Report

Week 50: FLASH bio

Anna Grebinyk for PPS, 21.12.23

BioPower

+ STRANGE STATISTIC:

- 2 sets of HDR, 2 cell lines
- 1 set of LDR, 1 cell line

Assays done:

- Comet assay – samples collected & frozen – analysis needs a few weeks – Paul
- Clonogenic assay – samples seeded – staining on 26.-28. Dec – Yuliia & Aleks & Anna
- MTT – samples seeded & read-out on 13.-15. Dec – Yuliia & Aleks
- Caspases – samples seeded & read-out on 13.-15. Dec – Anna & Aleks

→ analysis ongoing

Assays tested:

ROS:

- DCFH-DA – 13. Dec – Anna
- DHE – 13. Dec – Aleks
- H_2O_2 – 13. Dec – Paul

Antioxidant system:

- Gluth – 14. Dec – Paul

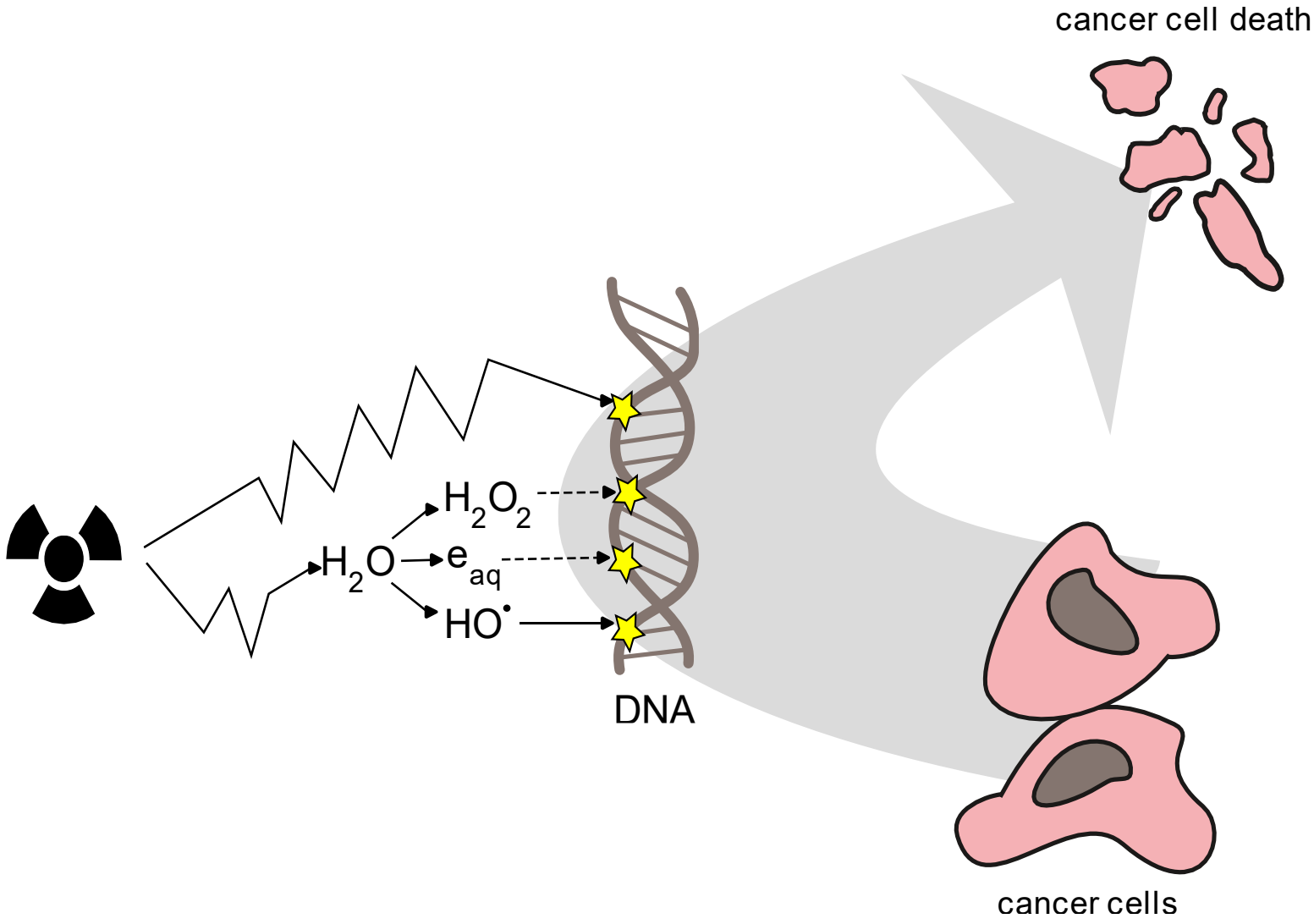
Biomolecules oxidation:

