Preparation of tumoroids for immunofluorescence

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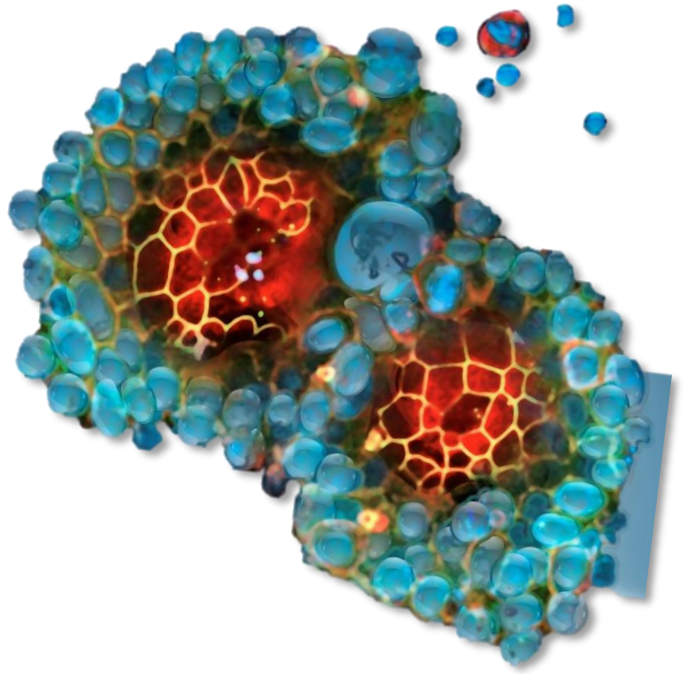
Introduction

Tumoroids, also known as tumoroid lines, cancer organoids, or patient-derived tumor organoids (PDTOs), are patient-derived cancer cells grown as 3D, self-organized multicellular structures.



Scientific literature suggests tumoroids are a promising tool for drug development studies and precision medicine applications because, compared to traditional 2D immortalized cancer cell lines, they:

- Better represent patient characteristics
- Support tumor heterogeneity
- Offer more clinically predictive results

Techniques such as immunofluorescence imaging, western blotting, and flow cytometry can be used to characterize tumoroids. However, the 3D structure of tumoroids and presence of basement membrane extract (BME), which is typically required for *in vitro* culture, can pose challenges when preparing these samples for downstream analysis. This protocol helps provide guidance on adapting tumoroids for immunofluorescence imaging.

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Required materials not supplied

Item	Cat. No.
Consumables	
Snap Cap Low Retention Microcentrifuge Tubes, 1.5 mL	3451PK
Snap Cap Low Retention Microcentrifuge Tubes, 0.6 mL	3446PK
Nunc Serological Pipettes, 10 mL	170356N
Nunc 15 mL Conical Sterile Polypropylene Centrifuge Tubes	339650
Nunc 50 mL Conical Sterile Polypropylene Centrifuge Tubes	339652
Nunc Cell Scrapers	179693
Nunc Non-Treated Multidishes, 6-well plate	150239
Reagents and buffers	
Pierce 16% Formaldehyde (w/v), Methanol-Free	28906
DPBS, no calcium, no magnesium	14190144
DMEM/F-12, GlutaMAX Supplement	10565018
Triton X-100 Surfact-Amps Detergent Solution	85112
BSA	–
BlockAid Blocking Solution	B10710
Counterstains	
Hoechst 33342 Solution (20 mM)	62249
Rhodamine Phalloidin	R415
SYTOX Green Nucleic Acid Stain	S7020
SYTOX Deep Red Nucleic Acid Stain	S11381
Equipment	
HulaMixer Sample Mixer	15920D
Room temperature rocker	–
Centrifuge	–
Confocal or fluorescent microscope	–

Items with a Cat. No. can be purchased through **thermofisher.com**.

Items without a Cat. No. can be purchased from **fishersci.com** or other laboratory suppliers.

Warning: Read the safety data sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. SDSs are available from **thermofisher.com/support**.

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Collect and wash tumoroids

Note

Tumoroids can be sticky and may adhere to culture vessels or pipettes. Pre-wetting with a culture medium or wash buffer containing BSA is recommended to minimize tumoroid loss during sample preparation. The protocol is written for staining tumoroids expanded in one non-treated (non-TC) T-25 flask. Scale volumes appropriately for the desired number of tumoroids and/or conditions to be tested using non-TC flasks or well plates.

