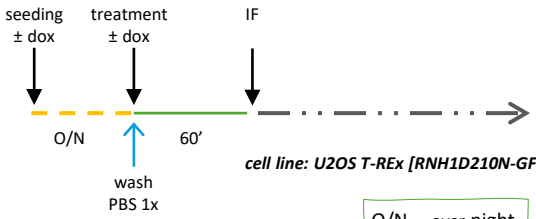
2. Monitoring the formation of R-loops in human cell



cell line: U2OS T-REx [RNH1D210N-GFP]

0 day

- seed cells 20 000 – 25 000 cells/cm^2; O/N
- to be at approx. 70% confluence next day;
- treatment with dox

1st day

1 well … 1 mL DMEM
… 2 mL 1xPBS

❁ pre-warm: DMEM, PBS
❁ cool down PBS (in fridge or on ice; approx. 4°)
❁ cool down 0.2 % Triton X-100 in PBS; (approx. 4°)
❁ cool down MeOH (-20°C)
❁ prepared all reagents and treatment

Cell culture room

- add 1 mL pre-warm DMEM to 6-well plate
- transfer CVs from plate to 6-well plate
- exchange DMEM for DMEM with treatment
- incubate (60 min; 37°C; 6% CO_2)
- wash cold PBS (2 mL) …. work in the lab on bench

wash
1. aspirate liquid
2. add washing solution
3. aspirate liquid
…… repeat …….
X. add desire liquid

O/N … over night

			calculate	
treatment	*c stock*	c final	µL / 1 mL	
dox	1 µg/mL	1 ng/mL	1 µL	
APH	2 mM	2 µM	1 µL	
CDDP	1 mM	10 µM	10 µL	
CPT	100 µM	100 nM	1 µL	

MM (Master mix)
in 15-mL falcon
dox 5 µL/ 5 mL of DMEM
from that prepare individual treatment (in eppendorph)

Keep cells on ice

Pre-extraction
- cold 0.2 % Triton X-100 in PBS (1mL; 10 min; on ice)
- wash with cold PBS (2 x 2 mL)

Fixation (work in the hood)
- 4 % (v/v) PFA in PBS (1mL; 15 min; r.t.)
- wash with PBS (3 x 2 mL)
- -20°C MeOH (1mL; 20 min; -20°C - freezer);
- wash with PBS (3 x 2 mL)

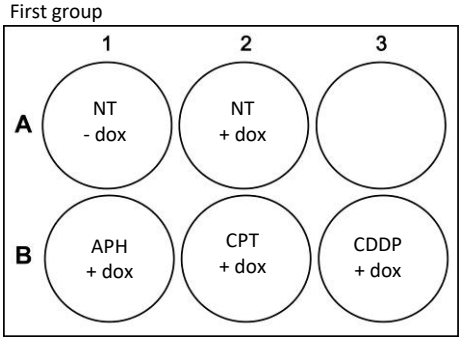
PFA
fixation via crosslinks

MeOH
dehydration and denaturation
for better access of PCNA epitope

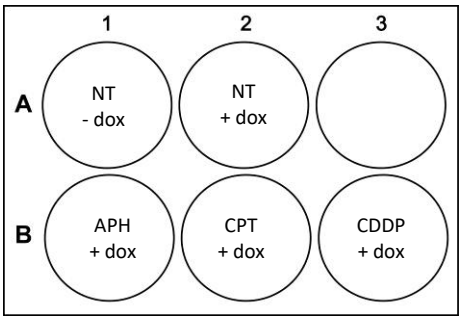
Blocking
- 3% BSA in PBS (1mL; 20 min)

3% BSA … 1.5 g … 50 mL

First group



Second group



❁ prepare wet chamber
❁ dilute primary antibody

❁ Primary antibody
- dilute the antibodies in blocking solution
  anti-PCNA (PC10) mouse 1:500
- 50 µL/CV
- place CVs on parafilm into wet chamber; cells facing up? ? ?
- incubation (1h; r.t.; wet chamber)

- wash with PBS (3 x 200 µL; 5min)

Secondary antibody
- dilute the antibodies in blocking solution in the one eppendorph
  anti-mouse cy3 (555nm) 1:500
  DAPI 1:1000
- 50 µL/CV
- incubation (30 min; r.t.; wet chamber - dark)

? ? ?
secondary antibody have to be kept in dark from on

- wash with PBS (3 x 200 µL; 5min)
- dip to H_2O
- dip to tissue to remove excess of liquid
- air dry (approx. 30 min; r.t.; in dark ? ? ?)

- mount with Fluoromont 4 – 4.5 µl/slide (cells facing down? ? ?)
- air dry (r.t.; in dark ? ? ?)

❁ ***option:*** incubation of primary antibodies O/N
- place CVs on parafilm into wet chamber; cells facing down? ? ?
- incubation (4°C; O/N; wet chamber)
- place CVs on parafilm into wet chamber; cells facing up? ? ?
- continue with wash etc. according to protocol ….

