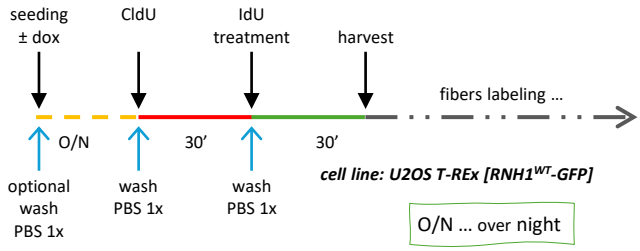
3. Analysis of the effect of R-loops on replication fork progression

0 day

- seed cells 3*10^5 cells/well; 6-well plate; O/N
- to be at approx. 70% confluence next day;
- treatment with dox

6-well plate: 1 well … 1 mL DMEM … 2 mL 1xPBS (PBS) … 400 µL Trp



O/N … over night

1st day

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

❁ pre-warm: DMEM, PBS
❁ cool PBS (in fridge or on ice)
❁ prepared all reagents and treatment
mix in 15-mL falcons or 1.5mL-eppendorpf tubes,
keep in water bath (37°C)

Cell culture room

- CldU 3 µL/mL
+ dox (±)
- incubate (30 min; 37°C; 6% CO_2)
- wash pre-warm PBS (2 mL)

MM 1A (Master mix) in 15-mL falcon: CldU 18 µL/ 6 mL

MM 2A in 15-mL falcon: CldU 15 µL/ 5 mL

❁ pre-warm: Trp
❁ cool down centrifuge (4°C)

- IdU 12 µL/mL
+ dox (±)
+ corresponding treatment
- incubate (30 min; 37°C; 6% CO_2)
- wash pre-warm PBS (2 mL)

MM 1B in 15-mL falcon: IdU 72 µL/ 6 mL

MM 2B in 15-mL falcon: IdU 60 µL/ 5 mL

❁ bucket with ice; label 1.5mL-eppendorpf tubes

- harvest cells
  - 400 µL Trp (approx.. 5 min; 37°C; 6% CO_2)
  - add 1 mL DMEM to neutralize Trp
- pipet ↑↓ (3x times)
- transfer cells to 1.5mL-eppendorpf tube (labeled)

Trp:
cells should look circular,
be de-attached – floating;
check progress under the microscope

⁉ overly pipetting can cause lysis of cells

Keep cells on ice

- spin (4 min; 4°C; 1500 rpm)
- aspirate supernatant
- resuspend 1 mL cold PBS, pipet ↑↓(3x times)
- spin (4 min; 4°C; 1500 rpm)
- aspirate supernatant
- resuspend cells 500 µL cold PBS µL;

cold PBS (approx. 4°C)

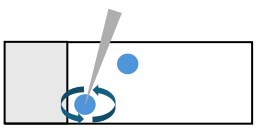
❁ wipe glass slides with dH_2O, label it with pencil
❁ CVs: 2 replicates / condition (well)
❁ prepare lysis buffer

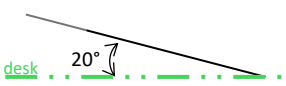
CVs:
First group: 10 CVs
Second group: 8 CVs

- briefly vortex cells before counting
- count cells
- adjust volume to 2.5*10^5 cells/mL

- 3 µL of cells + 9 µL lysis buffer
  - first the cell suspension – to approx. 2 CVs,
  - then add lysis buffer (*mix … start time)
- mix by gently with the tip (P200 pipette tip - yellow)
avoid creation of bubbles
- lysis 5 min at r.t.(starts after mixing);
- tilt the slide (15°- 45°)
- air dry the slides completely (15-20 min)
- place slides in to IF-cell(s)
- fix with fresh solution of methanol/acetic acid 3:1 (10 min; r.t.)
- dry O/N (keep the IF-cell open)

