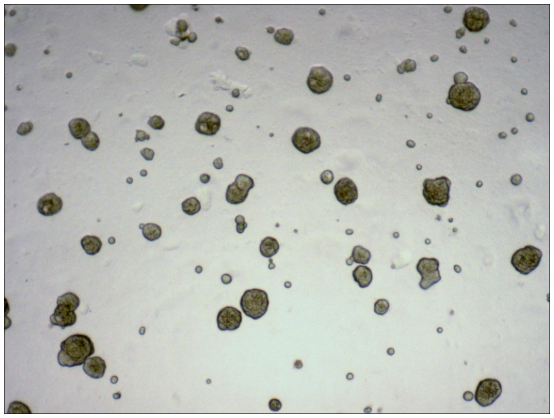
Imaging 3D cultures by brightfield microscopy Entered by Kevin Janes
Janes Lab Protocols 8/7/13

Notes before starting:

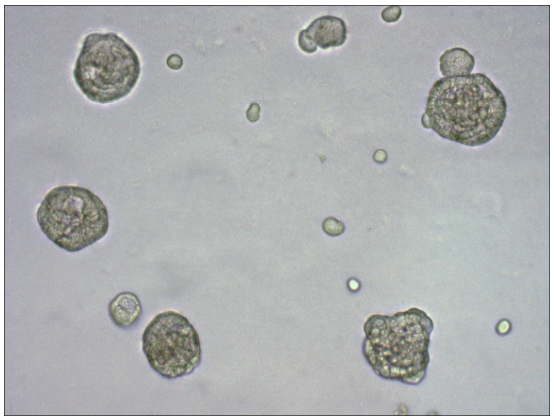
- These instructions are detailed for an Olympus CKX41 inverted brightfield microscope with a Q Color digital camera, but the principles should generalize to any inverted microscope and brightfield camera

1. Set up an eight-well chamberslide for 3D culture and allow to grow for the desired number of days.
   - *For any cell context or condition, allow for at-least four wells to generate one publication-quality field*
2. Select the desired objective for brightfield imaging.
   - *4× typically captures a large field of several dozen acini, whereas 10× captures a higher resolution view of 3–5 acini*
   - *High-quality imaging with a 20× objective is not possible because of excessive spherical aberration at this magnification*
3. Add the stage adaptor designed for holding microscope slides.
   - *Such an adaptor is important for getting the acini identically oriented when imaging over several days*
4. Remove the phase ring from the optical path.
   - *Phase contrast microscopy picks up too many irregularities in the Matrigel to generate high-quality images, and often the acinar lumena are too phase dense to visualize anything useful*
5. Increase the aperture stop so that there is sufficient contrast to view individual cells within each acinus
   - *The aperture stop specifies the angle of the cone of light that illuminates the specimen, providing depth of field to the image*
   - *On the CKX41, the "sweet spot" of the dial is halfway between the "AS" label and a fully closed aperture*
6. Plug in the Firewire cable to the Q Color camera and start up the Q Imaging software on the Mac Mini.
7. Remove the chamberslide from the 37ºC incubator and place it on the stage adaptor.
   - *Our stage adaptor does not hold the chamberslide snugly, so be sure to use a consistent orientation of the chamberslide within the adaptor (e.g., always rotated as far clockwise as possible, always aligned to be flush to the bottom of the adaptor, etc.)*
   - *Consistent orientation is important to generate reproducible quality images of the same acini over several days.*
8. Search the wells of interest to identify the flattest fields of view within each condition.
   - *Flat fields of view will have all the acini in the same focal plane, leading to the clearest and most visually-appealing images*
   - *Usually, the flattest fields are in the middle of each well on the top of the "dome" of Matrigel that was originally coated*
9. Select the most representative flat field and focus the specimen on the Q Color camera while it is acquiring live.
   - *The camera is not perfectly parfocal with the eyepiece, and so it is important to obtain the optimal focus on the digital image (i.e., the screen image)*
   - *To match the focus of a flat field from day to day, note the slight white or dark rings that form around acini that are slighly off the focal plane and attempt to mimic the same ring pattern from day to day (it is helpful to have the earlier images open for comparison when aquiring a new image)*
10. Set the proper exposure by altering the strength of the halogen source, the exposure time, and the white balance
    - *The strength of the halogen source and the exposure time are correlated—auto exposure often does not get the right level of grayscale, so it is easier to increase the halogen source than guess an exposure time*
    - *White balancing depends on the level of exposure of the image that is white balanced: dimmer exposures will look more blue after white balancing, whereas brighter exposure will look more yellow after white balancing*
    - *Auto exposure and white balancing can be done on the entire image or on a region of interest within the image (simply drag a box around the region where the correction is to be performed*

- *The ideal image has a uniform, light gray background:*



Example of a good exposure on a 4× objective (note the slightly yellowish tint from brighter illumination in the center, which was the best compromise for this field)



Example of a good exposure on a 10× objective (note the white ring around the upper-left acinus, which can be used for consistent focusing)

11. The RGB images can be converted to grayscale in Photoshop and resized as needed (be sure to back up the raw data on the server).