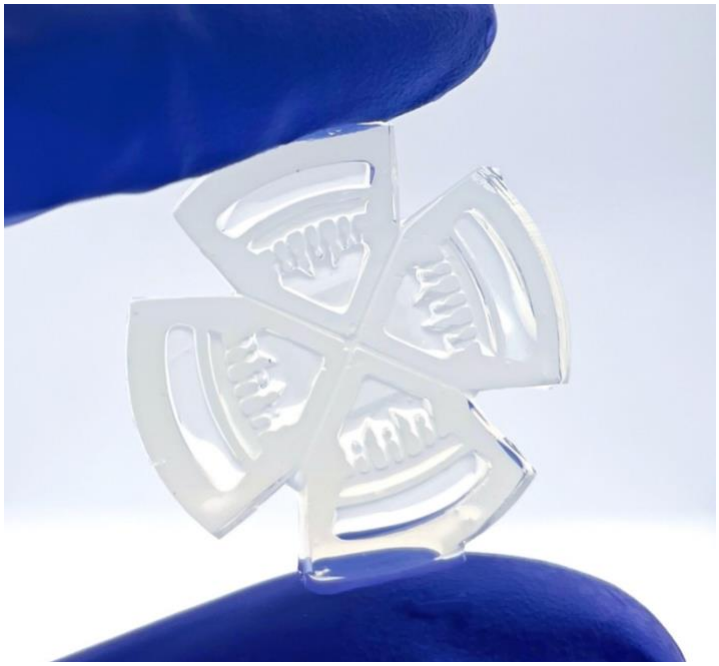
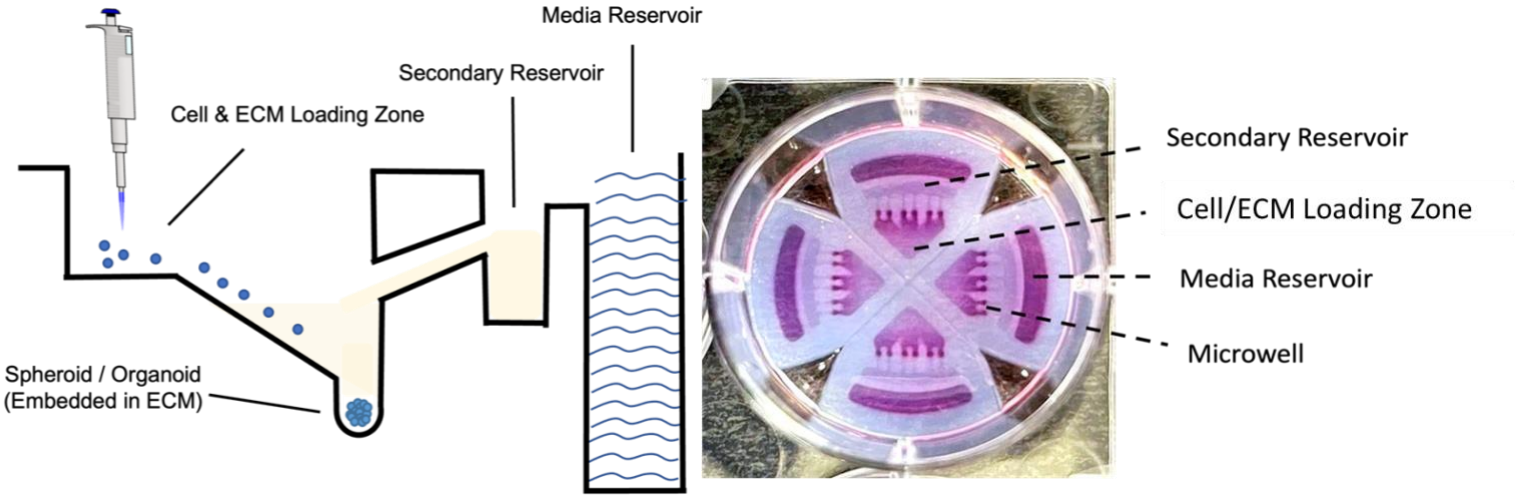
APRICELL BIOTECHNOLOGY

In-Situ Immunohistochemistry (IHC) Analysis of In-Vitro Models in the 3-in-1 Plate