- LPO – 14. Dec – Sergii

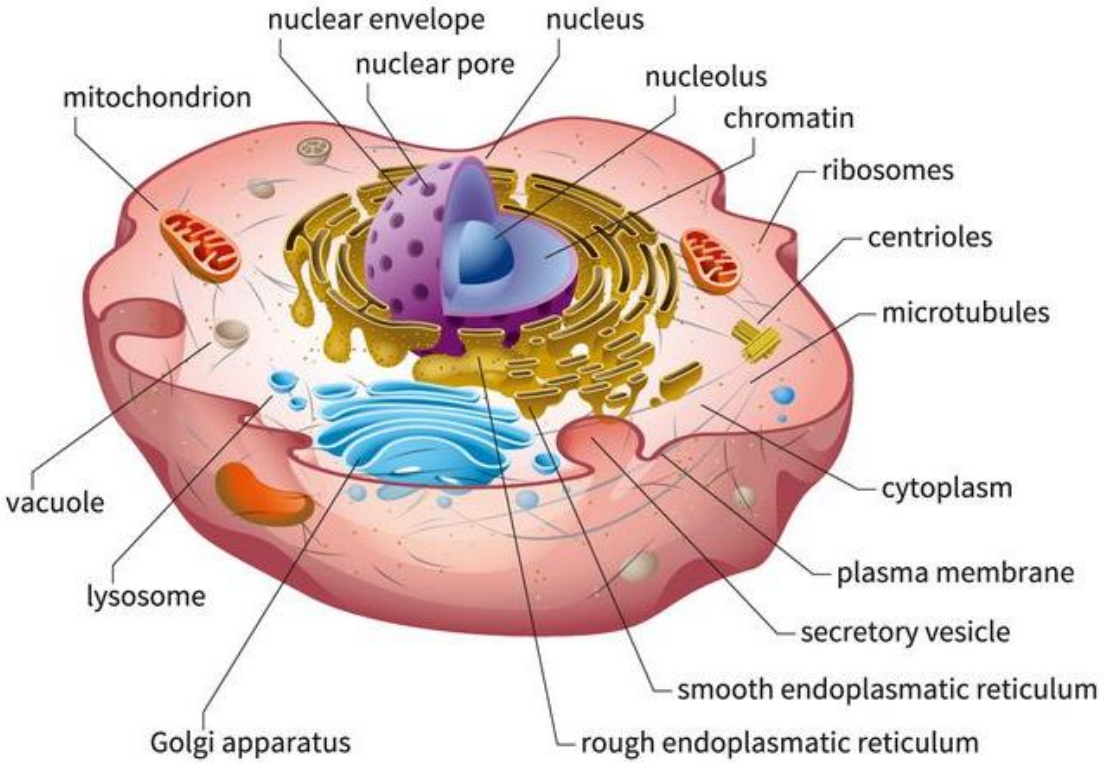
→ analysis ongoing, first look the assays are not ok with given conditions