1. Collect the tumoroids from a T-25 flask in a 15 mL or 50 mL centrifuge tube.
2. Add 5 mL of cold medium to the T-25 flask, rinse, and collect the rest of the tumoroids into the same tube.
3. Centrifuge at 400 x *g* for 5 min at 4°C.
4. Discard the supernatant, pre-wet a 10 mL pipette with cold DPBS + 0.1% BSA, and resuspend the pellet in 10 mL cold DPBS + 0.1% BSA to break the pellet into smaller clumps.
5. Centrifuge at 400 x *g* for 5 min at 4°C.

Note: Cold solutions may be stored at 4°C or on ice throughout the protocol.

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Fix and permeabilize tumoroids

1. Discard the supernatant, resuspend the pellet in 4 mL DPBS + 0.1% BSA, and transfer to a non-TC 6-well plate (2 mL per well). Add 2 mL of 8% formaldehyde (in DPBS) to each well (final formaldehyde concentration will be 4%), and incubate with gentle rocking at room temperature for 1 hr.

2. Collect the tumoroids in a prechilled 15 mL or 50 mL tube. Add 1–2 mL of cold DPBS + 0.1% BSA to the well, gently scrape the rest of the tumoroids with a cell scraper, and collect into the same tube.

3. Centrifuge at 400 x *g* for 5 min at 4°C.

4. Discard the supernatant, pre-wet a pipette in cold DBPS + 0.1% BSA, resuspend the pellet in 5 mL of cold DPBS + 0.1% BSA, triturate to break the pellet into smaller clumps, and centrifuge at 400 x *g* for 5 min at 4°C. Repeat this step once more.

5. Discard the supernatant, resuspend the pellet in 5 mL of permeabilization buffer (DPBS + 0.1% BSA + 0.5% Triton X-100), and incubate with gentle rocking at room temperature for 1 hr.

6. Centrifuge at 400 x *g* for 5 min at 4°C.

7. Pre-wetting the pipette each time, repeat step 4 twice to wash the tumoroids with cold DPBS + 0.1% BSA.

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Stain tumoroids

1. Resuspend the pellet in Invitrogen™ BlockAid™ Blocking Solution and incubate for 1 hour at room temperature.
   Note: Resuspend tumoroids in a volume of BlockAid Blocking Solution that is sufficient to transfer 100–500 µL of the suspension to a microcentrifuge tube for each staining condition. Depending on the number of tumoroids initially grown, multiple tubes can be used to serve as controls or stain for additional targets. Controls containing no primary antibody are recommended.
2. Add the primary antibody in BlockAid Blocking Solution. Adjust the volume of the staining solution to reach the desired final primary antibody concentration, accounting for the volume of BlockAid Blocking Solution from step 1.
3. Incubate at 4°C overnight on a rocking platform at ~25% speed.
4. The following day, centrifuge tumoroids at 400 x *g* for 5 min at 4°C, then discard the supernatant.
5. Resuspend in 1 mL (at least 5–10x volume of cell pellet) of cold DPBS + 0.1% BSA, centrifuge at 400 x *g* for 5 min at 4°C, then discard the supernatant. Repeat once more.
6. Add the secondary antibody in BlockAid Blocking Solution.
7. Incubate for 4 hr at room temperature (for tight-junction and membrane proteins) or overnight (for nuclear proteins, organelle-specific proteins, or other intracellular proteins) at 4°C on a rocking platform at ~25% speed.
   Note: An F-actin stain (e.g., 1:400 diluted rhodamine phalloidin) and nuclear counterstains (e.g., 1:10,000 diluted Invitrogen™ SYTOX™ Deep Red Nucleic Acid Stain or SYTOX™ Green Nucleic Acid Stain) can be added along with the secondary antibody.
8. Centrifuge the tumoroids at 400 x *g* for 5 min at 4°C, then discard the supernatant.
9. Resuspend in 1 mL (at least 5–10x volume of cell pellet) of cold DPBS + 0.1% BSA, centrifuge at 400 x *g* for 5 min at 4°C, then discard the supernatant. Repeat once more.
10. Proceed to imaging.

