RASTRUM

Protocol

In Situ Immunofluorescence Analysis of RASTRUM™ 3D Cell Models

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Introduction

This protocol outlines a method to detect and localise protein expression in RASTRUM 3D cell models. This protocol takes three days and is designed for 3D cell models printed into a 96-well plate.

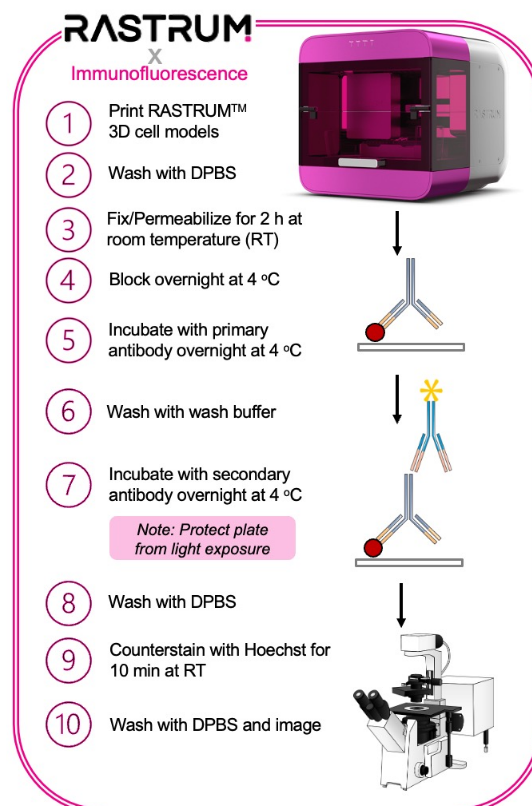
Storage and handling of reagents

Serum-containing buffers and counterstain buffer are stable at 4°C for 1 week after preparation. All other buffers are stable at room temperature for ~1 month.

Equipment and reagents required, but not provided

- RASTRUM 3D cell models
- Dulbecco's phosphate-buffered saline (DPBS)
- Wash buffer: 0.1% v/v Tween-20 in PBS (PBST)
- Fixation buffer: 4% paraformaldehyde (PFA) in DPBS
- Permeabilisation buffer: 0.1% v/v Triton-X-100 in DPBS
- Blocking buffer: 5% v/v bovine serum albumin (BSA) in PBST
- Primary antibodies (diluted in blocking buffer)
- Secondary antibodies (diluted in blocking buffer)
- Counterstain buffer: 5 µg/mL Hoechst 33342 (ThermoFisher, H3570) in DPBS
- 0.1% v/v sodium azide diluted in PBS
- Fluorescence microscope
- *Recommended:* Orbital shaker, or similar

Graphical Protocol



Protocol

Day 1

3D cell model preparation

1. Remove and discard the cell culture medium from RASTRUM 3D cell models.
2. Wash the 3D cell models by adding 150 µL of DPBS to each well and incubating the plate on an orbital shaker at 900 rpm for 5 min at room temperature (RT).

   Note: For all fluid removal steps, tilting the plate at a 45-degree angle to collect liquid at the bottom edge of the well will help maximise fluid removal from the plate. When aspirating media, be careful not to pierce or scratch the encapsulated cells and inert base with your pipette tip.
3. Remove and discard the DPBS.

Fixation and permeabilisation

4. Add 100 µL of fixation buffer to each well and incubate the plate for 20 min at RT.

   Note: If staining cells within a Large Plug or HTP model, we recommend increasing the incubation with fixation buffer to 1 h.
5. Remove and discard the fixation buffer, and wash each well with 150 µL of DPBS three times (with shaking at 900 rpm) for 5 min at RT.

   Note: Safely discard the fixation/permeabilisation buffer according to the EHS regulations of your organisation.
6. Add 100 µL of permeabilisation buffer to each well and incubate the plate for 30 min at RT.

   Note: If analysing both extracellular and intracellular proteins, we recommend using a mild permeabilisation reagent such as 0.1% saponin.

   Note: If desired, fixation and permeabilsation may be combined in a single step.
7. Remove and discard the permeabilisation buffer, and wash each well with 150 µL of DPBS three times (with shaking at 900 rpm) for 5 min at RT.