User Protocol

Subject area	Biochemistry, Genetics and Molecular Biology
More specific subject area	Immunohistochemistry of spheroids/organoids
Name of your protocol	Immunohistochemistry (IHC) staining of in-vitro cell-generated spheroids/organoids
Reagents/tools	Distilled water Ethanol Formalin solution, neutral buffered, 10% Dulbecco′s phosphate buffered saline (DPBS) Agarose 3-in-1 Plate hydrogel insert (Apricell biotechnology) Xylene Paraffin wax Biopsy cassette Disposable base molds Epredia™ HM 355S automatic microtome Microscope slides Cover slip Triton X-100 Bovine serum albumin (BSA) Entellan 4', 6-Diami-dino-2-phenylindole (DAPI) Monoclonal primary antibody Florescent tagged secondary antibody Alexa fluor™ 488 phalloidin
Experimental Design	Spheroids were fixed with 10% neutral buffered formalin, washed with DPBS and embedded in 2% agarose solution. The agarose-embedded spheroids were dehydrated by submerging in ethanol, followed by submerging in Xylene. Agarose blocks were then embedded in melted paraffin wax and placed in cassette for sectioning. Paraffin blocks were sliced using an automatic microtome device and then deparaffinized. Spheroid sections were then antibody stained, counterstained with DAPI, and imaged using fluorescent microscopy.
Value of the Protocol	Non-destructive detection of unstable proteins. Investigation of cancer biomarkers in the tumor microenvironment. Identification of the spatial distribution of target proteins throughout the spheroid.



Left image: side view schematic of one microwell of the 3-in-1 Plate.
Right image: birds-eye-view of a 3-in-1 Plate inside a well of a six-well plate.

Keep the insert inside the six well-plate and aspirate the media from the Media Reservoir of each quadrant of the 3-in-1 Plate. Follow the protocol below to prepare the slices from the spheroids inside the platform.

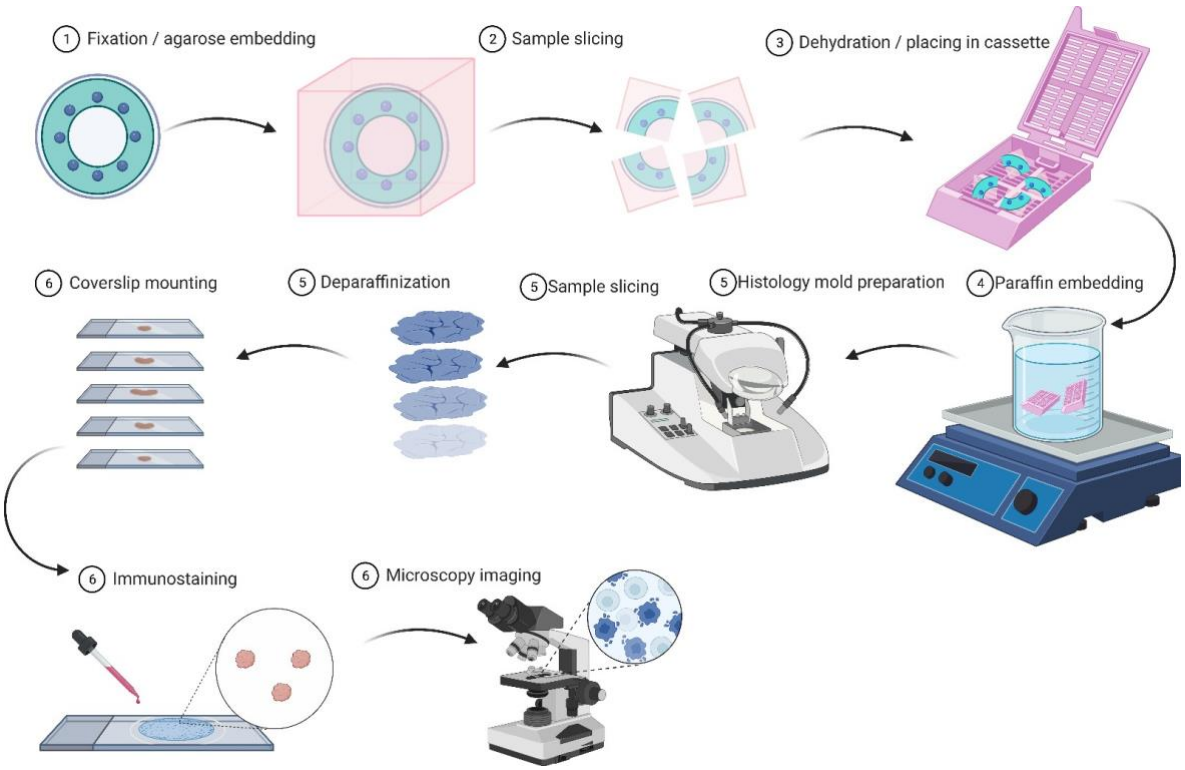


Fig.1 Schematic view of sample preparation steps for IHC analysis on spheroids in the 3-in-1 Plate.

