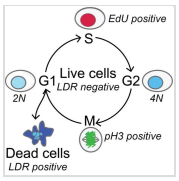

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Deep Dye Drop Protocol

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ABSTRACT

High-throughput measurement of cells perturbed using libraries of small molecules, gene knockouts, or different microenvironmental factors is a key step in functional genomics and pre-clinical drug discovery. However, it remains difficult to perform accurate single-cell assays in 384-well plates, limiting many studies to well-average measurements (e.g. CellTiter-Glo®). Here, a public domain “Dye Drop” method that uses sequential density displacement and microscopy to perform cell count and viability assays is described. Cell viability and DNA replication assays are followed by immunofluorescence imaging to collect single-cell dose-response data in the "Deep Dye Drop" version of the protocol. The resultant data can be used to calculate growth rate inhibition (GR) values and metrics. Dye Drop is rapid, reproducible, customizable, and compatible with manual or automated laboratory equipment. Dye Drop improves the tradeoff between data content and cost, enabling the collection of information-rich perturbagen-response datasets.

PROTOCOL CITATION

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High-throughput microscopy, live-cell assays, dose response, small molecule perturbation, viability, cell cycle, GR metrics

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MATERIALS TEXT

MATERIALS

Optiprep (Iodixanol) **Sigma Aldrich Catalog #D1556-250ML**

Odyssey® Blocking Buffer (PBS) **LI-COR Catalog #927-40000 927-40100**

Microseal® 'F' Foil **BioRad Sciences Catalog #MSF-1001**

Hoechst 33342 **Contributed by users Catalog #H3570**

Copper (II) sulfate pentahydrate **Sigma – Aldrich Catalog #209198**

Triton X-100 **Sigma Aldrich Catalog #X100**

LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation **Thermo Fisher Catalog #L34974**

1X PBS **VWR Scientific Catalog #75800-986**

Formaldehyde solution 37% **Sigma Aldrich Catalog #F1635-500ML**

EdU **Lumiprobe Catalog #10540**

Sulfo-Cy3 azide **Lumiprobe Catalog #B1330**

Phospho-Histone H3 Alexa 488 **Cell Signaling Technology Catalog #3465S**

Ascorbic acid **Sigma Catalog #A4544**

Stock solutions

- LIVE/DEAD Red- prepare according to directions (add **50 µL** DMSO per tube), store at **-20 °C** , limit

freeze/thaws

- EdU- prepare [M]**10 millimolar (mM)** working solution in DMSO, store at **-20 °C**

  25 mg EdU

  10 mL DMSO

- Sulfo-cy3 azide- prepare [M]**4 Molarity (m)** working solution in DMSO, store at **-20 °C**

  3 mg sulfo-cy3-azide

  1 mL DMSO

- Ascorbic acid- prepare **200 mg/ml** working solution in water ****fresh each time!****

- $CuSO_4.5H_20$- prepare [M]**200 millimolar (mM)** in water, store at room temperature, protected from light

  250 mg $CuSO_4S$

  5 mL water

- Triton X-100- prepare 10% working solution in 1X PBS

  10 mL Triton X-100

  90 mL PBS

1 Pulse cells with EdU+ stain dead cells with LDR

For 10 ml:

1 mL optiprep (10% final)

9 mL PBS

5 µL LDR (1:2000 final)

10 µL EdU (10 µM final)

384-well plate: Add **15 µL** per well along the edge of the wells using a multi-channel pipette and incubate for **01:00:00** (or desired pulse duration) @ **37 °C**

96-well Plate: add **60 µL** per well.

1.1

This file can be used for all solution calculations:

DDD.xlsx

2 Fix cells

For 10 ml:

2 mL Optiprep (20% final)

6.9 mL PBS

1.1 mL formaldehyde (4% final)

384-well plate: Add **20 µL** per well along the edge of the wells using a multi-channel pipette and incubate for **00:30:00** @ **Room temperature** in the dark (cover with foil)

96-well Plate: add **80 µL** per well.

