Immunohistochemistry of Non-Paraffin Embedded Tissue:

The following outline is the protocol used by Nano3D Biosciences, Inc. to perform immunohistochemistry without the need for paraffin embedding. Any standard fixation and immunohistochemistry protocols already used by your lab for the cell types and structures formed will also work with **Bio-Assembler™** 3D structures. Notice however, that "Step 1" listed below should be included in your usual protocol to prevent the normal loss of cells during all pipetting procedures. Another important point is that depending on the antibody used, a different type of fixative might be needed, i.e. Methanol, Paraformaldehyde, Formalin, or a combination of fixatives.

Materials Needed:

Recommended Fixatives: 4% Paraformaldehyde (in PBS) or Formalin.

- 16% Paraformaldehyde (PFA) can be purchased from Electron Microscopy Sciences (#15710) and diluted to 4% in PBS.
- 10% Histological Formalin Solution can be purchase from Fisher Scientific (#SF98) and used directly.

10 mM Phosphate buffered saline (PBS tablets can be purchased from Sigma Aldrich and dissolved in deionized water, P4417)

Antigen retrieval solution: Dilute 10X solution to 1X in PBS (Antigen Decloaker can be obtained from Biocare Medical, CB910M)

Permeabilization solution: 0.2% Triton X-100 in PBS (TRITON X-100 can be obtained from Fisher, AC32737-1000)

Donkey serum buffer: 10% donkey serum in PBS (Donkey Serum can be obtained from Sigma Aldrich, D9663)

Primary antibody solution: primary Ab (see manufacturer's directions for dilution) + 1% of 10% BSA, in PBS

Secondary antibody solution: secondary Ab (see manufacturer's directions for dilution) in PBS

VectaShield HardSet Mounting Medium ***with DAPI*** (Can be obtained from Vector Labs, H-1500)

Magnet: M3 magnet in a protective cover

Materials recommended:

A Pap pen or mini Pap pen can be purchased from Fisher (6505A or 6506A) or Newcomer Supply (NC9827128 or NC9204359).

Moisture chambers can be obtained from Evergreen Scientific (#240-9000-010 or 240-9020-Z10).

Part 1. 4% Paraformaldehyde (PFA) or Formalin Fixation:

Step 1: Remove the magnet from the lid and place it under the bottom of the petri dish. The exposed side of the magnet should now be oriented up (as shown in Figure 1), so the cells will be magnetically directed to the bottom of the dish. This will hold your cells at the bottom of the dish and you will be able to do all the washes without worrying about losing the cells during the procedure.

Figure 1. Magnet placed at the bottom of the petri-dish with the exposed side of the magnet oriented up.

Step 2: Gently pipette and discard media.

Step 3: Wash the cells 3 times with PBS.

Step 4: Add 1.5 ml of 4% PFA or 10% Formalin and incubate the cells for approximately 4 hours at room temperature. Note that the fixative and time of fixation may vary with the cell type, 3D structure, or target antigen. After fixation, the samples are ready to be embedded in paraffin and sectioned for the slides.

Step 5: Wash the cells 3 times with PBS.

Step 6: If desirable, transfer the cells (in PBS from step 4) into an Eppendorf tube (or other storage tubes) for storage at 4°C until paraffin embedding (see paraffin embedding procedure) is to be performed or direct staining of the 3D structures (see direct immunohistochemistry without paraffin embedding).

Note: Some cell structures can be very sticky to plastic pipettes, therefore, glass pipettes are better suited for transferring fixed 3D structures.

Part 2. Immunohistochemistry:

1. Select sections of the 3D grown tissue with a glass pipette and place each in a 35 mm diameter petri dish.
   a. If tissue sample is cohesive and of adequate size, it can be cut with a scalpel, but often parts of tissue can be separated by using a pipette.
   b. Be sure to include enough sections for proper controls
   c. In the meantime, heat water bath to 80°C

2. Add enough antigen retrieval solution to adequately cover each section in the dish and place in 80°C water bath for **20 minutes. Note:** Continue to monitor the temperature of the water bath so that it does not get to hot and melt the petri dish.
3. Place a magnet under each dish as shown in Figure 1.
4. Wash the structure while on the magnet 4x with PBS.
5. Add 500 µL of permeabilization solution to each petri dish for **15 min.**
6. Wash the structure while on the magnet 4x with PBS.
7. Transfer the tissue sections:
   Create wells on a cover slip, glass slide, or new 35 mm dish with a pap pen.