Fixation

1. Soak the spheroids/organoids in 10% neutral buffered formalin for 60 minutes by filling the Media Reservoir of the 3-in-1 Plate with 200 µL formalin.
2. Gently aspirate the formalin from the Media Reservoir and Cell/ECM Loading Zone and wash twice with DPBS. Spheroids/organoids can be stored in the refrigerator before embedding.

Histology Mold Preparation

Histology mold preparation includes the following steps:

1. Embedding in agarose

Prepare 2% (w/v) agarose solution by dissolving low-melting agarose powder in DPBS. Place a beaker on a hot plate at 100°C. Stir the solution until it is clear. If needed, cover the beaker with foil to avoid evaporation. Remove the fixed and DPBS-washed 3-in-1 Plate from the well by spatula and place it in a 3 cm Petri dish. Let the agarose solution cool down. Before it solidifies, gently pour it into the Petri dish to embed the spheroids/organoids. The height of agarose in the Petri dish must be smaller than the thickness of the cassette (see step 3).

Let the agarose embedded 3-in-1 Plate cool down to room temperature (10 min). Next, separate each quadrant by cutting the agarose embedded 3-in-1 Plate with a blade. Make sure each piece contains the full array of spheroids/organoids (6 per quadrant) and fits inside the cassette.

Note: Do not use hot agarose solution; high temperature may damage the spheroids/organoids and disintegrate the target proteins.

Next steps are optimized for samples with ~5mm thickness.

2. Dehydration

To dehydrate the samples, soak the agarose-embedded spheroids/organoid blocks in ethanol according to the following steps,

- 70% ethanol for 30 minutes.
- 90% ethanol for 30 minutes.
- 100% ethanol for 120 minutes (3X). Agarose blocks must look white at the end of this step. Repeat this step if there are clear spots in the samples.

To remove ethanol, soak in Xylene according to the following steps,

- Xylene for 30 minutes.
- Xylene for 30 minutes (clean wash).
- Xylene for 30 minutes (clean wash). Agarose blocks must become very clear at end of this step. Repeat this step if there are white spots in the samples.

Note: Long exposure to Xylene may damage the samples.

3. Embedding the agarose blocks in paraffin wax

- Prepare melted paraffin wax in a beaker and keep it on a hotplate at 65°C.
- Place the samples in cassettes. Label the cassettes according to your experimental conditions.
- Place the cassettes in melted paraffin wax for 120 min
- Refresh the paraffin wax and repeat the previous step.

4. Preparing histology mold

- Remove the cassette from the wax and remove the sample from the cassette.
- Place the cassette face down on the hot plate and place the agarose blocks in the histology mold on the bench. Each cassette can contain more than one agarose block depending on the number of

experimental conditions.

- Pour clean paraffin wax into the mold around the agarose blocks.
- Place the hot cassette on the mold and cover it with wax.
- Let them dry at room temperature for at least 30 minutes, and transfer to the fridge before slicing.

Slicing

Paraffin-embedded spheroids/organoids must be sliced to form monolayer cell distribution (sections) prior to antibody staining as follows,

1. Set the microtome thickness to 10 μm. **Note:** The slicing velocity can be optimized to the users specifications.
2. Hook the cassettes, containing paraffin blocks, to the clamp. Make sure the cassette is aligned vertically parallel to the blade holder.
3. Load a sharp blade, tighten the blade holder, and make sure the blade's zone of slicing covers the entire paraffin block during the vertical motion.
4. Leave a few millimeters gap between the paraffin block and the blade.
5. Turn on the device. Wait until the blade touches the paraffin block and starts the slicing. Use a tweezer to grab the sections. Gently place the sections on warm water. Continue slicing.
6. When the slicing is done, turn off the device, remove the blade and unhook the paraffin block. Note that paraffin block is still usable if a part of the spheroids/organoids remains (store in the freezer).
7. Label a glass slide, gently hold it underneath one section and grab it. The section sits and attaches on the glass slide. Leave the glass slide in slide holder to remove the excess water. Repeat this step with new glass slides until all the sections are collected. Leave the glass slides at room temperature overnight to dry out. A hot plate can be used for faster dehydration.

Deparaffinization

Deparaffinize by soaking glass slides in,

- Xylene (2 x 3 minutes)
- 100% ethanol (2 x 3 minutes)
- 95% ethanol (3 minutes)
- Distilled water (3 minutes)

Note: Don't let the sections dry out before staining.