→ for oxidant assays cells should be irradiated in PBS → time from Wildau-PITZ-Wildau ≤ max 2-3 h (to be tested)

Radiobiological effects



Animal cell



- basic structural & functional unit of life
- specified function & tasks
- capable of specialization & mobility
- *cytology* – study of cells

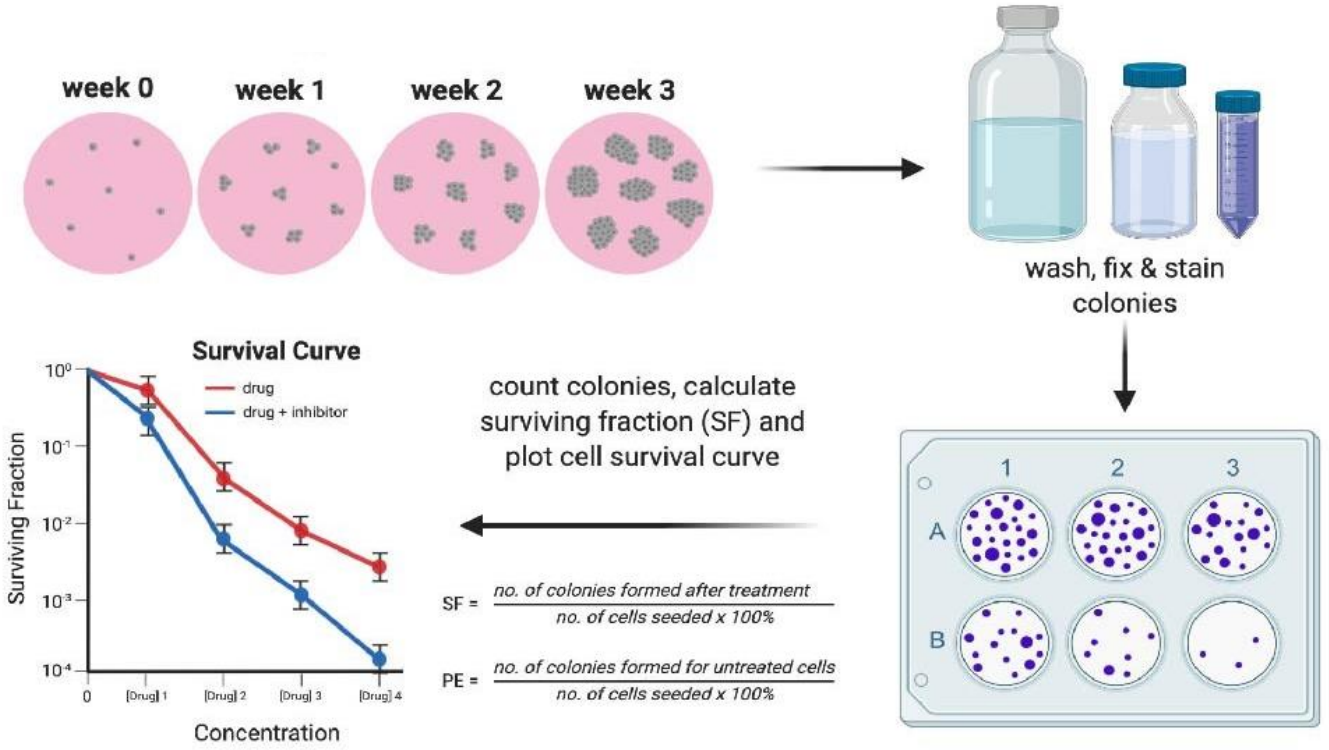
Life ?

