When referring to this protocol, please cite: Wang CC, Bajikar SS, Jamal L, Atkins KA, Janes KA. (2014) A time- and matrix-dependent TGFBR3–JUND–KRT5 regulatory circuit in single breast epithelial cells and basal-like premalignancies. *Nat Cell Biol*, 16, 345-56.

Before starting:
- Establish a 3D-compatible cell line that stably expresses a fluorescent reporter or fusion protein
- Obtain the appropriate phenol red-free culture medium for these cells to reduce background autofluorescence when imaging (media with bicarbonate-based buffers are discouraged unless the imaging setup has CO_2 control)
- Confirm that the laser-scanning confocal microscope is reserved for long-term (overnight) imaging on the day you plan to start; we typically reserve 3 pm to 9 am

I. **Seeding 3D overlay cultures in Matrigel**
1. Cut plastic coverslips (VWR #48376-049) to the size of the base of an 8-well chamber slide (BD Biosciences) and sterilize in 100% ethanol.
    - *Plastic coverslips provide solid support to remove the 3D cultures from the chamber slide before imaging*
    - *The coverslips must be smaller than the base, or else they will buckle and get stuck in the chamber slide; ideally, the coverslips are ~1 mm smaller than the base.*
    - *One 8-well chamber slide is required for one live-cell imaging experiments to account for variability in the quality of each imaging preparation*
2. Allow the ethanol to evaporate fully and then add the coverslips to the base of an 8-well chamber slide.
3. Coat the coverslipped chamber slides with Matrigel (Fisher #354230).
    - *Usually, 20–30 μl of matrigel is required to seal the border completely and then 45 μl of matrigel covers the top of plastic coverslip.*
4. Plate and culture cells according to established procedures for the desired number of days.

II. **Setting up sealed reservoir for live-cell imaging**
1. Transfer the conditioned medium from each chamber to a well on a 24-well plate.
2. Crack open the chamber slide and carefully move each plastic coverslip to its corresponding well (face up) on the 24-well plate.
    - *The 24-well plate acts as a staging area where the cultures can wait while individual imaging chambers are assembled*
3. Remove a plastic coverslip from the 24-well plate and flip it <u>face down</u> into a 6-cm culture dish with a fused glass coverslip (MatTek #P35G-1.5-14-C) that has been filled with conditioned medium from the same well of 3D culture.
    - *The goal is to get the inverted plastic coverslip as close to the fused glass coverslip as possible without distorting the 3D structures*
4. Sterilize 22 × 22 mm No. 1.5 coverslips (Fisher #12-541-B) in 100% ethanol. Allow the ethanol to dry, then apply a <u>thin</u> layer of high vacuum grease (Fisher #14-635-5D) around the margins of the glass coverslip.
    - *The vacuum grease is meant to seal the interface between the glass coverslip and the plastic of the 6-cm culture dish*
5. Place this glass coverslip on top of the inverted plastic coverslip to create an air-tight reservoir filled with conditioned medium and containing the inverted plastic coverslip with 3D structures.
6. Seal the borders of the glass coverslip with Valap and then overlay the sealed reservoir with light mineral oil (Fisher #0121-1) to prevent evaporation during imaging.
    - *Valap is solid at 37°C but liquid at 60°C; bring a heat block into the biosafety cabinet and use pipet tips with cut ends when handling Valap*
    - *See schematic on next page*

Top view   Side view
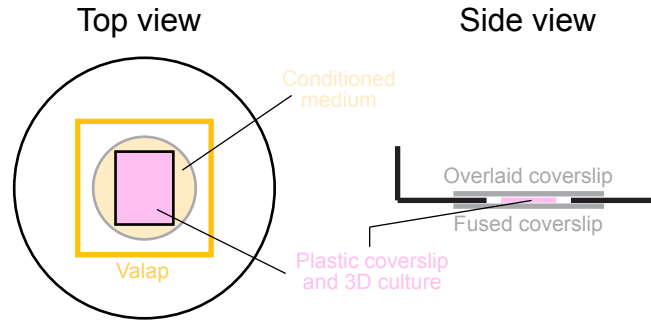
III. Time-lapse confocal imaging
1. Set up the confocal microscope for imaging.
    - *We use a Zeiss LSM 700 with an EC Plan-Neofluar 40x/1.30 oil-immersion objective and four diode lasers (5-10 mW) centered at 405, 488, 555 and 639 nm*
    - *Turn on in the following order: 1) extension cord that connected to all instruments, 2) X-cite light source, 3) incubator heater, 4) LSM 700 confocal laser scanning microscope, and 5) computer*
2. Equilibrate the 3D culture and stage at 37ºC for imaging.
3. Turn on the Zen 2009 software.
4. In the "Ocular" section, click "online" and use "brightfield on", "Red", "Green", or "Red" channel to find your target acini and focus. Then, click "offline".
5. In the "Acquisition" section, click "Smart-setup". In "Dye", choose fluorescent reporter proteins (Venus, EYFP, RFP, etc.). In "Proposal", choose "Best signal". For example, udsVenus was excited at 488 nm and its emission detected between 488 and 585 nm. RFP1 was excited at 555 nm and its emission detected above 582 nm.
6. In "Acquisition Mode", set "Averaging": Number 2, Bit Depth: 16 Bit. Set "Channels" to 1 Airy unit (29.0 for the 40x objective). Try to set laser powers at 2–5% to minimize photobleaching.
7. Click ""Auto Save" to store your data in your folder (e.g., D:\uva\Janes lab\)
8. Click "Time Series", set up the "cycles" and "interval" according to the desired imaging time. For example 65 cycles with 15 min interval for 16 hrs imaging. We typically acquire time-lapse images every 15 min for 15-20 hrs.
9. Click "Snap" to confirm the image parameter setting is good. Wait for 1 hr and click "Snap" again to confirm that target acini still at the same confocal plane on the microscope z-axis. Click "Start Experiment" to start the imaging.
10. After imaging, transfer data back to BME server.
11. Turn off "Auto Save"
12. Turn off in the following order: 1) computer, 2) LSM 700 confocal laser scanning microscope, 3) incubator heater, 4) X-cite light source, 5) extension cord that connected to all instruments.
13. Clean the objective and put on the microscope cover.

IV. Data collection and Analysis
1. Use Zen 2011 in MacPro (Room 2205, MR5)
2. In "Camera" section, choose "2D view".
3. Click "measure", "graphics", and then use "Draw Spline Contour" to draw the region of interest (ROI) (such as the whole cell, nuclear, or cytoplasmic subcompartments of a certain cell). The area and mean intensity of ROI will be measured and could be exported as Excel file format.

Reagent recipes

- **Valap** Store indefinitely at room temperature as 500 µl aliquots
  33% (w/w)   Vaseline (Fisher #19-086-291)
  33% (w/w)   Lanolin (Fisher #ICN15516683)
  33% (w/w)   Paraffin (Fisher #P31-500)