ANALYTICAL CYTOMETRY - PRACTICE 2018/2019

16. – 18. 1. 2019, IBP

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Day 1 (16.1.)	**A)**	**B)**
9 - 14 hod	Intro Hela 8 Fucci cells – analysis using flow cytometry (Verse) and CM MLN-4924 treatment	
14- 18 hod		Úvod Hela 8 Fucci cells – analysis using flow cytometry (Verse) and CM MLN-4924 treatment

Day 2 (17.1.)	**A)**	**B)**
9 - 12	Harvest and fixation of cells for proliferation and cell cycle analysis. Analysis using flow cytometry.	
12-15		Hela 8 Fucci – analysis on CM Harvest and fixation of cells for proliferation and cell cycle analysis. Analysis using flow cytometry.
14-18		

Day (18.1.)	**A)**	**B)**
9 - 13.30	Harvest of cells, immunophenotyping, analysis using flow cytometry	
13. 30 - 18		Harvest of cells, immunophenotyping, analysis using flow cytometry

Protokol 1
Fucci 8 cells – harvest, measurement, analysis of cell cycle using intracellular fluorescent proteins (both flow and CM)

Protokol 2
Simultaneous analysis of proliferation and cell cycle on DU145 cells after the treatment by inhibitor of neddylation.

Protokol 3
Immunophenotyping – staining of surface molecules CD24/CD44 and viability on DU145 cells.

Protokol 1

Model HeLa 8 Fucci cells – cell cycle analysis using fluorescent proteins

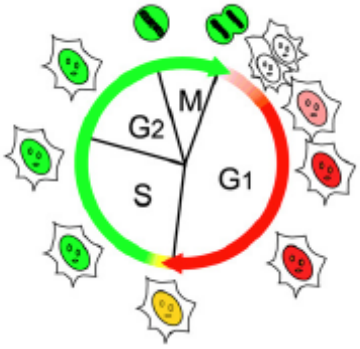
Aims

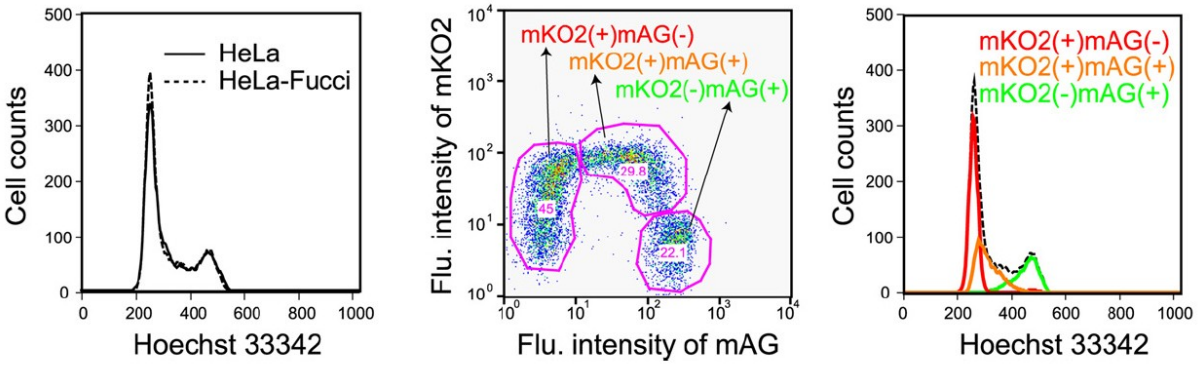
- to demostrate how to analyse cell cycle w/o any fixation and staining steps on living cells using flow cytometry and confocal microscopy
- analysis will be done on FACSVerse as one representative sample
- evaluation will be performed in FlowJo software

Theory

Buněčná linie HeLa 8 Fucci

- HeLa cells – human permanent cell line derived from cervical carcinoma
- one of the oldest and most common cell model used in cancer research
- Fucci probe (fluorescent ubiquitination-based cell cycle indicator) – enables visualisation of cell cycle progression in living cells
- cells in G1 phase emits red light, cells in S/G2/M green light
- find more info in PDF attached in your materials in IS





