**Cell Painting protocol v3:**

1. Seed U2OS cells into 384-well plate in growth media (40µL cell suspension/well) at a density of 1000 cells/ well.
2. Incubate for 60 min at RT in HTS stacks followed by incubation for ~20h at 37°C, 5 % CO2 and 95% rH to allow overnight recovery and growth of plated cells.
3. On the following day add 80nL test compounds (stock 5mM, to achieve 10uM assay concentration), DMSO into assay plates, then incubate the plates at 37°C, 5% CO2 and ~95% rH for 48 hours.
4. Do NOT remove media! Add 20µl of 3 fold diluted MitoTracker staining solution and incubate 30 minutes at 37C, 5% CO2, and 95% rH
5. Dispense fixation solution into all wells to achieve final concentration of 4% PFA.
6. Incubate for 20 minutes at RT.
7. Remove supernatant and wash 2x with 1X HBSS, 60µL/well
8. Permeabilization/staining of cells with 20µL of Phalloidin, Concanavalin A, Hoechst, WGA and SYTO 14, containing 0.1% TritonX-100 and 1% BSA for 30 min at RT
9. Discard solution and wash 2x with 1X HBSS, 60µL/well
10. Fill the wells with 60µL of 1X HBSS/ 0.05% Sodium azide
11. Seal plates tightly with adhesive seals

**Final dye concentrations in each well:**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Dye (stock concentration)** | **Phalloidin568 (6.6µM)** | **ConcanA (2mg/ml)** | **Hoechst (1mg/ml)** | **SYTO14 (5mM)** | **WGA 555 (0.15mg/ml)** | **MitoTrackerRed (1mM)** |
| Final concentration in well | 8.25nM | 0.005mg/mL | 1µg/mL | 6µM | 1.5µg/mL | 0.5µM |