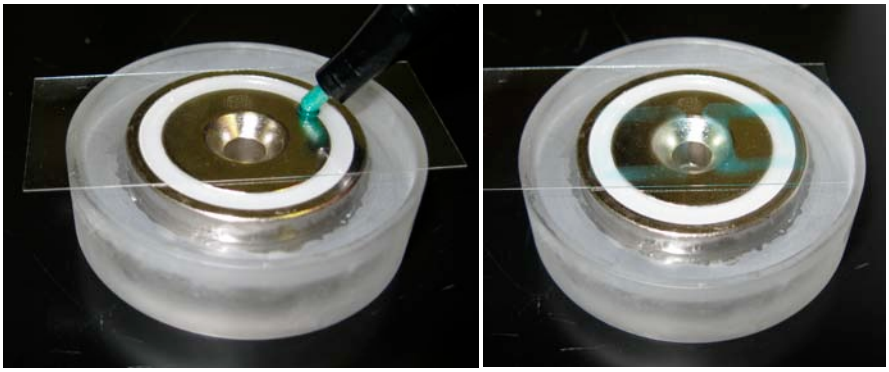

Figure 2. (left) Photograph of a well being drawn with a pap pen on a cover slip. (right) Green region is the well generated using pap pen (the wells are centered over the magnet region).

a. Transfer the tissue sections to wells. We recommend the use of glass pipettes to avoid the cells sticking to plastic pipettes which can cause the loss of cells.

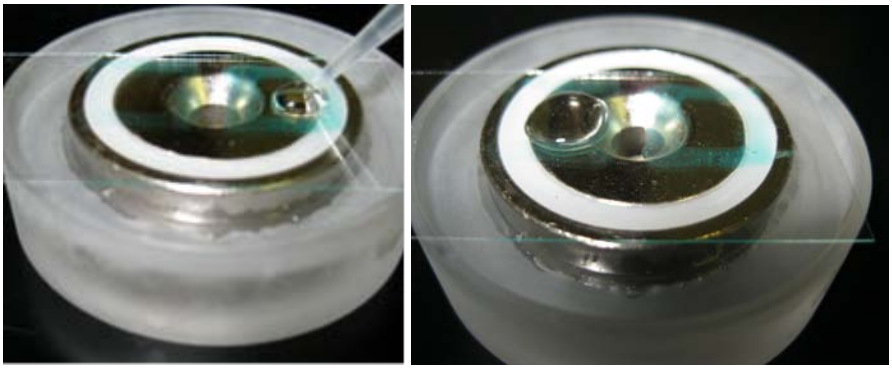

Figure 3. Photograph of sample pipetted on pap pen generated wells.

b. If available, place in a moisture chamber.

8. On each well, add 50-100 µL of donkey serum in PBS for **1 h** at **room temperature.**
9. For experimental sections, remove the serum and add primary antibody solution (see manufacturer's directions; on top of magnet) for either **1 h** at **37°C** (in incubator) or **overnight** at **4°C** (in fridge). **Note:** Controls have goat/donkey serum buffer left on. Longer incubation time allows for better antibody binding.
10. Wash the structure while on the magnet 4x with PBS.
11. Add enough secondary antibody solution to fill the well (see manufacturer's directions) for **1 h** at **room temperature.**
12. Wash the structure while on the magnet 4x with PBS.
13. While keeping cover slip on top of the magnet, add one drop (~25 µL) of VectaShield HardSet Mounting Medium ***with DAPI*** per section, and a small line of DAPI on the edge facing you. VectaShield HardSet Mounting Medium ***with DAPI*** can be obtained from Vector Labs (H-1500). **Note:** Do not add too much DAPI as this will cause tissue sections to shift when placing on slide.
14. Place slide onto cover slip line side first.
15. Leave the slides overnight in the fridge. **Note:** Make sure slides are open to air so they can dry properly. DO NOT leave in moisture chamber as they will not dry.