(Sakaue-Sawano et al., 2008; materials)

1) Flow cytometry analysis of cell cycle

Material

- **HeLa 8 Fucci** cell line
- solution of **PBS+EDTA** – disturb cell-to-cell junctions
- **trypsin** – pancreatic enzyme
- non-**nesteril media with serum** – for trypsin inactivation
- **PBS** – for washing steps

Process:

Cell harvest and sample preparation

- soak up the media from the dish
- add 3mL of PBS+EDTA – 1-2 minutes than remove
- add 0,5 mL of Trypsin – let incubate in termostat (37^{o}C) until the cells release from dish surface (cca 1-2 mins)
- add 2,5 mL of media with serum
- wash the dish with 1 mL PBS, add to the suspension into tube
- centrifuge 200g, 5 mins
- soak up supernatant
- resuspend pelet in v 1 mL PBS
- centrifuge 200g, 5 mins
- soak up supernatant
- resuspend pelet in 300 μl PBS and measure

Results

Describe the proces of measurement and analysis of cell cycle in Hella 8 Fucci. Attach results (plots) acquired from FlowJo evalutation.

2) Confocal microscopy analysis

Process:

Day 1: Seeding of HeLa 8 Fucci cell for CM analysis.

Day 2: Treatment

MLN-4924 (stock 10 mM, working concentration 1 μM)
TRAIL (100 ug/ml stock, 50 ng/ml working concentration)
Mitomycin (stock 1 mg/ml, working concentration 1 μg/ml)

Add notes and descriptions to all drugs used - MLN-4924 (see protocol 2), TRAIL a Mitomycin (drug type, mechanism of action).

Count dilutions and volumes which will be used for the treatment.

Day 2-3: Analysis of cell cycle using confocal microscopy

Describe the analysis of cell cycle using CM. Describe the changes in cell cycle, observed after various treatments.

Protokol 2

Analysis of cell cycle, proliferation and cell viability on DU145 cells after the treatment by inhibitor of neddylation

Aims

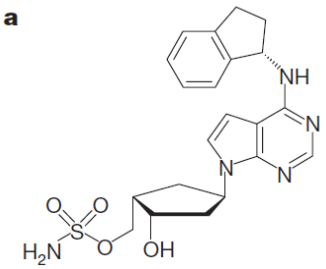
- to describe the effect of neddylation inhibitor (MLN-4924) on DU145 cells
- to use FACSVerse cytometr for sample analysis

