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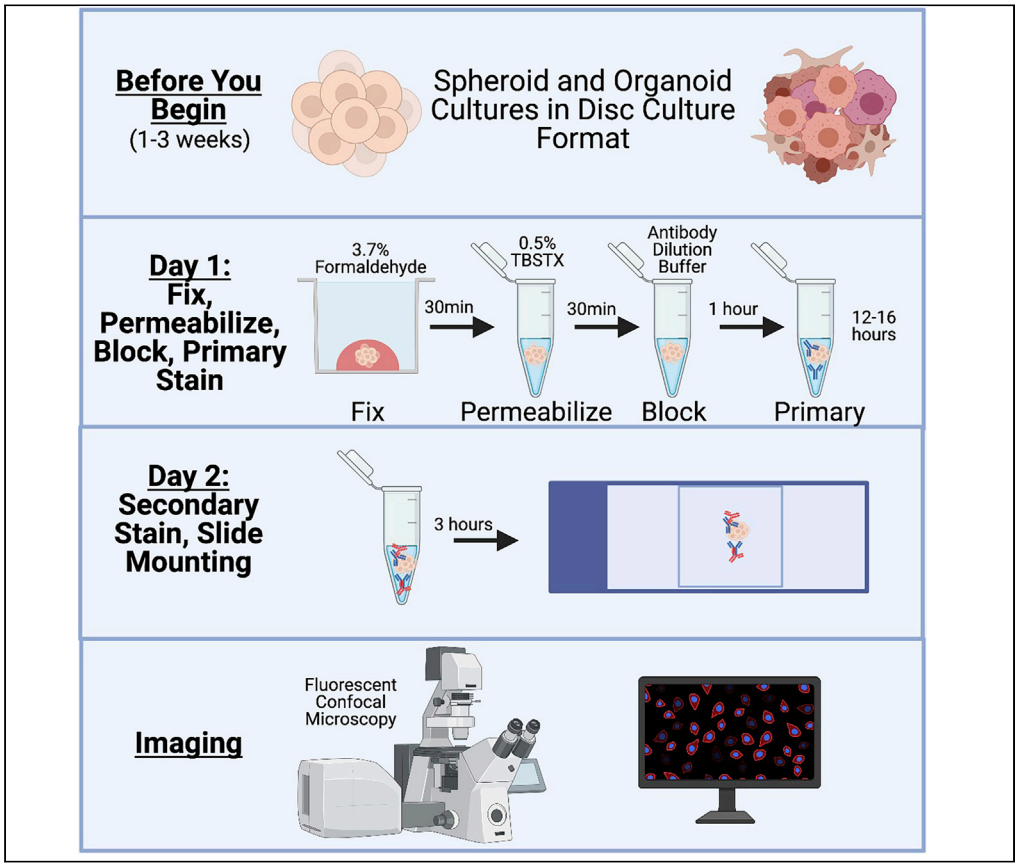
Protocol

Immunofluorescent staining of cancer spheroids and fine-needle aspiration-derived organoids



Our organoid generation technique has allowed for the development of downstream organoid applications. Here, we detail an accessible, straightforward protocol for immunofluorescent staining and imaging of thyroid cancer organoids, particularly those with tumor de-differentiation. Immunofluorescence is a powerful tool to help understand the localization of cell types within organoids and determine the interactions between those cells. As organoids have been shown to recapitulate patient tumor morphology, immunofluorescent staining and imaging of organoids allows for enhanced understanding of near *in vivo* structures.

Kensey N. Bergdorf, Courtney J. Phifer, Matthew E. Bechard, Mason A. Lee, Oliver G. McDonald, Ethan Lee, Vivian L. Weiss

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Highlights

Protocol for fixation and permeabilization of intact spheroids and organoids

Immunofluorescent staining of both spheroids and organoids

Identify fibroblasts and cancer cells in 3D cultures from de-differentiated tumors

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Immunofluorescent staining of cancer spheroids and fine-needle aspiration-derived organoids

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SUMMARY

Our organoid generation technique has allowed for the development of downstream organoid applications. Here, we detail an accessible, straightforward protocol for immunofluorescent staining and imaging of thyroid cancer organoids, particularly those with tumor de-differentiation. Immunofluorescence is a powerful tool to help understand the localization of cell types within organoids and determine the interactions between those cells. As organoids have been shown to recapitulate patient tumor morphology, immunofluorescent staining and imaging of organoids allows for enhanced understanding of near *in vivo* structures.

