

**When referring to this protocol, please cite:** Wells CJ, Labban N, Showalter SL, Przanowska RK, Janes KA. (2026) Fast learning-free organoid quantification and tracking with OrganoSeg2. *Sci Rep*, 16, 7928.

1. Initiate organoid cultures with as many replicate wells for each dose of radiation as will be tested.
  - *For organoid setup, see “Initiation of primary luminal breast tumor organoids from tumor scrapes.”*
  - *Use stripwell plates (Corning 9102) and arrange replicates in their own column, so that all replicates belonging to each experimental condition can be removed from the plate during irradiation.*
2. On culture Day 2, image the cultures with the diffusion condenser slider using brightfield, GFP, and BFP channels, and refeed.
  - *Make sure that the diffusion condenser slider is in for flat-field illumination at 2x or 4x magnification*
  - *Recommended settings for fluorescence (which will be dark at Day 2):*
    - *GFP: 0.2–0.3 Power, 0.15–0.25 Exposure, 5–8 Gain*
    - *BFP: 0.2 Power, 0.15 Exposure, 5 Gain*
3. On radiation Day 1, refeed cultures. For at least one replicate per experimental condition, add NucView 488 in PBS (Sigma SCT100) at a dilution of 1:1000 (i.e., 0.1 µl for 100 µl of growth medium).
  - *Radiation Day 1 could be culture Day 4, 5, or 6 depending on the day of surgery and organoid initiation.*
  - *Make all the media + NucView stain in one batch, mix, then add to each well.*
4. Incubate the cultures for 15 minutes at 37°C.
5. Image the cultures for brightfield, GFP, and BFP exactly as in Step 2.
6. Remove each stripwell of the plate and place it in its own stripwell rack. Place all stripwell racks into a covered box (with biohazard labels) for transport to the CellRad+ cell irradiator (currently housed in the Abbas lab in the West Complex, Room 7141).
  - *If radiating multiple patient cases, consolidate strips on stripwell racks based on shared radiation dose, but be sure to label each strip carefully.*
7. If the irradiator is not already turned on, follow the written directions kept near the instrument to turn it on and warm it up.
  - *If a reservation has been made ahead of time on the shared Abbas lab calendar, typically someone in the Abbas lab will turn on the instrument before the reserved time slot.*
  - *Note that the instrument can take between 20 and 60 minutes (depending on how long it has been turned off) to warm up, so it is best to turn it on in advance if an Abbas lab member will not be able to do so (e.g. on weekends).*
8. Using the “auto-dose control” function of the instrument, assign the desired radiation dose and irradiate one plate at a time. Turn off the instrument if no one else is signed up to use it for the rest of the day by following the written directions kept near the instrument.
  - *We typically deliver 1 Gy and/or 6 Gy but have delivered up to 12.5 Gy.*
  - *Make sure the shelf position is set to #5 for a microplate, the plate is within the #5 circle, and the turntable is on to ensure uniform dosing*
9. Transport the samples back to the lab and reorder stripwells if desired.
10. Repeat Steps 5–9 on five consecutive days. On radiation Day 3, supplement the cultures with 40 µl of medium (do not replace the existing medium, simply add fresh medium to the wells).
11. After radiation Day 5, refeed the cultures with growth medium ± NucView in PBS (diluted 1:1000 as before) according to what each well had received on radiation Day 1.
12. Two or three days after radiation Day 5, image the cultures for brightfield, GFP, and BFP exactly as in Step 2 and supplement with 40 µl of media exactly as in Step 10.
  - *This time point could be culture Day 11, 12, or 13 depending on the day of surgery and organoid initiation.*
13. At the endpoint on culture Day 14, image the cultures for brightfield, GFP, and BFP exactly as in Step 2 then add 0.1 µg/ml DAPI in PBS with NucView 488 (DMSO) at 1:400.
  - *The terminal NucView 488 was not in the published protocol but has been added to ensure the highest sensitivity quantification of apoptotic cells at the endpoint by adding fresh dye at a higher concentration*

## **Organoid irradiation and cell death staining**

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14. Incubate the cultures for 1 hour at 4°C then image the cultures again for brightfield, GFP, and BFP exactly as in Step 2.
15. At this point, additional endpoint measurements (such as RNA extraction) can be performed if desired.