IF-cell … 75 mL

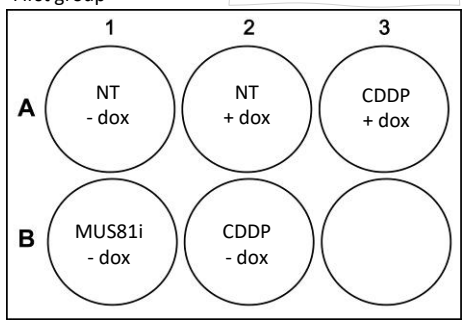




	calculate		
treatment	*c stock*	c final	µL / 1 mL
dox	1 µg/mL	1 ng/mL	1 µL
MUS81i	5 mM	5 µM	1 µL
CDDP	1 mM	10 µM	10 µL
CPT	100 µM	100 nM	1 µL
CldU	19 mM	57 µM	3 µL
IdU	28.24 mM	338 µM	12 µL

MM 1C
2.5 µL dox / 2.5 mL MM-DMEM

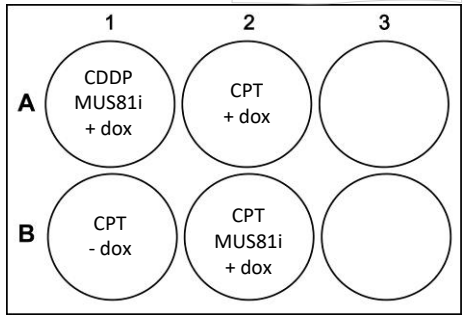
First group



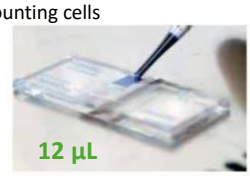
page 5

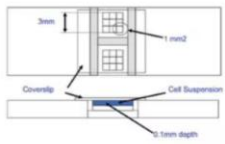
MM 2C
3.5 µL dox / 3.5 mL MM-DMEM

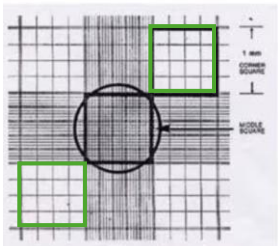
Second group

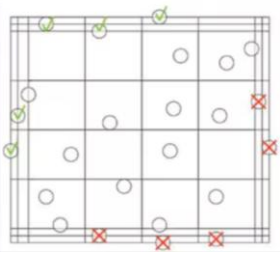


Counting cells









Lysis buffer				
solution	1 mL	*solution*	1 mL	2 mL
SDS 10%	50 µL	SDS 20%	25 µL	50 µL
TrisHCl pH 7.4 -7.5	200 µL	TrisHCl pH 7.4 -7.5	200 µL	400 µL
EDTA 500 mM	100 µL	EDTA 500 mM	100 µL	200 µL
H_2O sterile	650 µL	H_2O TK	675 µL	1350 µL

$$cells/mL = \frac{\#\ of\ cells\ counted}{\#\ of\ big\ squares\ counted} \times dilution\ factor \times 10\,000$$

$$V_1c_1 = V_2c_2$$

$$total\ volume = (calculated\ cells/mL\ \ x\ current\ volume)/\ 2.5 \times 10^5\ cells/mL$$

$$volume\ to\ add = total\ volume\ -\ current\ volume\ in\ epp.$$

3. Analysis of the effect of R-loops on replication fork progression

2nd day

- rehydrate slides in 1xPBS (2 x 5 min)
- denature DNA in 2.5 M HCl (1 h; r.t.)

❄ prepare and filter blocking solution
❄ calculate antibodies dilution
+ keep some filtered blocking solution for antibody dilution
❄ prepare 1L PBST 0.2%

- wash PBS (5 x 3 min)

Blocking
- blocking solution (recipe bellow)
- blocking in IF-cell (20 min)

❄ can be reuse multiple times (collect, keep at 4°C)

Primary antibody
- drain slides briefly dipping on a tissue
- dilute the antibodies in blocking solution (mix in the one ependorph)

anti-rat CldU 1:500
anti-mouse IdU 1:100
65 µL/CV; covered by cover slips
incubation (2 h; r.t.; the black staining box filled with H_2O)

- wash in PBST 0.2% (5 x 3 min)
- blocking solution (leave while preparing the sc. Ab)

Secondary antibody
- dilute the antibody in blocking solution (mix in the one ependorph)