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Troubleshooting

Problem/question	Solution/answer
How large should the tumoroids be prior to preparing for immunofluorescence imaging?	Tumoroids can be fixed at any time during growth, depending on the application of interest. Most tumoroid lines reach a size of 200–400 µm in diameter in 7–10 days after plating.
The tumoroids are sticking to the side of the pipette tip.	Add 0.1% BSA to wash buffers and DPBS to prevent tumoroids from sticking to the walls of pipette tips.
The tumoroids are breaking apart during bulk processing.	Use a wide-bore pipette tip or cut the end off a regular pipette tip with scissors or a razor blade to use when processing tumoroids. Also, after adding buffers, flick the tube to gently resuspend the tumoroids. **Note**: Do not pipette up and down.
There is no signal for my antigen, or it is very dim.	For low-expression antigens, if optimizing the concentration of the primary antibody does not yield a suitable signal, incubate the tumoroids with the secondary antibody overnight. For antigens expressed toward the core of the tumoroid, use a nanobody (e.g., alpaca secondary antibody), as they have improved penetrating capability compared to conventional secondary antibodies.
How long can fixed tumoroids be stored?	It depends on the antigen being stained. It is best to image the tumoroids within 2–3 days after staining. However, they can be kept fixed for up to 2 weeks at 4°C. **Note**: Do not do this for low-expression antigens/proteins expressed toward the core.
The images of the tumoroids are not crisp.	Use confocal imaging and Z-stacking to get the best resolution.
The images have background staining.	1. Use a validated antibody. 2. Use BlockAid Blocking Solution to reduce background signal.
There is no signal from my nuclear stain, or it is very dim.	Add nuclear counterstains in DPBS (with no BSA) in an additional staining step following incubation with secondary antibodies. Incubate for 3–4 hours at room temperature, wash tumoroids 2 times with cold DPBS + 0.1% BSA, and proceed to imaging.

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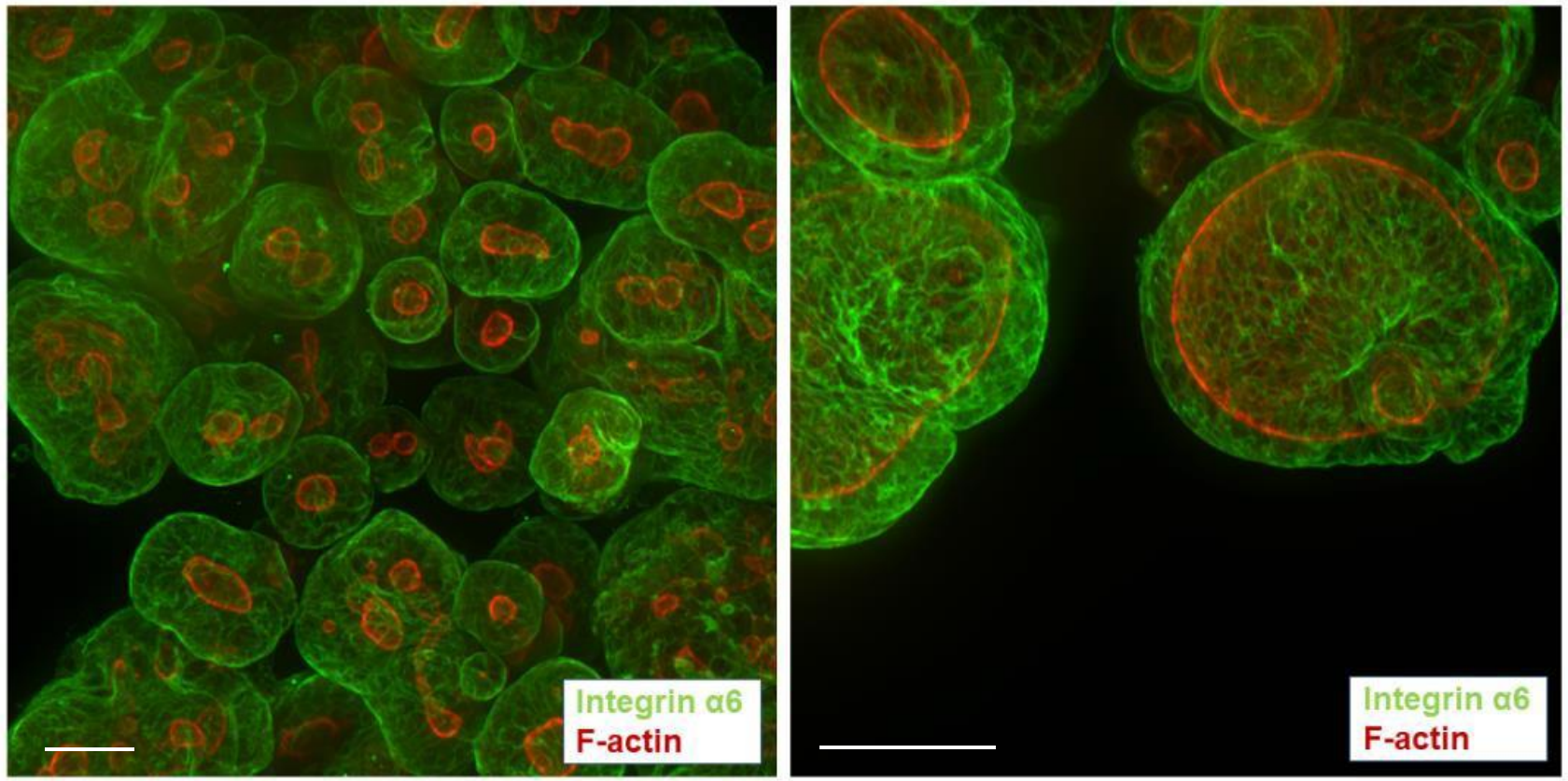
Representative images