Theory

MLN-4924

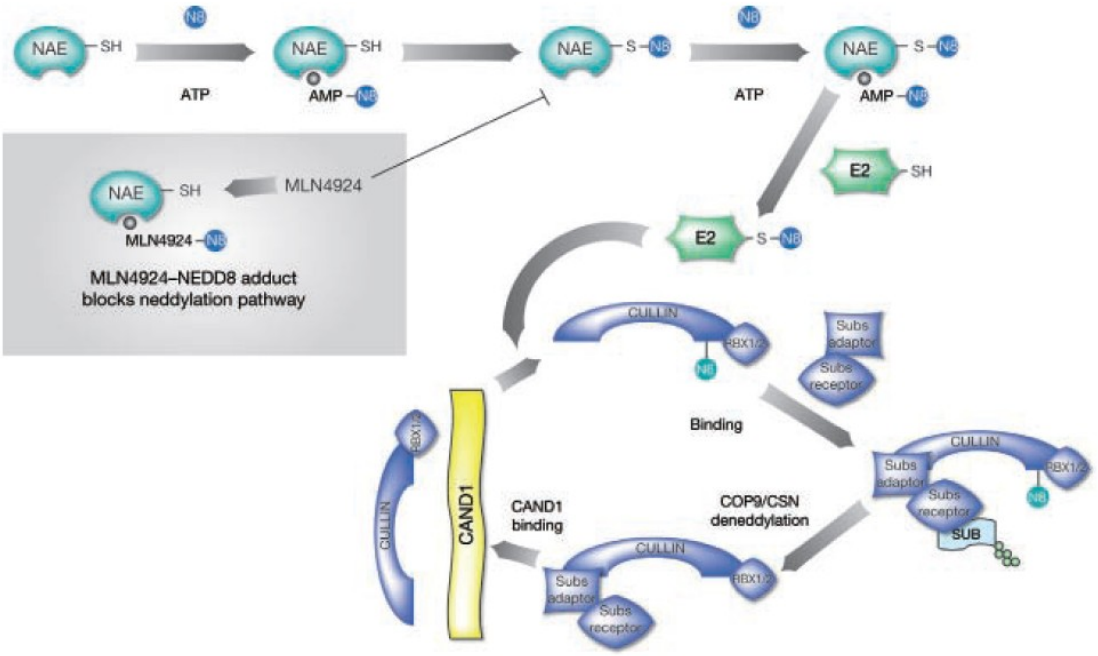
- ATP competitive inhibitor
- Phase I of clinical trials for lymphoma, myeloma, AML, ALL, melanoma and other non-hematological cancers
- creates stable aduct between NEDD8 and MLN-4924 which leads to the arres of neddylation pathway (figure **Soucy et al., 2010)**.
- proces of neddylation is necessary for the ubiquitine ligase $Skp2^{SCF}$ aktivity which play role in the regulation of various cell cycle processes
- one of the most important binding substrates are proteins regulating cell cycle ($p27^{Kip1}$, $p21^{cip1}$) or replication (Cdt1).

Structure of MLN-4924 (Soucy et al., 2009)



MLN4924

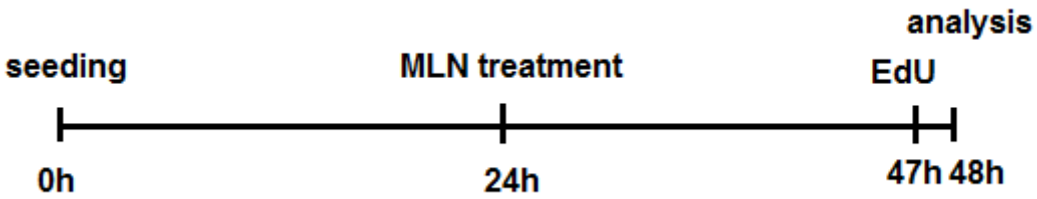
Neddylation pathway arrest
(Soucy et al., 2010)



Analysis of proliferation and cell cycle

Material

- DU 145 cell line (untreated vs treatred)
- PBS+EDTA trypsin
- nonsterile media with serum
- nonsterile FACS tubes
- PBS + 1% BSA
- Live Dead Fixable stain kit Red
- Edu click-iT AF488 kit
- PO-PRO-1



Process

1. Cell harvest and sample preparation

- soak up the media from the dish
- add 3mL of PBS+EDTA – 1-2 minutes than remove
- add 0,5 mL of Trypsin – let incubate in termostat (37^{o}C) until the cells release from dish surface (cca 1-2 mins)
- add 2,5 mL of media with serum
- wash the dish with 1 mL PBS, add to the suspension into tube
- centrifuge 200g, 5 mins
- soak up supernatant
- resuspend pelet in v 1 mL PBS
- centrifuge 200g, 5 mins
- soak up supernatant

2. Viability stain

- dilute viability marker in PBS (1:1000)
- add 100 µl/sample, incubate 15 mins, 4°C
- add 1 ml PBS + 1% BSA, centrifuge (200g, 5 min), soak up supernatant

2. Fix

- resuspend cells in 100 µl 4% PFA
- incubate 15 mins, RT
- add 1 ml PBS + 1% BSA, centrifuge (200g, 5 min), soak up supernatant