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

anti-mouse Alexa-488 1:300
anti-rat cy3 1:150
65 µL/CV; covered by cover slips
incubation (1h; r.t.; the black staining box filled with H_2O)

- wash in PBST 0.2% (5 x 3 min)
- 1xPBS (2 x)
- pour milliQ-H_2O to the IF-cell and immediately take in out
- let slides air dry completely (15-20 min)
- mount with Fluoromont 30 µl/slide
- dry (approx. 1h)

Microscopy
- DM6000; oil 63x
- 7 fibers/image → 150 fibers …. approx. 40 images (20 + 20 from each replicates)

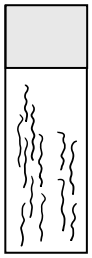
Analysis
- 150 fibers (150 green + 150 red = 300 measurements)

2.5 M HCl
32% HCl → 25ml/100ml; 37% HCl → 20ml/100ml, 35% HCl → 22ml/100ml

acids general: pour acid into water

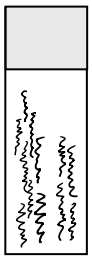
Blocking solution			
solution	**70 mL**	**90 mL**	***140 mL***
1xPBS	68.6 mL	88.2 mL	137.2 mL
BSA	1.4 g	1.8 g	2.8 g
Tween 20	70 µL	90 µL	140 µL
2% BSA, 0,1% Tween 20, 1xPBS, 0,22 µL filtered; can be reused			

PBST 0.2 %	
solution	
1xPBS	1 L
Tween 20	2 mL



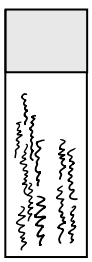
Fixation
The preservation of biological tissues from decay. It terminates any ongoing biochemical reactions; may also increase mechanical strength or stability.

denaturation



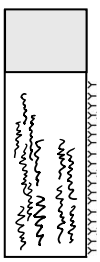
DNA Denaturation
The compact structure of the double helix is maintained by the hydrogen bonds between base pairs and the van der Waals interactions between the stacked bases. Various agents (heat, strong alkalis, urea, and formamide) weaken such forces and promote the separation of the strands. The resulting unwound polynucleotide strands adopt a random arrangement.

blocking



Blocking
reduce non-specific background noise by covering exposed binding sites.

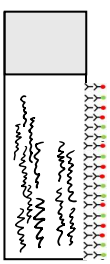
primary ant



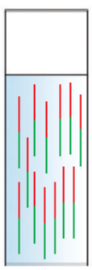
A primary antibody
an immunoglobulin that binds directly to a specific target antigen (protein or biomolecule) to detect, quantify, or locate it within a sample. They are produced in various host species (e.g., mice, rabbits) and come as monoclonal (single epitope) or polyclonal (multiple epitopes) types.

anti-rat CldU & anti-mouse IdU
- both is antibody against BrdU
- each has different affinity to CldU or IdU

secondary ant



Secondary antibodies
essential research tools that bind to primary antibodies, allowing visualization or detection of target antigens. They are conjugated with labels (fluorescent, enzymatic, or biotin) for signal detection and enhance sensitivity by amplifying the signal, as multiple secondaries can bind one primary antibody.



