**FACS Propidium Iodide (PI) – cell cycle analysis**

1. Collect cells (1-2 mln cells) on ice
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Wash cells in 5 ml ice-cold PBS and resuspend
5. Spin down for 5 min, 150g @ 4⁰C
6. Remove supernatant
7. Add 5 ml 70% ice cold ethanol dropwise while vortexing
8. Cells can now be stored at 4⁰C for weeks
9. Spin down for 5 min, 150g @ 4⁰C
10. Remove supernatant
11. Wash cells in 5 ml PBS
12. Spin down for 5 min, 150g @ 4⁰C
13. Remove supernatant
14. Add 1 ml of PI staining solution for each sample and transfer to FACS tube
15. Incubate 30 min at room temperature. **Keep in the dark!**
16. Spin down for 5 min, 150g @ 4⁰C
17. Remove supernatant
18. Add 200 µl PBS 0.5% FCS
19. Transfer sample to the flow cytometer and measure cell fluorescence. Maximum excitation of PI bound to DNA is at 536 nm, and emission is at 617 nm. Blue (488 nm) or green light lines of lasers are optimal for excitation of PI fluorescence. Count 50.000 events

Preparation PI staining solution

Dissolve PI 100 mg in 100 ml MQ

Stock = 1 mg/ml

PI staining solution is:

1 µg/ml PI

100 µg/ml RNAse A

0.1% TritonX-100 in PBS

10 mL:

1 mg RNAse A (drawer Rianne)

10 ml PBS

10 µL stock PI

10 µL of Triton-X100

**FACS gamma-H2AX-FITC – ds breaks (DNA damage)**

1. Collect cells (1-2 mln cells) on ice
2. Spin down at 150 *x g* for 5 min at 4⁰C
3. Remove medium and resuspend cells in 5 mL of ice cold of PBS
4. Spin down at 150 *x g* for 5 min at 4⁰C
5. Remove PBS and resuspend cells in 1 mL of ice cold 1% PFA (hood!)
6. Incubate for 15 min on ICE
7. Spin down at 150 *x g* for 5 min at 4⁰C
8. Remove PFA (hood!)
9. Wash 1x with ice cold PBS
10. Spin down at 150 *x g* for 5 min at 4⁰C
11. Remove PBS (hood!)
12. Add 5 ml 70% ice cold ethanol dropwise while vortexing
13. Cells can now be stored at -20⁰C for weeks (at least 2 hours)
14. Spin down at 150 *x g* for 5 min at 4⁰C
15. Remove supernatant
16. Resuspend cell pellet in 2 mL of BSA-T-PBS
	1. BSA 1%, Triton X100 0.2% - store weeks at 4°C
17. Spin down at 150 *x g* for 5 min at 4⁰C
18. Remove supernatant
19. Resuspend cell pellet in 2 mL of BSA-T-PBS
20. Keep on RT for 5 min
21. Spin down at 150 *x g* for 5 min at 4⁰C
22. Remove supernatant
23. Resuspend cell pellet in 100 µL of BSA-T-PBS + 1-2 µg mouse gamma-H2AX-FITC
	1. Control: mouse IgG-FITC
24. Incubate at 4°C overnight
25. Add 2 mL of BSA-T-PBS incubate for 2 min
26. Spin down for 5 min, 150g @ 4⁰C
27. Remove supernatant
28. Add 2 mL of BSA-T-PBS incubate for 2 min and transfer to FACS tube
29. Spin down for 5 min, 150g @ 4⁰C
30. Remove supernatant
31. Add 200 µl PBS 0.5% FCS

