Hybridization (overnight, @ 37 ℃)
SAFETY: Hybridization buffer contains formalin, if spilled clean up and soak area in water, formalin extremely volatile and nasty, do not inhale
Cut ⅛ of filter piece and place on slide according to which sample it is (3 filter pieces can go on a slide)

Slides are labeled with shapes and colors to help identify like so

Apply hybridization buffer and probe (9:1)

We multiply by two to get 18 µL buffer and 2 µL probe

Probe lives in small white box in the fridge labeled "ALV01", if you run out more aliquots

Can be found in -80 near atria. (Let Taylor or I know if there are less than 6 vials because we need to order it from Germany)

Buffer lives in -20 in lab on a shelf inside the door labeled "Hybridization Buffer"

If doing 6 samples: make 1 extra 18:2 buffer and probe
If doing 12 samples: make 2 extra 18:2 buffer and probe
Mix buffer and probe in a 1.5 mL Eppendorf tube
Apply 20 µL to filter pieces along the "inner part of the pizza" - very viscous
Use the edge of a pipette to lightly spread the mix around to break the surface tension

Making hybridization chambers
Fold Kimwipe in fourths "hot dog" way and slide into 50 mL Falcon tube labeled on caps with "C"
Add slide with probe on top **make sure slide is horizontal at all times**
Add 1 mL of buffer in gap between slide and Kimwipe saturating the wipe

Place all the hybridization chambers in the incubation oven horizontally

To prevent from rolling around place a tray to keep tubes from rolling around

Wash buffer steps (2x @ 3 mL in wells @ 46 ℃)
Mix Wash Buffer according to number of samples ***see Reagents for CARD-FISH***
Add 3mL per well
Gently remove the filter pieces handling on the crust of the filter and place into corresponding well
Place the saturated Kimwipe, slide, and uncapped hybridization chambers on Kimwipes in hood (formalin needs to evaporate and cannot be disposed of in trash until evaporated)
Place the wells with filters into the oven at 46 ℃ for 30 minutes
Transfer filters from first wash into second wash and replace in oven at same temp and time duration

TNT Buffer Wash (performed in wells for 15 minutes in dark at room temperature)
Add 3 mL of TNT Buffer into each well (check to make sure TNT Buffer is not cloudy or has precipitate, sign of bacterial growth)

Transfer filters to corresponding well and incubate for 15 minutes in the dark at room temperature

TSA and Fluoroscene (incubate in dark at room temp for 30 minutes)
Remove filters from TNT Wash and place on a slides (3 to a slide) in the previous order
    Make sure the side of the filter with the sample is facing up!!
TSA and Fluoroscene lives in box in lab fridge
Add TSA and Fluoroscine system & amplification reagent in a 1.5 mL Eppendorf tube
    For 6 samples: 2.5 µL system & 125 µL amplification reagent
    For 12 samples: 5 µL system and 125 µL amplification reagent
Add 20 µL per filter on slide in same arrangement as probe application and incubate in dark at room temp for 30 minutes

TSA and Fluoroscine system comes as a solid and needs to dissolved in DMSO first, tell Taylor if running low and she will dissolve another vial. Keep an eye on reagent amounts so we can reorder

TNT Wash (2x at 55 ℃)
Add 3 mL of TNT Buffer to each well
Place filters from TSA slides into the wells
Incubate for 20 minutes at 55 ℃, repeat once in different wells with same amount of TNT Buffer

Calcafuor *stains cellulose in cells blue*
Remove the filters from the TNT wash and place on a slide (3 to a slide) with the sample side facing up
Add 20 µL of Calcafuor to each filter piece
    Calcafuor lives in a centrifuge tube in a beaker in a shelf in the lab fridge
Incubate in room temperature in the dark for 7 minutes

Millique Rinse (2x @ room temp for 1 minute each)
Fill wells with 3 mL Millique water
Remove the filters from the slides and place into wells, let sit at room temp for a minute
Transfer to next wash, wait for a minute

Citifuor/PI *Citifluor is a mounting reagent and PI (propidium iodide) stains the nucleus of the cell*
Remove filter pieces from the Millique rinse and place on final mounting slides with sample side up (**only 2 filter pieces to a slide)**
Add 20 µL of Mounting Reagent (**refer to Reagents for CARD-FISH**) to each filter piece
Place coverslip over the filter making sure there is no air bubbles
Place mounted slides horizontally on a flat surface (well plates work well) in the lab fridge

Labeling slides

Label slides with corresponding shape and number, “Salt Pond”, date of sample, cast and depth,filter pore size, volume filtered