CldU 555 nm
IdU 488 nm

Acquire images and analyse

replication – normal speed

replication – slowed down

3. Analysis of the effect of R-loops on replication fork progression

3rd day

ImageJ/Fiji

Protocol in detail in Analysis_Fiji_ImageJ

3. Analysis of the effect of R-loops on replication fork progression

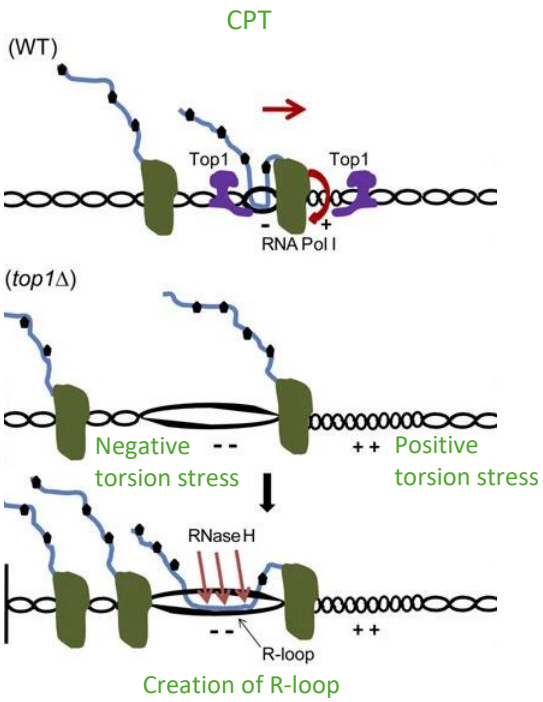
Treatment

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

MUS81i
- inhibitor of MUS81
- block transcription-replication conflict resolution
- MUS81 … endonuclease
  cut DNA strand → release of supercoil → replication can continue

dox
- doxycyclin
- induction of RNAse H1 wild type – overexpression
- for conflict resolution



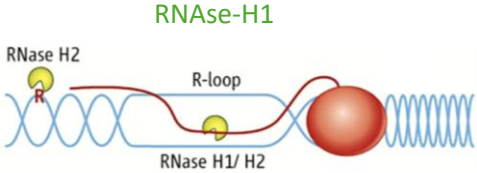


Figure 1. RNA/DNA hybrids and single rNMPs in DNA are substrates for RNase H2.

DNA is in blue, RNA is red line and letter R. The red oval represents RNA polymerase.

Reagent & solutions

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

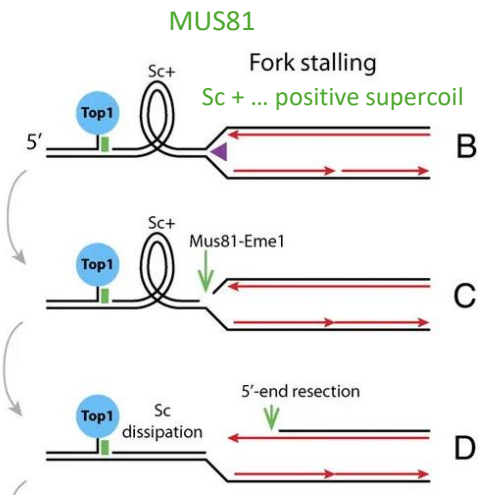
PBST
- PBS + tween 20 (0.2%)
- tween 20 … detergent
- to improve washes

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

Trp
- trypsin/EDTA (in this case)
- to detach adherent cells from culture surfaces (trypsinization) and dissociate tissues into single-cell suspensions. a protease, breaks down adhesion proteins
- EDTA, a chelating agent, removes calcium/magnesium to weaken cell-to-cell adhesion and boost enzyme activity
  + stabilization of Trp

BSA
- bovine serum albumin

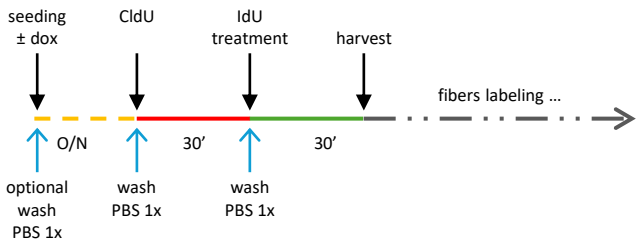
Primary antibodies
anti-rat CldU & anti-mouse IdU
- both is antibody against BrdU – with different affinity to CldU or IdU
- CldU, IdU, BrdU … thymidine analogs



Source of images:
DOI:10.1101/gad.573310
DOI:10.1083/jcb.201104003https://annualreport.nichd.nih.gov/2013/crouch.html