HuCo1044 (left) and HuCo21320 (right) were stained for integrin α6. After overnight incubation with a primary antibody, the tumoroids were incubated with an Invitrogen™ Alexa Fluor™ Plus 488 secondary antibody. F-actin was stained with rhodamine phalloidin. Images were acquired on the Invitrogen™ CellInsight™ CX7 LZR Pro High-Content Screening Platform in confocal mode (scale bars = 100 µm).

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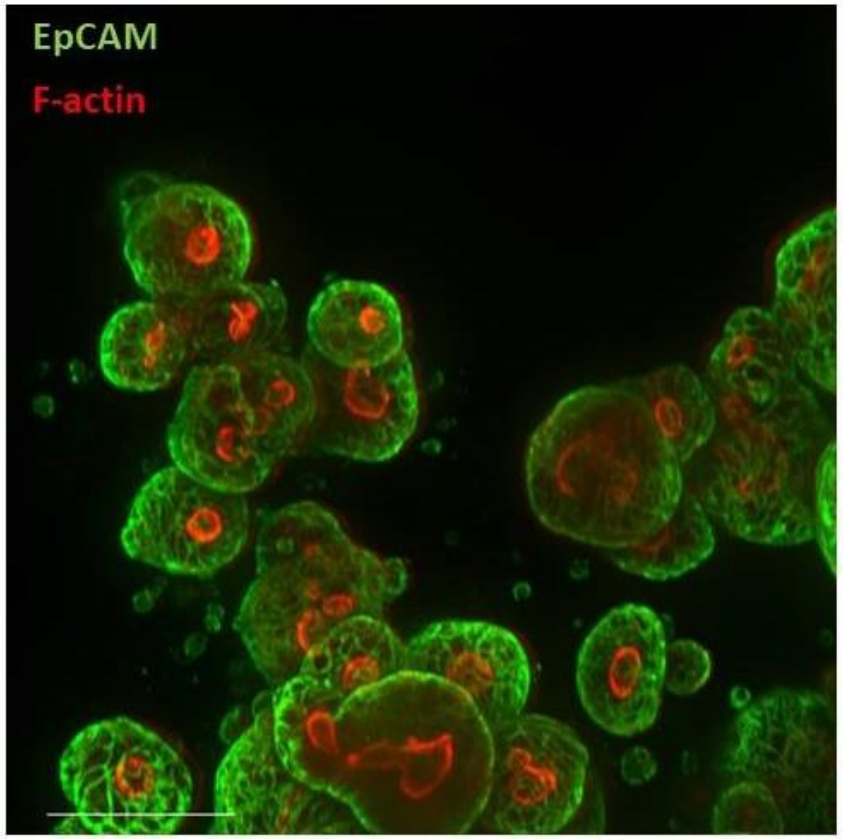