For complete details on the use and execution of this protocol, please refer to Lee et al. (2020) and Vilgelm et al. (2020).

BEFORE YOU BEGIN

Reagent preparation

Timing: 30 min

1. Prepare cytoskeleton buffer, 0.1% TBSTX, 0.5% TBSTX, and Antibody Dilution Buffer as described in materials and equipment.
   a. Store 0.1% TBSTX, 0.5% TBSTX, and Antibody Dilution Buffer at 4°C. Cytoskeleton buffer must be prepared fresh.

Spheroid or organoid growth

Timing: 5–14 days

2. Spheroids/organoids should be grown in 75% Matrigel disc cultures as described in Phifer et al., 2021.

Note: Spheroid/organoid size will vary drastically between cultures, so there is no recommended size or density to have prior to immunofluorescent staining. However, we generally recommend that there be more than one organoid to be stained, due to the potential for loss. This protocol may also work using the semi-solid culture method, but we find the disc method preferable for downstream immunofluorescent staining.

Optional: This protocol is effective for baseline characterization of cultures in addition to analyzing treatment effects. Drugs or supplements can be added to the media surrounding the Matrigel discs prior to performing this protocol.

Note: Many published organoid/spheroid immunofluorescence protocols grow their cultures and stain in sterile chamber slides. We found that this method complicated long-term culture of some slow-growing organoids, as it made media changes more cumbersome and frequently led to disc detachment. Additionally, because we could not centrifuge the chamber slides easily, extra time was needed for organoids/spheroids to settle to the bottom of the chamber between each step. This greatly increased the length of the staining protocol and loss of cultures. For these reasons, we recommend growing the cultures in normal disc-culture conditions and performing all staining and fixation in microcentrifuge tubes prior to transferring to a slide.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-alpha smooth muscle actin	Abcam	ab5694
Mouse Anti-Beta Catenin	Vanderbilt Antibody and Protein Resource	VAPRCAT
Guinea Pig Anti-Cytokeratin 8+18	Abcam	ab194130
Goat Anti-Guinea Pig IgG (H+L) Alexa Fluor 647	Abcam	ab150187
Donkey Anti-Mouse IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific	A-21202
Donkey Anti-Rabbit IgG (H+L) Alexa Fluor 555	Abcam	ab150074
Alexa Fluor 568 Phalloidin	Thermo Fisher Scientific	A12380
Hoechst 3342	Thermo Fisher Scientific	H3570
Chemicals, peptides, and recombinant proteins		
Matrigel	Fisher Scientific	CB-40234
Fetal bovine serum (FBS)	Thermo Fisher Scientific	26140079
Bovine serum albumin (BSA)	Research Products International	A30075100
37% Formaldehyde	VWR	97064-606
1× Phosphate-buffered saline (PBS)	Corning	MT21040CV
2-Morpholinoethanesulfonic acid (MES)	Research Products International	M22050-100.0
Potassium chloride (KCl)	Research Products International	P41000500
Magnesium chloride ($MgCl_2$)	Fisher Scientific	AC223211000
Ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA)	Fisher Scientific	O2783-100
Sucrose	Fisher Scientific	57-311-305KG
10× Tris-buffered saline (TBS)	Corning	MT46012CM
Triton X-100	Fisher Scientific	TX15681
ProLong Gold Antifade Mountant without DAPI	Thermo Fisher Scientific	P36930
ProLong Gold Antifade Mountant with DAPI	Thermo Fisher Scientific	P36931
Other		
Clear nail polish	n/a	n/a
Fisherbrand Superfrost Plus Microscope Slides	Fisher Scientific	12-550-15
Corning Square and Rectangular Cover Glasses	Fisher Scientific	12-533-464

MATERIALS AND EQUIPMENT

Alternatives: We image our organoid immunofluorescent staining with a Nikon Spinning Disk Imaging Microscope. This platform is equipped with a Yokogawa CSU-X1 spinning disk head, Andor DU-897 EMCCD, a high-speed piezo stage, automated and encoded X/Y stage, a four-line high-power solid-state laser launch, and many objectives. Any confocal microscope equipped with fluorescent imaging capabilities should work for this protocol.