2. Permeabilitation

- resuspend cells in 100 µl 0,15% Tritonu X-100
- incubate 15 min, RT
- add 1 ml PBS + 1% BSA, centrifuge (200g, 5 min), soak up supernatant

2. Click-iT reaction

- divide saples into two tubes (ISO and SP)
- prepare click-iT reaction solution based on recipe bellow
- add 125uL of PBS + 1% BSA into ISO tubes; and 125uL of click-iT reaction solution into SP tubes
- incubate 30 mins, RT, dark
- than add 1 ml PBS + 1% BSA, centrifuge (200g, 5 min), soak up supernatant

	1 reaction
PBS	109,5 µl
CuSO4	2,5 µl
Fluorescent dye azide	0,625 µl
Reaction buffer additive (dilluted)	12,5 µl
Total reaction volume	125 µl

2. Cell cycle staining

- dilute PO-PRO-1 in PBS (1:10 000)
- add 500 µl/vzorek
- incubate 30 mins, RT, dark

Results

Describe the process of measurement and analysis of results acquired using flow cytometry. Attach result plots from FlowJo evaluation.

Protokol 3
Analysis of DU145 cell phenotype

Aim

- to analyse DU145 cell phenotype using two surface molecules CD24 and CD44 (primary Ab) conjugated with fluorescent probes on living cells

Theory

- DU-145 model is epithelial cell line derived from prostate cancer brain metastasis
- CD24 and CD44 are characteristic markers of cancer stem cells (CSC) in prostate cancer
- CSC – cancer cell subpopulations responsible for progression of disease and metastasis
- CSC traits – self-renewal, increased expression of antiapoptotic molecules, expression of molecules responsible for multidrug resistance (ABC transporters) etc.

CD44

- surface molecule associated with proliferation, differantiation, migration and angiogenesis processes
- associated with worse prognosis in various types of malignancies
- ligands – osteopontin, fibronectin, collagen, hyaluronate
- in prostate cancer considered as a marker of cancer but also normal stem cells

CD24

- surface molecule
- marker of nondiferentiated hematopoetic cells
- play role in cell adhesion
- acts as receptor for P-selectin
- increased expression shown in breast, ovarium and prostate cancer

Material

- DU-145 cells
- solution of PBS+EDTA
- trypsin
- nonsterile media with serum
- nonsterime FACS tubes
- PBS + 1% BSA
- antibodies – table bellow

Count:

for 10 ml 1% BSA add ml 20 % BSA into ml PBS

Antibodies:

antibody	fluorochrom	provider, cat. number	dilution
CD24			
CD44			
viabilita			
IgG2a κ			
IgG2b			

Samples:

- 2 samples:
  specific (SP)
  isotype control (ISO)

Process:

1. Sample preparation

- soak up the media from the dish
- add 3mL of PBS+EDTA – 1-2 minutes than remove
- add 0,5 mL of Trypsin – let incubate in termostat (37^{o}C) until the cells release from dish surface (cca 1-2 mins)
- add 2,5 mL of media with serum
- wash the dish with 1 mL PBS, add to the suspension into tube
- centrifuge 200g, 5 mins
- soak up supernatant
- add 1 ml PBS+1% BSA
- both sample divide into 2 tubes
- centrifuge 200g, 5 mins
- soak up supernatant

2. CD24 a CD44 staining

- add 100uL of antibodies or isotype controls diluted in PBS+1% BSA

Count:

1. tube ISO – into 50 μl PBS+1% BSA add

μl IgG

μl IgG

2. tube SP – into 50 uL of PBS+ 1% BSA add

μl CD44

μl CD24

- pippete the sample up and down twice
- incubate 20 mins in 4C
- add 1 ml PBS + 1% BSA
- centrifuge 200g; 5mins
- soak up supernatant

3. viability staining

- rozsuspend in 500 μl PBS
- add Propidium iodide (1:200)
- measure

Results

Describe the process of measurement and analysis of results acquired using flow cytometry. Attach result plots from FlowJo evaluation.