half a mm if possible on a side. This is quite laborious, make sure to stretch your wrist and hand muscles well.
    c. Using a wide bore pipette tip, aspirate ~ 500uL of digest mix, use it to wash your cutting surface and aspirate up the bits into a 2mL Eppendorf tube. Do this again, making sure to aspirate as much of the chunky bits as possible for a final volume of 1mL in the Eppendorf.
    d. Place tube sideways in a shaker @ 37C, and let incubate 20', vortexing in between at the 10' mark.

e. At the end, strain over a 100 um filter, spin down, resuspend in buffer and then pass this over a 40 or 70 um filter depending on the cell sizes of interest. Typically immune cells are small so we do 40 um.
f. Proceed directly to encapsulation or sorting of live/labeled cells

16. Follow manufacturers instructions for scRNA-Seq encapsulation, RT etc. Proceed until setting up the cDNA amplification step. Then refer to next guide ‘ZipSeq Library construction, sequencing, and analysis’