Note: 0.1% TBSTX, 0.5% TBSTX, fixative, and cytoskeleton buffer can be stored at 4°C for up to one year. Antibody Dilution Buffer can be stored at 4°C for up to one month.

Cytoskeleton buffer		
Reagent	Final concentration (mM or μM)	Amount
1 M MES, pH 6.1	10 mM	1 mL
1 M KCl	138 mM	13.8 mL
1 M $MgCl_2$	3 mM	0.3 mL
125 mM EGTA, pH 7.1	1.25 mM	1.0 mL
Milli Q H_2O	n/a	83.9 mL
Total		**100 mL**

0.1% TBSTX		
Reagent	Final concentration (%)	Amount
1× TBS	n/a	500 mL
Triton X-100	0.1%	500 μL
Total		**500 mL**

0.5% TBSTX		
Reagent	Final concentration (%)	Amount
1× TBS	n/a	100 mL
Triton X-100	0.5%	500 μL
Total		**100 mL**

Antibody dilution buffer		
Reagent	Final concentration (%)	Amount
BSA	2%	2 g
0.1% TBSTX	n/A	100 mL
Total		**100 mL**

3.7% Formaldehyde fixative

Dilute 37% formaldehyde 10-fold in 1× PBS to create the fixative for this protocol.

If using phalloidin, dilute 37% formaldehyde in cytoskeleton buffer plus 0.144 g/mL sucrose. The 3.7% formaldehyde in cytoskeleton buffer can be kept at 4°C for up to a year, but the sucrose must only be added to aliquots immediately before use.

⚠ CRITICAL: Formaldehyde is a potent skin and respiratory irritant. Gloves should always be worn while handling formaldehyde and extra care should be taken to avoid any inhalation while measuring.

STEP-BY-STEP METHOD DETAILS

Fixing and permeabilization

Timing: 1.5 h

Organoids must be fixed prior to staining to preserve protein localization and structure, and to prevent degradation. Permeabilization disrupts the cell membrane to allow for intracellular staining.

1. Fix the organoids.
   a. Aspirate media from the wells of disc culture, leaving only the Matrigel discs containing the spheroids/organoids.
   b. Put 2 mL of fixative in each well and incubate plate at 4°C for 15 min. After 15 min, swirl the plate by hand and place back at 4°C for another 15 min.
   c. Pipette to break up the Matrigel and transfer into a labeled 2 mL microcentrifuge tube.

⚠ CRITICAL: Unless otherwise stated, all steps that involve mixing of organoids will use a P1000 tip with the tip cut off at the end. This is done to prevent mechanical disruption of the organoids. Troubleshooting 1

2. Wash and permeabilization
   a. Wash: Centrifuge the organoids at 2300 × *g* for 5 min in a microcentrifuge. Aspirate off the supernatant, paying careful attention to not disrupt the pellet. Resuspend the pellet in 1 mL of 0.1% TBSTX with a P1000 and repeat centrifugation and aspiration.
   b. Resuspend each pellet in 1 mL 0.5% TBSTX.
   c. Place tubes on rotator at room temperature (20°C–22°C) to permeabilize for 30 min.

Blocking and staining

Timing: 1.5 days

Blocking prevents non-specific staining before any antibodies are added. The primary antibody will detect the protein of interest, and the secondary antibody will detect the primary antibody and allow for fluorescent imaging.

3. Blocking and primary immunostaining
   a. Repeat wash step in 2a.
   b. Resuspend pellets in 1 mL Antibody Dilution Buffer.
   c. Place on rotator for 1 h at room temperature (20°C–22°C) to block.