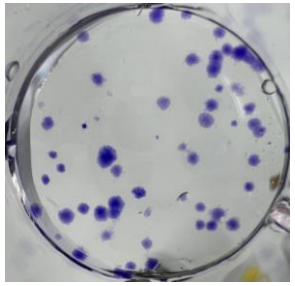
- reproduction
- heredity
- cellular organization
- growth and development
- response to stimuli
- adaptation through evolution
- homeostasis and metabolism

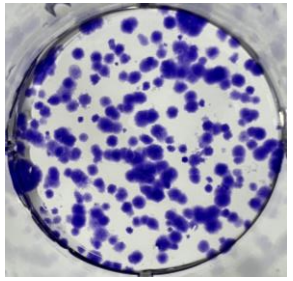
Clonogenic assay

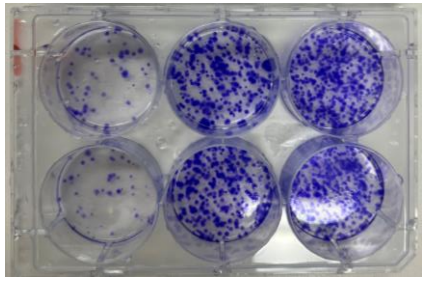
Cell proliferation

- colony formation assay is an in vitro cell survival assay based on the ability of a single cell to grow into a colony
- initially used in the field of radiobiology
- has become a standard tool in cancer research to evaluate cellular growth and the cytotoxic or genotoxic effects