2.1 Run DeepDyeDrop1 protocol on the plate washer (aspirate all but **10 µL** , dilute with **80 µL** PBS, stop, store @ **4 °C** . Aspirate all but **10 µL** if continuing with permeabilization right away).

3 Permeabilize:

For 10 ml:

1 mL Optiprep (10% final)

8.5 mL PBS

500 µL 10% Triton X-100 (0.5 % final)

384-well plate: Add **15 µL** per well along the edge of the wells using a multi-channel pipette and incubate for **00:20:00** @ **Room temperature** in the dark.

96-well plate: add **60 µL** per well.

4 Click Reaction:

For 10 ml:

Combine ***in order***

7 mL PBS

2 mL optiprep (20% final)

100 µL 200mM CuSO4 (2mMfinal)

10 µL 4mM sulfo-cy3-azide (4µM final)

1 mL 200 mg/ml ascorbic acid (20 mg/ml final)

384-well plate: Add **20 µL** per well along the edge of the wells using a multi-channel pipette and incubate for **00:30:00** @ **Room temperature** in the dark.

96-well Plate: add **80 µL** per well.

4.1 Run DeepDyeDrop1 protocol on the plate washer (aspirate all but **10 µL** , dilute with **80 µL** PBS).

Important note: Stopping point if not performing immunofluorescence staining. Include **2 µL** Hoechst 33342 (1:5000 final) in the click reaction mix

5 Immunofluorescence:

384-well plate: Add **40 µL** Odyssey blocking buffer per well and incubate @ **Room temperature** for **01:00:00** in the dark on a plate rocker at the slowest setting.

96-well plate: Add **160 µL** per well.

5.1 Run DeepDyeDrop2 protocol on the plate washer (aspirate all but **10 µL**)

Antibodies:

5.2

For 10 ml:

10 mL odyssey blocking buffer

2 µL Hoechst 33342 (1:5000 final)

5 µL pH3-A488 antibody (1:2000 final)

384-well plate: Add **15 µL** antibody solution per well, incubate **Overnight** @ **4 °C** in the dark, on a plate rocker at the slowest setting.
96-well plate: add **60 µL** per well.

5.3 Run DeepDyeDrop3 protocol on the plate washer (wash once with PBST, twice with PBS, leave **80 µL** PBS per well). Seal plates with Microseal 'F' foil seal and store @ **4 °C** until imaging.

6 Image Acquisition:

On the Operetta:

Hoechst (ex 360-400 em 410-480)
EdU-cy3 (ex 520-550 em 560-630)
pH3-A488 (ex 460-490 em 500-550)
LDR-A647 (ex 620-640 em 650-700)

On the IXM-C:

DAPI
FITC
TRITC
Cy5

Important note: Focus height will vary with plate type and cell line, but expect to image the pH3 higher than the others since cells ball up during mitosis

7 Image Analysis

1. Apply flat field correction if available
2. Segment nuclei based on Hoechst signal
3. Define a ring around the nuclei, be sure this does not include any of the nuclear area (it will be used to subtract the local background from each channel)
4. calculate the **nuclear area**
5. for each channel, calculate the average intensities within the nuclear mask, and within the surrounding ring
6. **for each channel, subtract the ring intensity from the nuclear intensity**
7. multiply the nuclear area by the Hoechst intensity for the **DNA content**
8. calculate **SER spot pixel size 8 texture feature** for the LDR signal within the nuclear area
9. output features (nuclear area,DNA content, Hoechst, EdU, pH3, LDR background corrected intensities, LDR texture feature)in a .tsv file
10. identify cells in mitosis through pH3 intensity
11. identify dead cells though LDR intensity and/or texture- threshold independent of cell line
12. identify additional dead cells through size (small) and intensity (high)- optimize per cell line
13. identify population of cells in each phase of the cell cycle based on DNA content and EdU intensity
14. identify additional dead cells based on size (small) and intensity (high)

8 Data Analysis