Pause point: Organoids can be left in the Antibody Dilution Buffer for blocking overnight (12–16 h).

   d. For fibroblast/tumor cell staining: add both the anti-smooth muscle actin antibody and the anti-cytokeratin 8/18 antibody at a dilution of 1:250 each, directly to the Antibody Dilution Buffer.
   For β-catenin staining: add the anti-β-catenin antibody to a final dilution of 1:500 directly to the Antibody Dilution Buffer.
   e. Place on rotator at 4°C for 12–16 h.

Optional: Staining can be done for shorter time periods at room temperature (20°C–22°C) or 37°C, but this will need to be optimized for each antibody and may increase background staining. Troubleshooting 2

4. Secondary immunostaining
   a. Remove from rotator and repeat wash step in 2a.
   b. Resuspend pellets in 1 mL Antibody Dilution Buffer.
   c. For fibroblast/tumor cell staining: Add anti-guinea pig and anti-rabbit secondary antibodies, each at a dilution of 1:250, directly to the Antibody Dilution Buffer/organoid suspension.
   For β-catenin staining: Add anti-mouse secondary antibody to a final dilution of 1:500 in Antibody Dilution Buffer.
   d. Place on rotator in the dark at room temperature (20°C–22°C) for 3 h.

Slide preparation and imaging

Timing: 1 h (preparation)

Slide preparation is the final step that must be done before slides can be stored indefinitely. Imaging and analysis can occur days or weeks after the slides have been prepared and sealed.

5. Wash and mount on slide
   a. Repeat wash step in 2a

   Optional: Resuspend pellet in 0.1% TBSTX. Add Hoechst at a 1:1000 dilution directly to 0.1% TBSTX, rotate for 20 min at room temperature (20°C–22°C) in the dark and repeat wash step in 2a.

   Optional: Resuspend pellet in 0.1% TBSTX. Add phalloidin at a 1:100 dilution directly to 0.1% TBSTX, rotate for 20 min at room temperature (20°C–22°C) in the dark and repeat wash step in 2a.
   b. Remove as much of the supernatant as possible without disturbing the pellet.
   c. Resuspend organoids in 50 uL PBS with a P200 with the end of the tip cut off and pipette the organoid suspension onto a labeled slide.
   d. Allow the organoids to settle. Troubleshooting 3
   e. Tip the slide slightly so that the PBS pools in one spot, leaving the majority of the organoids in place. Aspirate the PBS, taking care not to disturb the organoids.
   f. Pipette 50 uL Prolong Gold Antifade onto the slide.

   Note: If using Hoescht, use Prolong Gold without DAPI. If not using Hoescht, use Prolong Gold with DAPI. Prolong Gold enables long-term storage of slides prior to imaging, as long as the slides are sealed and properly stored.
   g. Place a coverslip over the slide without creating bubbles.
   h. Seal the edges with clear nail polish and allow to cure in the dark for 5–10 min.

   Pause point: Store in a dark box at 4°C until ready to image.

6. Image
   a. On the microscope, use the focusing adjustment knobs to adjust the z-plane down to the bottom of the organoid of interest and select it as "bottom."
   b. Adjust the z-plane up to the top of the organoid and select it as "top."
   c. Use the recommended step size (typically 1–2 um).
   d. Select "create Z-stack."
   e. Once finished, save file.

   Note: If imaging multiple color channels, make sure to image from the longest wavelength to the shortest wavelength (i.e., image 561 then 488 then 405) to prevent bleaching. Samples will