FITC

Ex: 496 nm

Em: 518 nm

**FACS Edu-Pacific Blue + PI – s-phase (dividing fraction)/cell cycle**

* Allow vials to warm up at RT
* Prepare 1% BSA in PBS (pH = 7.1-7.4)
* Prepare 10 mM solution of Edu
	+ Add 4 mL of DMSO to component A (mix well)
	+ Store at -20°C
* Prepare 500 mL of 1x Click-iT saponin based permeabilization and wash reagent
	+ Add 50 mL of component E (hood!) to 450 mL of 1% BSA in PBS (1X)
	+ Smaller amounts: dilute component E 1:10 with 1% BSA in PBS (10X)
	+ Store at 4°C (1X = 6 months, 10X = 12 months)
* Prepare 10X stock Click-iT EdU buffer additive (component G)
	+ Add 2 mL of MQ to the vial
	+ Mix well
	+ Store at -20°C

Label cells with EdU

1. Seed cells (1-2 mln cells) in culture dishes
2. Add 10 µM of EdU to culture medium
3. Incubate for 1 hour at 37°C

Fix and permeabilize

1. Collect cells in 15 mL tube
2. Spin down at 150 *x g* for 5 min at RT
3. Remove medium
4. Wash 1x with 3 mL of 1% BSA in PBS
5. Spin down at 150 *x g* for 5 min at RT
6. Remove PBS
7. Add 100µL of Click-iT fixative (component D), mix well
8. Incubate for 15 min at RT in DARK
9. Wash 1x with 3 mL of 1% BSA in PBS
10. Spin down at 150 *x g* for 5 min at RT
11. Remove supernatant
12. Resuspend cells in 100 µL of 1x Click-iT saponin-based permeabilization and wash reagent and mix well
13. Incubate for 15 min at RT

Click-iT reaction

1. Prepare 1x Click-iT Edu buffer additive
	1. Dilute 10X in MQ
2. Prepare Click-iT Plus reaction cocktail:

|  |  |
| --- | --- |
| components | # of reactions (µL) |
| 1 | 2 | 5 | 10 | 15 | 30 | 50 |
| PBS  | 438 | 875 | 2190 | 4380 | 6570 | 13200 | 21900 |
| Copper protectant (F) | 10 | 20 | 50 | 100 | 150 | 300 | 500 |
| Fluorescent dye picolyl azide  | 2.5 | 5 | 12.5 | 25 | 37.5 | 75 | 125 |
| Reaction buffer additive  | 50 | 100 | 250 | 500 | 750 | 1500 | 2500 |
| Total volume  | 500 | 1000 | 2500 | 5000 | 7500 | 15000 | 25000 |

1. Add 500 µL of Click-iT Plus reaction cocktail to each tube and mix well
2. Incubate for 30 min at RT (in DARK!)
3. Wash 1x with 500 µL of 1x Click-iT saponin based permeabilization and wash reagent
4. Spin down at 150 *x g* for 5 min at RT
5. Remove supernatant
6. Add ribonuclease A (RNase A) + DNA stain to tube – transfer to FACS tube
7. Incubate for 30 min at RT (in DARK!)
8. Spin down for 5 min, 150 *x g* at RT
9. Remove supernatant
10. Add 200 µl PBS 0.5% FCS

Pacific Blue

Ex: 405 nm

Em: 450 nm

**FACS Cell ROX FAR RED/Dihygrorhodamine 123 (DHR) – ROS levels**

1. Collect (1-2 mln cells) in 15 mL tube
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Wash 1x with PBS (1 mL) – transfer to Eppendorf
5. Spin down for 5 min, 150g @ 4⁰C
6. Remove supernatant
7. Incubate with H2O2 range (serum free medium) for 15 min at 37°C
	1. stock = 26 µmol/µL
	2. 0-12.5-25-50-100mM H2O2
	3. 100 mM = 4 µL in 1 mL

Cell ROX

1. Wash 1x with PBS
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Add 200 µL of Cell ROX (500X diluted in serum free medium) – final: 5 µM
5. Incubate for 30 min at 37°C in DARK
6. Wash 3x with PBS and transfer to FACS tube
7. Spin down for 5 min, 150g @ 4⁰C
8. Remove supernatant
9. Add 200 µl PBS 0.5% FCS

Cell ROX Far RED

Ex: 644 nm

Em: 665 nm

* Use APC (633, 660)