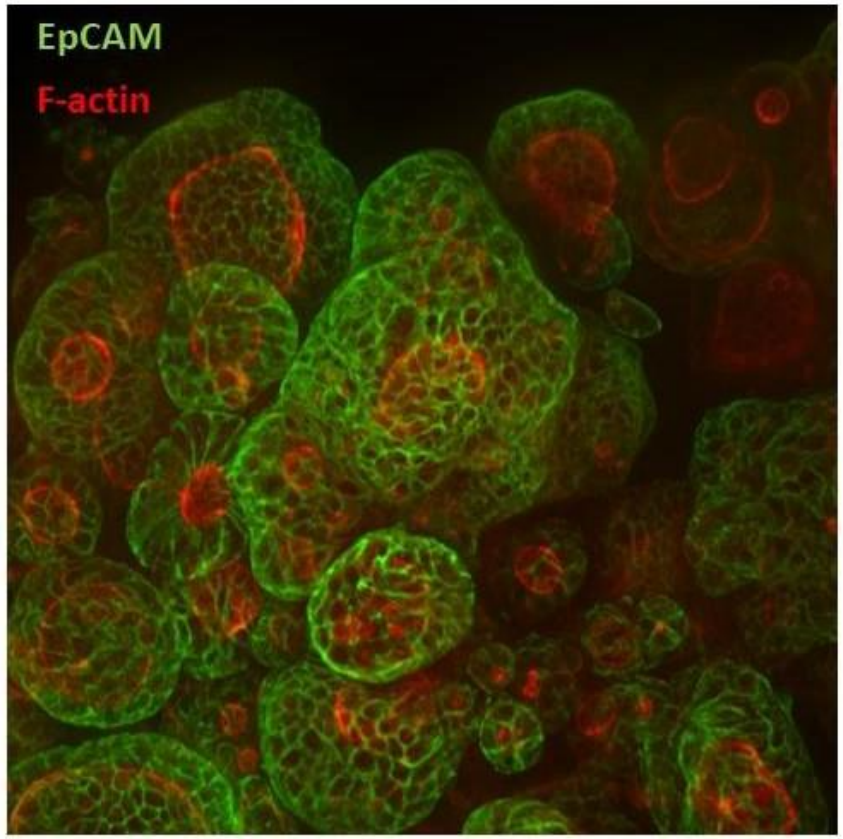

HuCo1044 (left) and HuCo21320 (right) were stained for epithelial cell adhesion molecule (EpCAM). After overnight incubation with a primary antibody, the tumoroids were incubated with an Invitrogen™ Alexa Fluor™ Plus 488 secondary antibody. F-actin was stained with rhodamine phalloidin. Images were acquired on the CellInsight CX7 LZR Pro HCS Platform in confocal mode (scale bars = 100 µm).