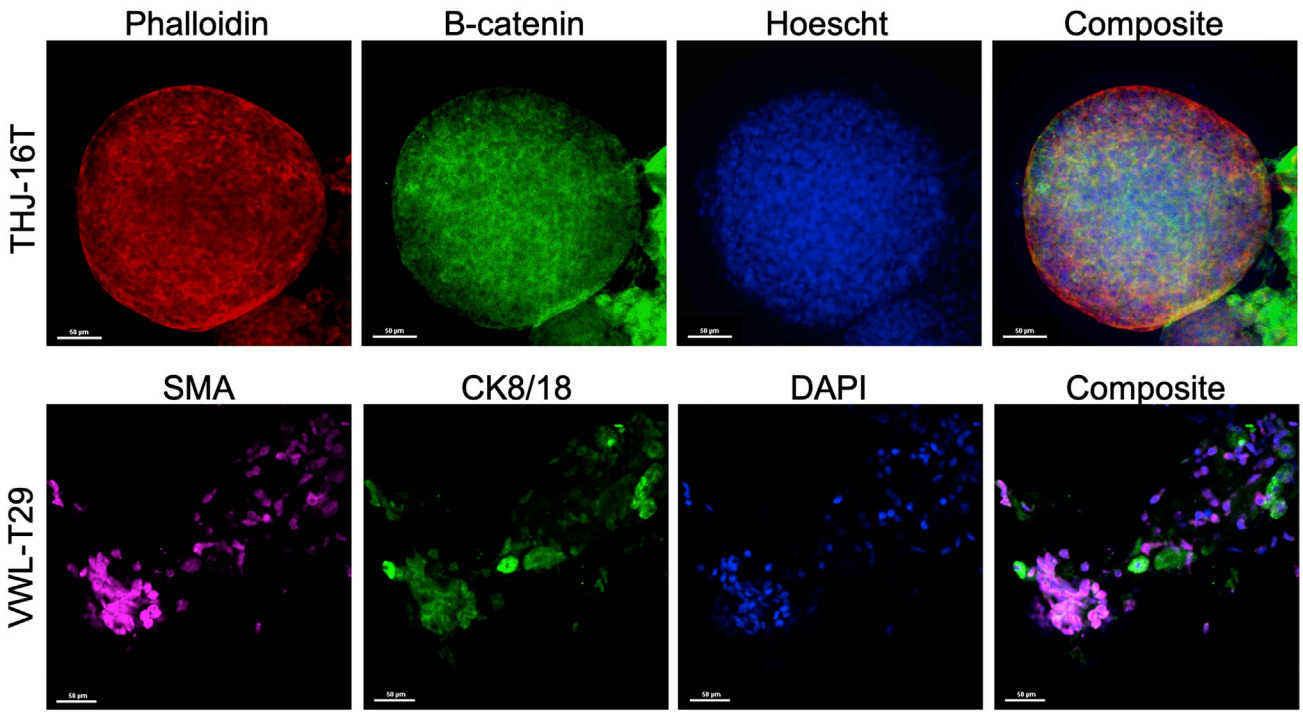

Figure 1. Example of results

THJ-16T, a thyroid cancer cell line, was grown in spheroid format then stained with phalloidin (actin filaments-red), β-catenin (green), and Hoechst (nuclei-blue), reprinted with permission from Lee, et al., 2020. VWL-T29 is a primary patient organoid obtained as described in Phifer et al., 2021. This line was stained for smooth muscle actin (SMA, fibroblasts, magenta), cytokeratin 8/18 (CK8/18, tumor cells, green), and DAPI (blue), reprinted with permission from Vilgelm et al., 2020. Scale bars are 50 um.

bleach faster at higher magnifications, so always start with low magnifications before going to higher magnifications.

f. Perform analysis of your choice.

EXPECTED OUTCOMES

Expected outcomes are demonstrated in Figure 1

LIMITATIONS

While we have had great success in staining thyroid cancer organoids for smooth muscle actin, cytokeratin 8/18, α-tubulin, and β-catenin, these methods may not work for all antibodies in all organoid types. Some organoids have a dense extracellular matrix that will retain antibodies and cause high levels of background staining, while preventing the antibody from reaching the specific target within the cells. Additionally, due to the frequent centrifugation and aspiration, it is expected that some cell/organoid loss will occur. We recommend performing this protocol with robust cultures (many organoids or large organoids) to ensure that many organoids are available for imaging.

