**Cell painting protocol:**

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 40nL test compounds, 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 |