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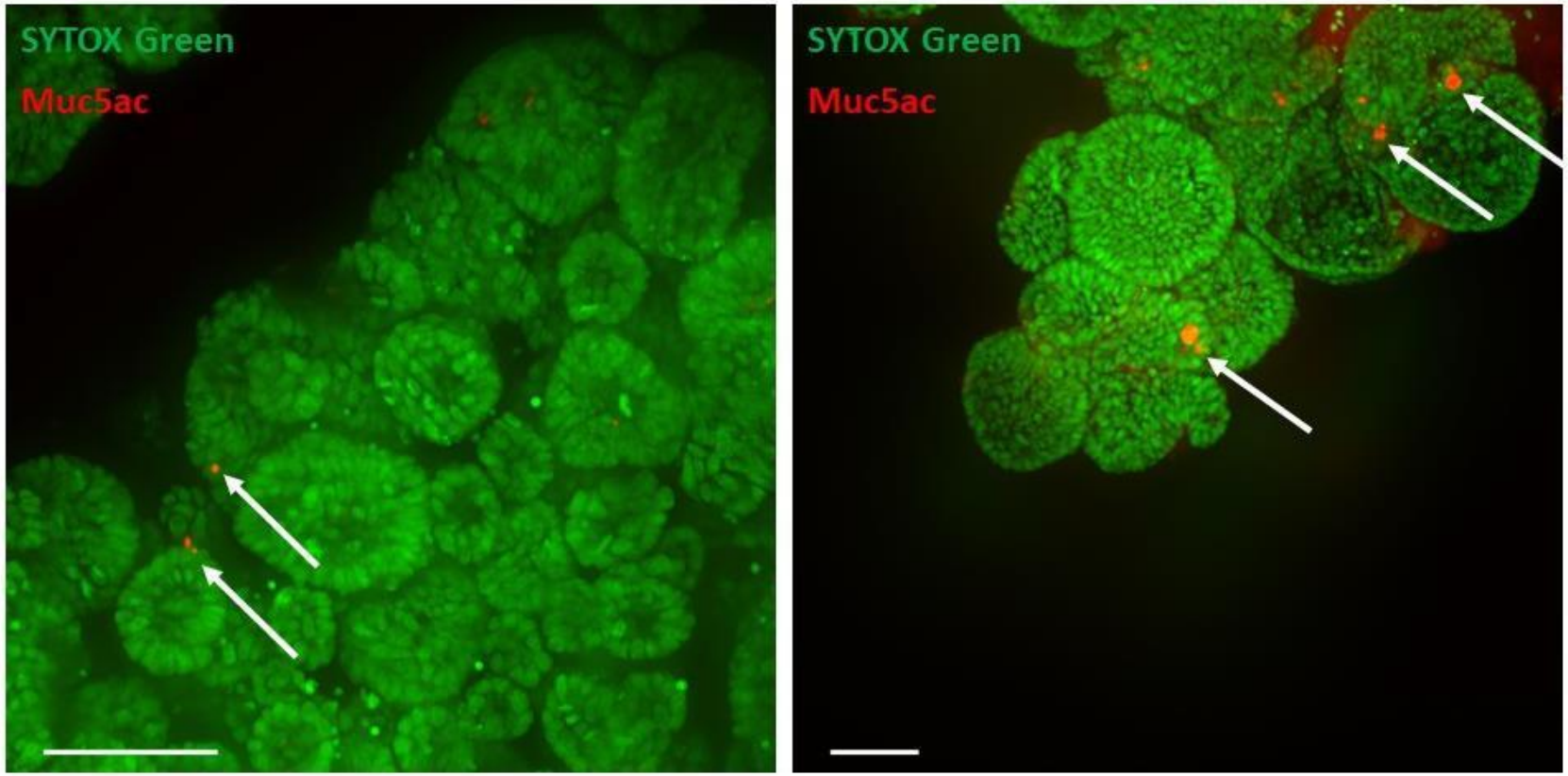
Representative images



HuCo1044 (left) and HuCo21320 (right) were stained for Mucin 5AC. After overnight incubation with a primary antibody, the tumoroids were incubated with an Invitrogen™ Alexa Fluor™ 647 alpaca anti-mouse nanobody overnight at 4°C. The following day, nuclei were counterstained with SYTOX Green Nucleic Acid Stain. Images were acquired on the CellInsight CX7 LZR Pro HCS Platform in confocal mode (scale bars = 100 µm).