Organoids are certainly more difficult to stain and image consistently due to the range of cell types, where spheroid cultures will only contain one cell type. Tumors collected by fine needle aspiration may also demonstrate a range of de-differentiation, particularly those from unresectable, widely metastatic, or advanced disease. Indeed, this is one of the clear benefits of FNA, that more aggressive lesions can be sampled than those that undergo surgical resection, However, de-differentiation or sarcomatoid transformation of the tumor can make distinction of tumor cells from fibroblasts more challenging. To help identify de-differentiated or sarcomatoid tumor cells, this protocol utilizes a

stain for cytokeratin 8/18. In contrast to conventional AE1/3 staining, which detects both high- and low-molecular weight keratins, cytokeratin 8/18 staining is only for low-molecular weight keratins. Multiple studies suggest that cytokeratin 8/18 provides more sensitive detection of epithelial origin, and thus improved detection of more undifferentiated or spindled tumors (Ordóñez, 2013; Pradniwat and Treetipsatit, 2013; Kodack et al., 2017). However, a remaining limitation is that some highly spindled carcinomas and some myofibroblasts from bladder biopsies may still express low levels of smooth muscle actin and cytokeratin 8/18 (Tamas and Epstein, 2007). Fortunately, primary bladder FNAs are uncommon in clinical practice and are unlikely to be performed for research tissue acquisition.

TROUBLESHOOTING

Problem 1

Organoids are no longer intact/have broken apart.

Potential solution

Ensure that all pipetting that involves transferring or mixing the organoids is performed with a P1000 tip that has been cut to make the opening larger. For large organoids, more of the tip will need to be removed before pipetting to prevent any shearing.

Problem 2

Matrigel dissociation and background staining.

Potential solution

Failure to dissociate the Matrigel, inadequate blocking, and/or the conditions that the primary and secondary antibodies were incubated can result in background staining. By fixing the organoids at 4°C and mixing during the fixation time, the Matrigel dissociates. If the Matrigel isn't dissociating, then fixing for a longer period of time or using cold reagents for the fixative solution should assist. Blocking and incubating the primary antibody overnight (12–16 h) at 4°C are the best ways to limit background staining. However, incubation conditions can be optimized for each antibody.

Problem 3

Organoids clumping together.

Potential solution

If the organoids are clumping together on the slide, they will be more difficult to image. To prevent clumping, make sure that the organoids are well-mixed in the PBS before transferring to the slide. If they are particularly sticky organoids, you may suspend them in a higher volume of PBS to mix and provide more separation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vivian Weiss, (Vivian.l.weiss@vumc.org).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

This study did not generate or analyze any datasets or code.

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AUTHOR CONTRIBUTIONS

C.J.P., M.E.B., and M.A.L. developed the protocol. K.N.B. and C.J.P. prepared the manuscript, with revisions and comments provided by all authors. Funding was provided by O.G.M., E.L., and V.L.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Lee, M., Bergdorf, K., Phifer, C., Jones, C., Byon, S., Sawyer, L., Bauer, J., and Weiss, V. (2020). Novel three-dimensional cultures provide insights into thyroid cancer behavior. Endocrine Relat. Cancer *27*, 111–121.

Phifer, C., Bergdorf, K., Bechard, M., Vilgelm, A., Baregamian, N., McDonald, O., Lee, E., and Weiss, V. (2021). Obtaining patient-derived cancer organoid cultures via fine-needle aspiration. STAR Protoc. *2*, 100220.

Vilgelm, A., Bergdorf, K., Wolf, M., Bharti, V., Shattuck-Brandt, R., Blevins, A., Jones, C., Phifer, C., Lee, M., Lowe, C., et al. (2020). Fine-needle aspiration-based patient-derived cancer organoids. iScience *23*, 101408.

Ordóñez, N. (2013). Broad-spectrum immunohistochemical epithelial markers: a review. Hum. Pathol. *44*, 1195–1215.

Pradniwat, K., and Treetipsatit, J. (2013). A practical approach to undifferentiated tumors: a review on helpful markers and common pitfalls. Asian Arch. Pathol. *9*, 123–136.

Kodack, D., Farago, A.F., Dastur, A., Held, M., Dardaei, L., et al. (2017). Primary patient-derived cancer cells and their potential for personalized cancer patient care. Cell Rep. *21*, 3298–3309.

Tamas, E.F., and Epstein, J.I. (2007). Detection of residual tumor cells in bladder biopsy specimens: pitfalls in the interpretation of cytokeratin stains. Am. J. Surg. Pathol. *31*, 390–397.