Blocking

8. Add 100 µL of blocking buffer to each well to block non-specific antibody binding.

   Note: While we have recommended 5% BSA as a blocking agent, it is also acceptable to use 10% serum from the host species in which the secondary antibodies are derived, and assuming the same host species for all secondary antibodies.
9. Incubate the plate overnight at 4°C.

Day 2

Primary antibody staining

10. Prepare the primary antibodies by diluting them in blocking buffer to a predetermined optimal working concentration.

    Note: Prepare working concentrations of primary antibodies fresh on the day of staining. After preparation, diluted antibodies are stable at 4°C for up to 3 days. We recommend using unconjugated primary antibodies for the detection of low abundance proteins.
11. Remove and discard the blocking buffer.

12. Add 100 µL of primary antibodies to each well, cover the plate with aluminium foil and incubate overnight at 4°C.
    Note: Primary antibody staining may be performed at RT for 1 h. For best results, always incubate overnight at 4°C.

Day 3

Secondary antibody staining

13. Prepare the secondary antibodies by diluting them in 5% BSA to a predetermined optimal working concentration.
    Note: Prepare working concentrations of secondary antibodies fresh on the day of staining. After preparation, diluted antibodies are stable at 4°C for up to 3 days.
14. Remove and discard the primary antibodies, and wash each well with 150 µL of wash buffer three times (with shaking at 900 rpm) for 5 min at 4°C.
15. Add 100 µL of secondary antibodies to each well, cover the plate with aluminium foil and incubate overnight at 4°C.
    Note: Secondary antibody staining may be performed at RT for 1 h. For best results, always incubate overnight at 4°C.

Day 4

Counterstaining

16. Remove and discard the secondary antibody, and wash each well with 150 µL of DPBS three times (with shaking at 900 rpm) for 5 min at 4°C in the dark.
17. Add 100 µL of counterstain buffer and incubate the plate at RT for 10 min in the dark.
18. Remove and discard the counterstain buffer, and wash each well with 150 µL of DPBS three times for (with shaking at 900 rpm) 5 min.
19. Add 150 µL of 0.1% sodium azide/PBS.
    Note: Fixed and stained 3D models can be stored in 0.1% sodium azide/PBS at 4°C in the dark for up to 1 week.
20. Proceed to imaging with a fluorescence microscope.

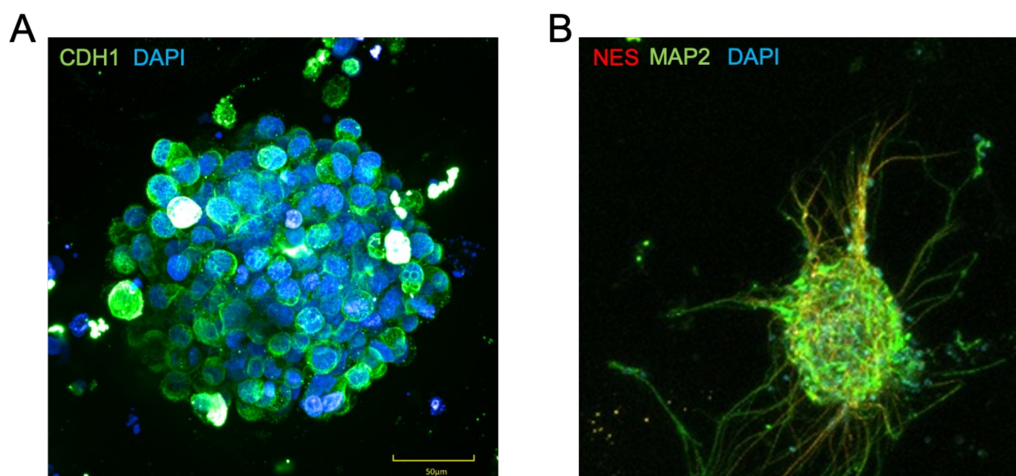


Figure 1: **Immunofluorescence staining of RASTRUM 3D cell models.**
(**A**) Trophoblast organoid stained with intercellular junction marker E-cadherin (green) and DAPI (blue). Data generated by the McClements group from the University of Technology Sydney. (**B**) Neurosphere 30 days after printing neural progenitor cells stained with Nestin (red), MAP2 (green) and DAPI (blue).

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