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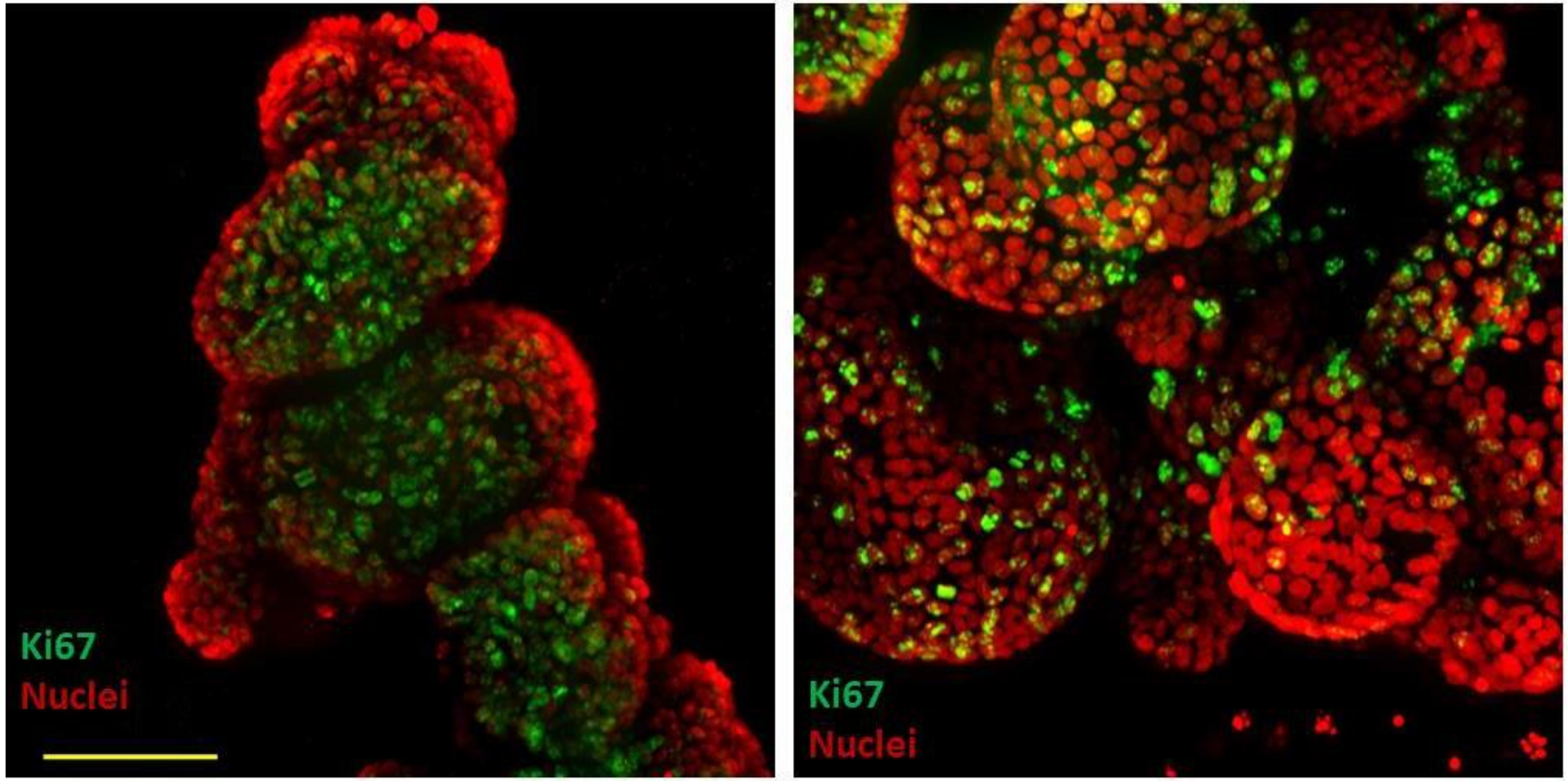

HuCo1044 (left) and HuCo21320 (right) were stained with a FITC-conjugated primary antibody against Ki-67 (green) to visualize proliferating cells. Nuclei were counterstained with SYTOX Deep Red Nucleic Acid Stain. Images were acquired on the CellInsight CX7 LZR Pro HCS Platform in confocal mode (scale bar = 100 µm).

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Antibodies used for immunofluorescence staining of tumoroids

Primary antibody*	Supplier	Cat. No.	Conc.	Secondary antibody Cat. No. (1:500 conc.)	Secondary antibody incubation time	Comment
Cytokeratin 20	Abcam	AB76126	1:100	A32731	4 hours	Added secondary antibody along with rhodamine phalloidin
Integrin β4	Abcam	AB110167	1:25	A48262	4 hours	NA
Integrin α6	BD Biosciences	555734	1:25	A48262	4 hours	NA
MUC5AC	Thermo Fisher Scientific	MA5-12178	1:50	SMS1AF647	Overnight	Nuclear counterstain added the next day for 4 hours
CEA	Abcam	AB133633	1:200	SRBAF488-1-100	Overnight	Phalloidin added the next day for 4 hours
E-cadherin	Thermo Fisher Scientific	MA5-14458	1:50	A32731	4 hours	Added secondary antibody along with rhodamine phalloidin
EpCAM	Thermo Fisher Scientific	14-9326-82	1:50	A32723	4 hours	Added secondary antibody along with rhodamine phalloidin
Ki67	Thermo Fisher Scientific	11-5698-82	1:100	NA, as primary antibody is FITC-conjugated		

* All primary antibodies were incubated overnight.

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Thank you

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