3. Analysis of the effect of R-loops on replication fork progression

Treatment schedule and scheme

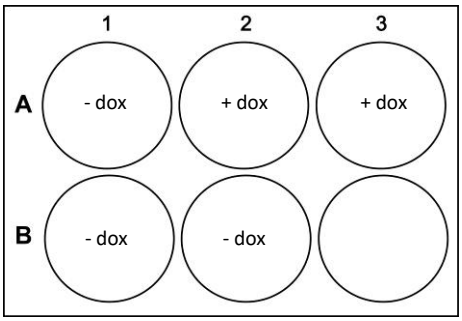


1 well … 1 mL DMEM
… 2 mL 1xPBS (PBS)
… 400 µL Trp

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

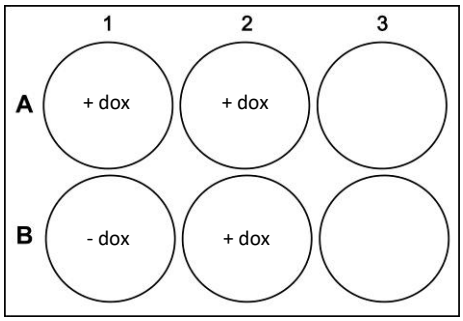
seeding
± dox

First group



+ dox: 2.5 µL dox / 2.5 mL DMEM
- dox: only DMEM

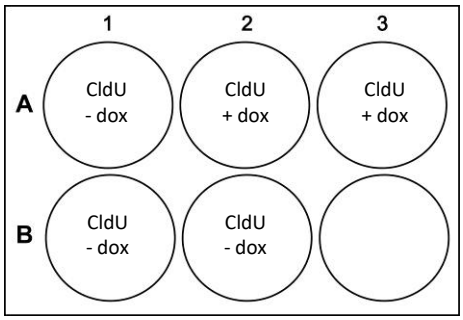
Second group



+ dox: 4 µL dox / 4 mL DMEM
- dox: only DMEM

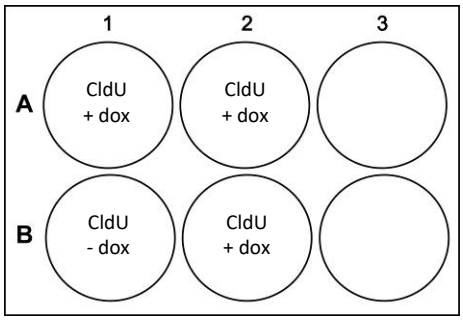
CldU
± dox

First group



CldU: 18 µL CldU / 6 mL DMEM → + dox: 2.5 µL dox / 2.5 mL CldU-DMEM
→ - dox: 3.5 mL CldU-DMEM

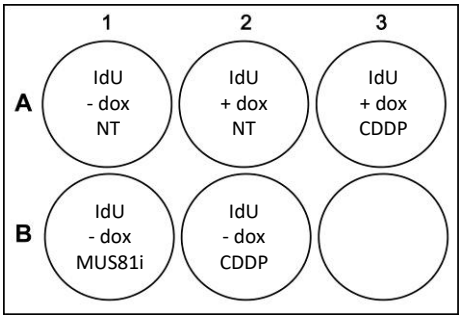
Second group



CldU: 15 µL CldU / 5 mL DMEM → + dox: 3.5 µL dox / 3.5 mL CldU-DMEM
→ - dox: 1.5 mL CldU-DMEM

IdU
± dox
treatment

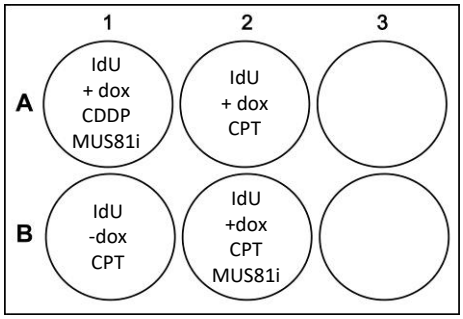
First group



IdU: 72 µL CldU / 6 mL DMEM → + dox: 2.5 µL dox / 2.5 mL IdU-DMEM
→ - dox: 3.5 mL IdU-DMEM

⇩ treatment

Second group



CldU: 72 µL IdU / 6 mL DMEM → + dox: 3.5 µL dox / 3.5 mL IdU-DMEM
→ - dox: 1.5 mL IdU-DMEM

⇩ treatment