DHR 123

1. Wash 1x with PBS
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Add 200 µL of DHR 123 (500X diluted in serum free medium) – final: 20 µM
5. Incubate for 30 min at 37°C in DARK
6. Wash 1x with PBS and transfer to FACS tube
7. Spin down for 5 min, 150g @ 4⁰C
8. Remove supernatant
9. Add 200 µl PBS 0.5% FCS

Dihygrorhodamine (DHR) 123

Ex: 508 nm

Em: 529 nm

* Use Viobright FITC (494, 522)

**FACS Cell Mitotracker DEEP RED – # mitochondria**

1. Collect (1-2 mln cells) in 15 mL tube
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Wash 1x with PBS (1 mL) – transfer to Eppendorf
5. Spin down for 5 min, 150g @ 4⁰C
6. Remove supernatant
7. Add 200 µL of Mitotracker (1:4000 diluted in serum free medium) – final: 250 nM
8. Incubate for 30 min at 37°C in DARK
9. Wash 1x with PBS and transfer to FACS tube
10. Spin down for 5 min, 150g @ 4⁰C
11. Remove supernatant
12. Add 200 µl PBS 0.5% FCS

Mitotracker DEEP RED

Ex: 644 nm

Em: 665 nm

* Use APC (633, 660)

**FACS Mitotracker RED CM-H2XROS– ROS in mitochondria**

1. Collect (1-2 mln cells) in 15 mL tube
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Wash 1x with PBS (1 mL) – transfer to Eppendorf
5. Spin down for 5 min, 150g @ 4⁰C
6. Remove supernatant
7. Add 400 µL of H2O2
	1. 4 µL from bottle fridge in 1000 µL of serum free medium
	2. Dilute stock 1:32 – 12.5 µL in 387.5 µL
8. Incubate for 15 min at 37°C in DARK
9. Spin down for 5 min, 150g @ 4⁰C
10. Wash 1x with PBS and transfer to FACS tube
11. Spin down for 5 min, 150g @ 4⁰C
12. Remove supernatant
13. Add 200 µl of MitoROS
	1. Stock: 1 mM – dilute 1:4000 (final 250 nM) in serum free medium
14. Incubate for 30 min at 37°C in DARK
15. Add 1 mL of PBS
16. Spin down for 5 min, 150g @ 4⁰C
17. Wash 1x with PBS
18. Spin down for 5 min, 150g @ 4⁰C
19. Remove supernatant and add 200 µl of PBS 0.5% FCS

MitoROS

Ex: 579 nm

Em: 599 nm

* Use PE (633, 660)

**FACS Annexin V-SYTOX AADvanced**

* Prepare 1x Annexin Binding Buffer:
	+ 10 assays: 1 ml of 5x buffer to 4 ml MQ
* Prepare 500 μM SYTOX AADvanced working solution
	1. Add 200 μL of DMSO to vial
	2. Mix well
	3. Store at 4°C
* Count total cells (day 4 – viability should be similar)
1. Collect (1-2 mln cells) in 15 mL tube
2. Spin down for 5 min, 150g @ 4⁰C
3. Remove supernatant
4. Wash 1x with PBS (1 mL) – transfer to Eppendorf
5. Spin down for 5 min, 150g @ 4⁰C
6. Remove supernatant
7. Resuspend the cells in 1x Annexin Binding Buffer at 1x106 cells/mL
8. To each 100 μL of cells in Annexin Binding buffer (transferred to FACS tube):
	1. Add 5 μL of Pacific Blue Annexin V
	2. Add 1 μL of 500 μM SYTOX ADDvanced
9. Incubate for 30 min at RT in DARK
10. Add 400 μL of 1x Annexin Binding Buffer
11. Mix gently and keep samples on ICE

Pacific Blue Annexin V

Ex: 415

Em: 455

* Use …………………. (….., …..)

SYTOX ADDvanced

Ex: 546

Em: 647

* Use …………………. (….., …..)