❁ **? ? ? Choosing of secondary antibodies**
⇨ based on their excitation and emission spectrum
- DAPI: emission 461 nm
- Green Fluorescent Protein (GFP): emission 488 nm
  fused with RNAseH1
- PCNA: has to have different emission (wavelengths), e.g. 555 nm, 647 nm

2nd day

Microscopy
- DM6000 (objective lenses: 63x, 1.42 NA, oil)

Note:
theory for fixation, blocking, primary and secondary ant is in protocol for Fibers assay (3)

U2OS T-REx [RNH1D210N-GFP]
- derivative of the human osteosarcoma cell line U2OS
- expresses conditionally a catalytically-inactive form of RNase H1 (RNH1) fused C-terminally to green fluorescence protein (GFP)
- the RNH1D210N mutant can avidly bind to R-loops, but fails to degrade them and hence stabilizes these structures and can serve as a tool for their detection

Pre-extraction
- wash away soluble, non-chromatin-associated proteins before fixing the cell sample
- specifically bound or structural proteins by reducing background noise and improving the signal-to-noise ratio
- particularly for nuclear proteins

2. Monitoring the formation of R-loops in human cell

2nd-3rd day

ImageJ/Fiji

Protocol in detail in Analysis_Fiji_ImageJ

2. Monitoring the formation of R-loops in human cell

Treatment

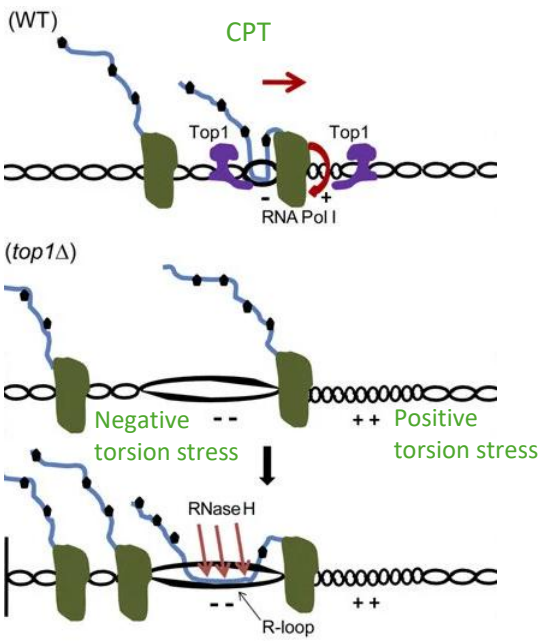
CPT
- camptothecin
- fast, the most common
- inhibition of Top 1 → it leaves nicks; ↑ gH2AX; run off; SSB → DSB during replication
- ↑ negative torsion stress → ↑ R-loops serve as obstacle for replication
- (CPT works similar as depletion of Top1)

CDDP
- cisplatin
- adducts + oxidative stress; resolved by translesion synthesis and repriming

APH
- aphidicolin
- inhibition of of replicative polymerases;
- low concentration (0.2 µM) slow down; high block (2 µM)

dox
- doxycyclin
- induction of RNAse H1 mutant – expression
- RNAse H1 foci for R-loops visualization

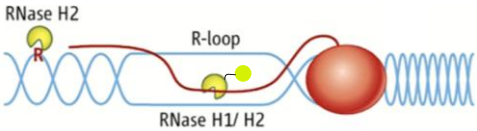
PCNA
- proliferating cell nuclear antigen
- DNA clamp that acts as a processivity factor for DNA polymerase
- essential for replication
- can be used to distinguish of cells in S-phase



RNAse-H1 wt - GFP:
cell line: U2OS T-REx [RNH1WT-GFP]
- cuts R-loop
- fluorescent for visualization of RNAseH1

RNAse-H1 mutant - GFP:
cell line: U2OS T-REx [RNH1D210N-GFP]
- doesn't cuts R-loop, only binds
- fluorescent for visualization of R-loops

Tet ON system: in the introduction



Reagent & solutions

PBS
- Phosphate-Buffered Saline
- 10x PBS … stock; 10x concentrated
- 1x PBS … as working solution, can be written as PBS in protocol

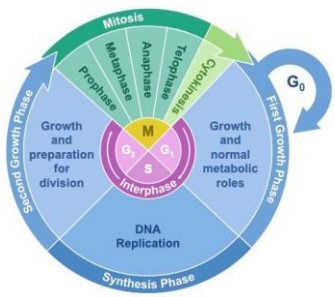
DMEM
Dulbecco's Modified Eagle Medium
a widely used basal medium for supporting the growth of many different mammalian cells

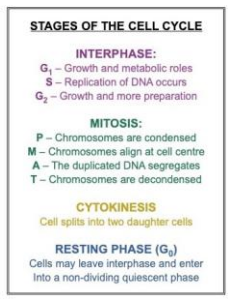
BSA
- bovine serum albumin

PFA or FA
- Paraformaldehyde

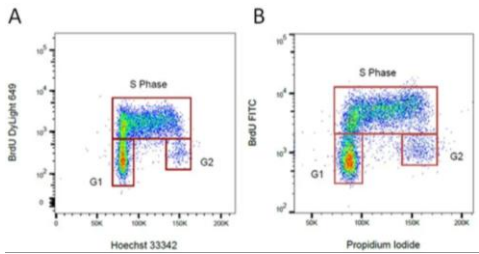
Triton-X
- a nonionic surfactant
- commonly used for membrane solubilization

Cell cycle

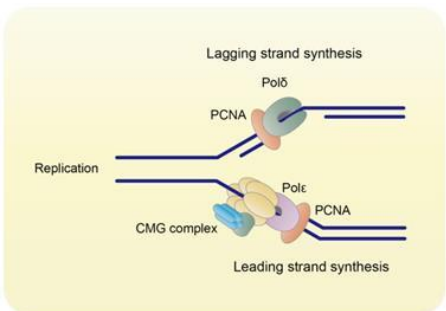




Visualization of cell cycle (S-phase)
- proteins or nucleotide analogues (e.g. thymidine: EdU, IdU, CldU, BrdU …) which are specific for S-phase
  + DNA contend (G1 … N; G2 … 2N) … staining with DAPI or Hoechst or Propidium Iodide



PCNA



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