Antibody Staining

Antibody staining is carried out in the following steps,

1. **Permeabilization**
   - Incubate once in TBS + 0.3% Triton-X100 for 10 minutes at room temperature to permeabilize nuclei (improves intracellular staining).
2. **Blocking**
   - Add to each section: 3% bovine serum albumin (BSA) and 0.3% Triton X-100 in TBS and keep it at room temperature for 20 minutes. Note that all BSA solutions must be 0.2µm syringe filtered before use.
3. **Primary antibody staining**
   - Dilute at 1:200 to 1:1000 in 1% BSA, 0.3% Triton X-100 in TBS (follow the antibody instruction if they have specified dilution factors for IHC). Incubate overnight at 4°C in the humidity chamber. (Alternatively, incubate for 2-4 hours at room temperature).
   - After the incubation, wash 3x in TBS for 5 minutes each.

4. Secondary antibody staining

- Dilute the secondary antibody at 1:500 in 1% BSA, 0.3% Triton X-100 in TBS. (follow the antibody instruction if they have specified dilution factors for IHC). Incubate for 1 hour at room temperature.
- After the incubation, wash 3x in TBS for 5 minutes each.

5. Counter-stain with DAPI

- Dilute the 5 mg/mL stock solution down to 1 ug/mL in TBS by diluting by a factor of 5000 (i.e., 10 mL TBS for a 2μL aliquot).
- Incubate at room temperature for 5 minutes.

  Wash 3x in TBS for 5 minutes each.

6. Mount with Entellan

- Use a plastic dropper to put 3 drops of Entellan on each glass slide. Gently place a rectangular coverslip on top and gently press out any small bubbles.
- Wait at least 30 minutes to partially dry before imaging.

To demonstrate the applicability of the presented protocol, different target proteins were antibody stained.

Figure 2 shows IHC staining of actin, Hif1-α, and LC3 as well as counter-staining with DAPI in human glioblastoma (hGB) U251-derived spheroids. First, to show the cytoskeleton of cells, U251 spheroids were IHC stained with actin antibody, shown in Fig. 2(A). The cytoskeleton of a cell regulates different cellular activities such as growth, migration, and structural support, hence, is essential to determine the structure of tumor cells.

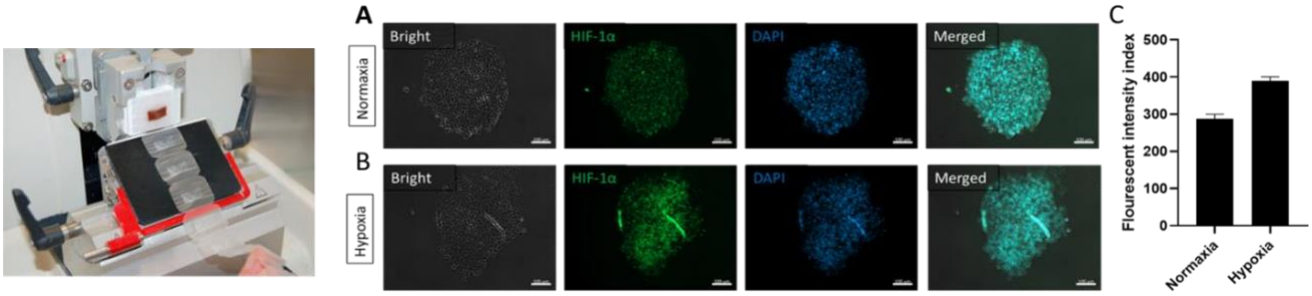


Fig 2. IHC stained slices of glioblastoma spheroids in a 3-in-1 Plate in A) Normaxia and B) Hypoxia conditions. C) Quantified image analysis of the immune-stained slices.

Hypoxia is one of the key regulators of cancer cell metabolism that can trigger tumor hallmarks such as invasion and angiogenesis [8]. To induce hypoxia, the spheroids were kept in a hypoxic chamber for 24 hours and IHC stained with Hif1-α antibody and quantified through an image analysis software, shown in Fig. 2 (B, C).

Important Notes

1. After deparaffinization, do not let the slides dry out! Incubate the primary and secondary antibodies in the airtight glass container with snap lid. Put a wet paper towel and 3-4 mm of water at the bottom. Carefully break a serological pipette into 1/3 segments and place them on the paper towel to hold the slides level and above the water.
2. It is best to perform all steps in the fume hood. Xylene and Entellan fumes are toxic.
3. Using the optimal form of heat induced epitope retrieval (HIER) can help increase signal intensity. If it is listed in the antibody datasheet.
4. Make sure that the primary antibody is a suitable target for the secondary antibody (i.e. if using a

rabbit primary, the secondary should be anti-rabbit).

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