**PFA Cell Fixation with the BlueWasher**

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**Reagents Preparation**

Instruments:

1. Break-Safe ampule opener.

Reagents:

1. 32% PFA in 10 ml amber vials on HiTIF Shelf (Room D304)
2. DAPI stock at 2.5mg/ml (1,000X) in HiTIF –200C (Room XYZ)

Important Safety Considerations:

* **PFA is extremely toxic and a carcinogen. All steps below must be performed inside the chemical fume hood.**
* Wear gloves and PPE.
* Keep chemical fume hood sash below the green validation sticker found on the RIGHT side of the sash rail.
* Break 32% PFA ampules with the Break-Safe ampule opener. Discard ampule remains into the sharps collection bottle in the Chemical fume hood.
* Discard all plasticware that came in contact with PFA into the round black chemical waste container.
* Discard any remaining PFA liquid into the PFA waste bottle. Alternatively, use the Prime single line to dispense all PFA into waste carboy via the BlueWasher liquid discard port.

Procedure:

1. Calculate the necessary volume of 8% PFA in PBS to prepare: minimum 40 ml for a single (first) 384-well plate, then 20 ml for each additional 384-well plate. 20 ml are needed for the initial priming of the tubing, 20 ml are needed for each 384 well plate to be fixed (assuming the addition of 40ul 8% PFA to 40 ul of medium + cells for each well).
2. To prepare PFA 8% in PBS add 10 ml 32% PFA and 30 ml 1X PBS for 40 ml of 8% PFA for a single 384 well plate. Scale up the volume of PFA 8% to be prepared according to the calculations above.
3. Calculate the necessary volume of DAPI solution (2.5 ug/ml in PBS) to prepare. minimum 40 ml for a single (first) 384-well plate, then 20 ml for each additional 384-well plate. 20 ml are needed for the initial priming of the tubing, 20 ml are needed for each 384 well plate to be fixed (assuming the addition of 50 ul DAPI to empty plate for each well).
4. Add 10 ul of DAPI stock to every 10 ml of 1X PBS. Scale up the volume of DAPI solution to be prepared according to the calculations above. DAPI is light sensitive. Keep protected from light by wrapping the solution container with aluminum foil.

**Bluewasher Setup**

Instruments:

1. Bluewasher plate washer/dispenser (Chemical fume hood Room D304)

Reagents:

1. 8% PFA solution

Important: The Bluewasher uses centrifugation to evacuate liquid from your plate. Therefore, you MUST have the correct balance plate in the Bluewasher, and you must place the dummy working plate back onto the tray when you finish with the QuickClean program to clean the Bluewasher.

Procedure:

1. Initialize the Bluewasher
2. Have the BlueWasher present the Balance Plate to ensure that it matches the plate(s) you will be processing.
3. Have the BlueWasher present the dummy Working Plate. This plate needs to be present whenever the BlueWasher is primed and/or cleaned.
4. Check that the liquid volumes in the bottles connected to the **GREEN** line tubing (PBS 1X) and the **BLUE** line tubing (ddH2O) are sufficient to perform the experiment (I.e., at least 50% full of liquid for each bottle)
5. Place the **WHITE** line tubing into the 8% PFA solution. Ensure that the end of the tubing is at the bottom of the 8% PFA solution container.
6. Locate the protocol named in `PFAFIX:1\_Prime\_Water\_PBS\_PFA` in the protocol curtain menu.
7. Select the protocol and then click on the GREEN arrow to start it. This protocol will prime the Water (Blue), PBS (Green) and PFA 8% (White) lines, in this order.

**Fixation**

Procedure:

1. Important: Swap out the dummy **WORKING** plate on the BlueWasher with your plate containing cells and media.
2. Important: remove the lid from the plate.
3. Ensure that the carrier tray is attached to the magnetic ram.
4. Locate the protocol named in `PFAFIX:2\_Add40ul\_PFA` in the protocol curtain menu.
5. Select the protocol and then click on the GREEN arrow to start it. This protocol will add 40 ul of PFA 8% per well from the white line to the plate containing cells and media. The final concentration of PFA in the plate will be 4%.
6. Re-lid the plate.
7. Remove the lidded plate from the Bluewasher tray. Leave the removed plate inside the chemical fume hood.
8. Repeat steps 1 – 8 for other plates, if necessary.
9. Incubate the plate at RT for 15 minutes.
10. During the incubation step, remove the tubing from the PFA bottle and place the tubing into a small bottle containing water. There are two white gallon jugs labeled “White Line water”. Use these to re-fill your bottle of water.
11. Place the dummy **WORKING** plate on the BlueWasher tray.
12. Locate the protocol named in `PFAFIX:3\_Rinse\_PFA\_Line` in the protocol curtain menu.
13. Select the protocol and then click on the GREEN arrow to start it. This protocol will flush out the PFA from the white line tubing.

**PBS 1X Washes and DAPI Staining**

1. Once the PFA incubation step is finished, swap the dummy **WORKING** plate with your plate on the BlueWasher tray.
2. Important: remove the lid from your plate.
3. Ensure that the carrier tray is attached to the magnetic ram.
4. Locate the protocol named in `PFAFIX:4\_Wash\_3X\_PBS` in the protocol curtain menu.
5. Select the protocol and then click on the GREEN arrow to start it. This protocol will wash the plate three times with PBS 1X and leave the plate empty at the end.
6. Re-lid the plate.
7. Remove the plate from the BlueWasher tray when complete.
8. Repeat steps 1 – 2 for other plates, if necessary.
9. Place the White Line tubing into the DAPI solution tube.
10. Locate the protocol named in `PFAFIX:5\_Prime\_DAPI` in the protocol curtain menu.
11. Select the protocol and then click on the GREEN arrow to start it. This protocol will prime the white line with DAPI solution.
12. Place an empty plate on the BlueWasher tray.
13. Important: remove the lid from your plate.
14. Locate the protocol named in `PFAFIX:6\_ADD\_DAPI` in the protocol curtain menu.
15. Select the protocol and then click on the GREEN arrow to start it. This protocol will add 50 ul with DAPI in PBS to the empty plate.
16. Re-lid the plate.
17. Remove the plate from the BlueWasher tray when complete.
18. Repeat steps 12 – 17 for other plates, if present.
19. Place the dummy **WORKING** plate on the Bluewasher tray.

**Clean Up**

Reagents:

Alufoil (Where you can find it)

Procedure:

1. Remove the white line tubing from the DAPI tube and place it in the small water bottle.
2. Locate the protocol named in ` PFAFIX:7\_Rinse\_All\_Lines` in the protocol curtain menu.
3. Select the protocol and then click on the GREEN arrow to start it.
4. Allow program to fin.
5. Start the Quick Clean protocol.
6. Run through all the prompts and let the Quick Clean protocol run.
7. Seal the plate wells with AluFoil.
8. Store the plate at 4oC until ready for imaging.