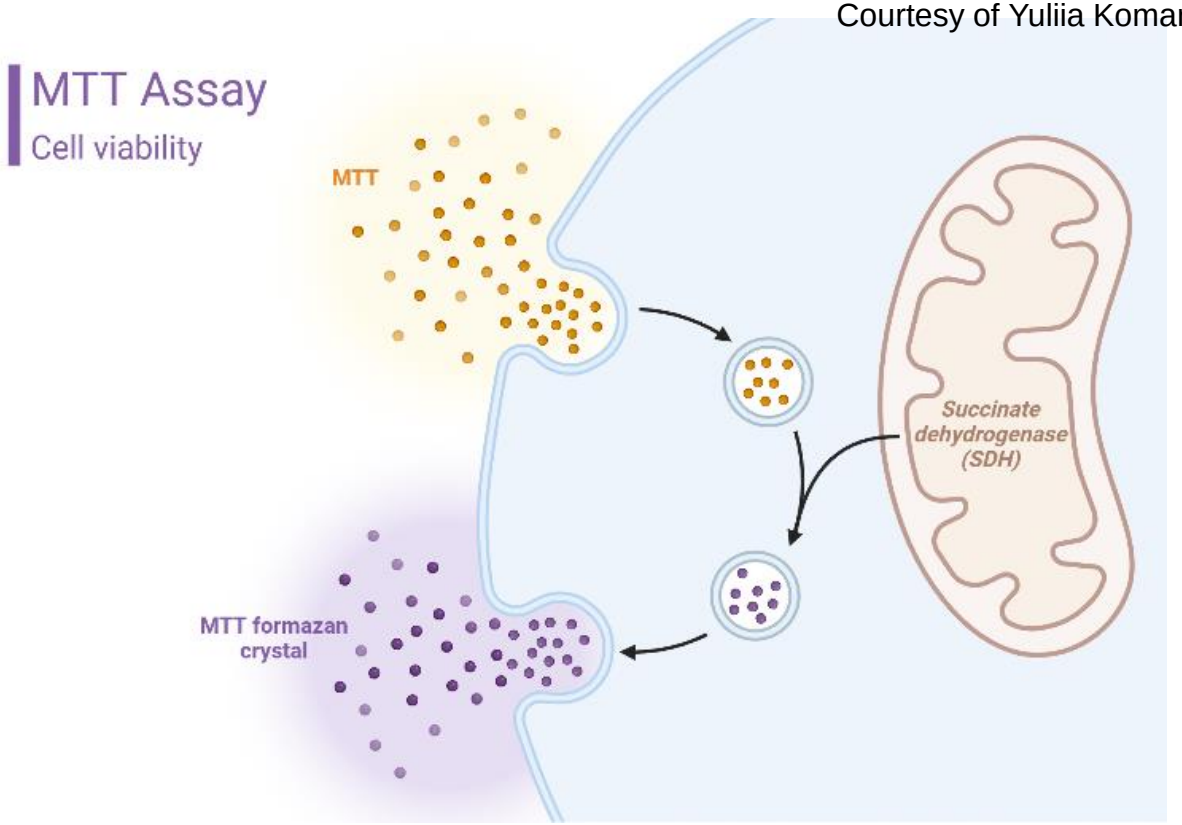


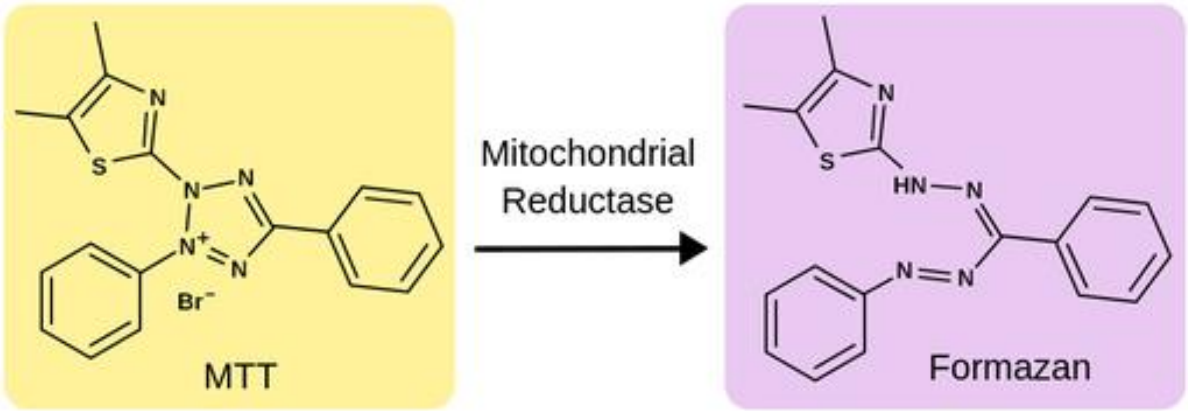
Courtesy of Yuliia Komar

MTT Assay

Cell viability

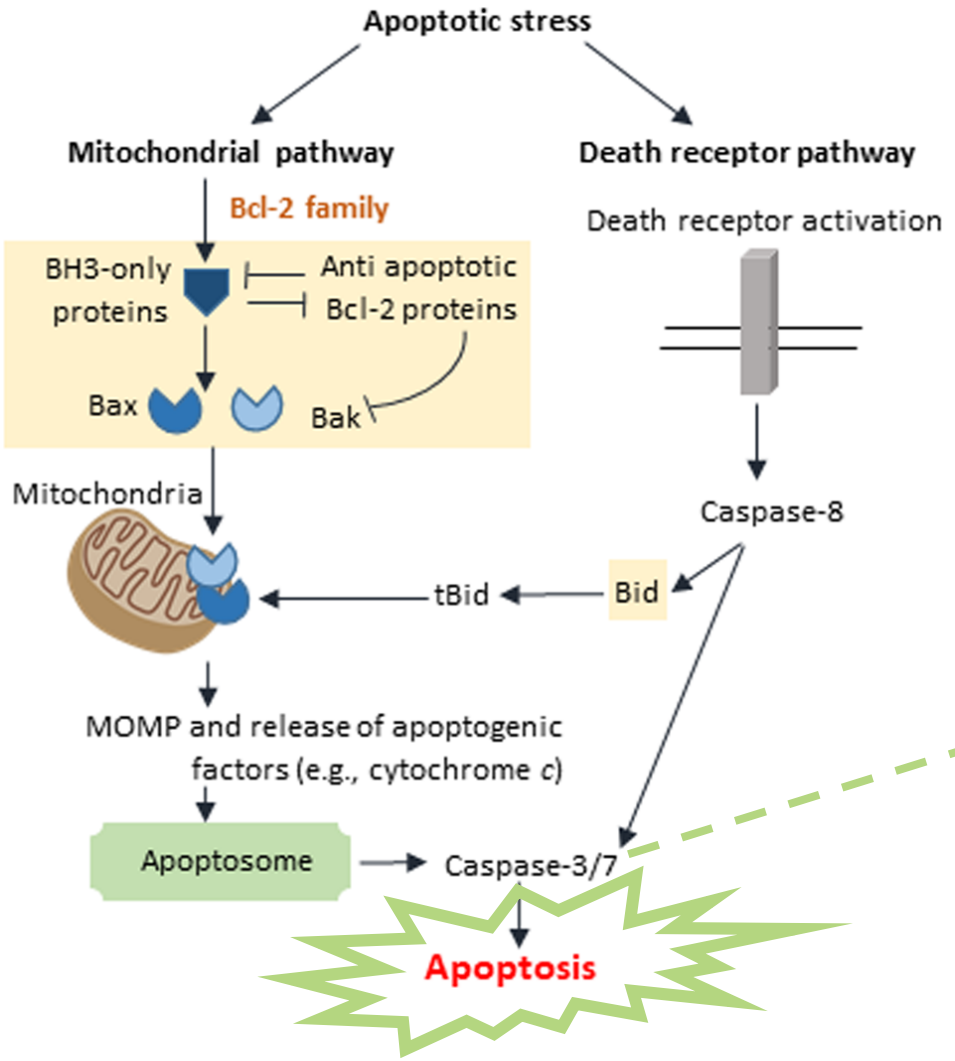
- colorimetric assay for assessing cell metabolic activity
- NAD(P)H-dependent cellular oxidoreductase enzymes reflect the number of viable cells
- Reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its insoluble formazan, which has a purple color
- used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence)



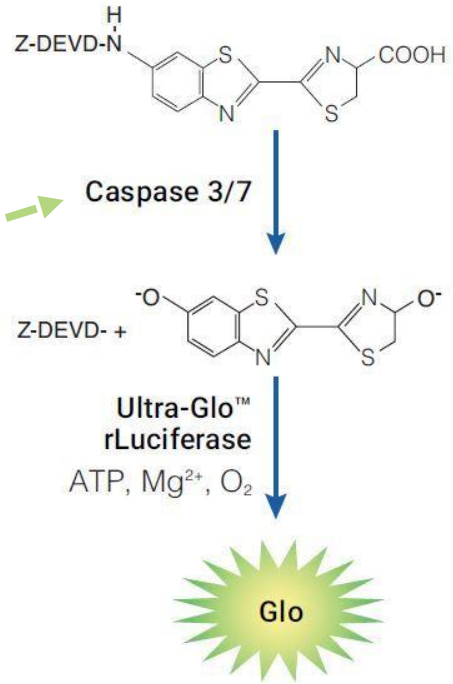


The Caspase-Glo® 3/7 assay

Homogeneous Assay Measures Caspase-3/7 Activity



- Apoptosis – programmed cell death, biochemical events that lead to characteristic cell changes (morphology) and death
- In contrast to necrosis, which is a form of traumatic cell death that results from acute cellular injury, apoptosis is a highly regulated and controlled process



- Adding the Caspase-Glo® 3/7 Reagent results in cell lysis, followed by caspase cleavage of the substrate
- This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal

Courtesy of Paul Borchert

Comet Assay (Single-Cell Gel Electrophoresis)

DNA damage

What is measured?

- comet assay is used measure DNA damage of individual cells
- assay sensitivity to DNA damage is modified by selecting electrophoresis solution:
  - TBE electrophoresis solution: ssDNA breaks and dsDNA breaks
  - alkaline electrophoresis solution: ssDNA breaks, dsDNA breaks, majority of AP sites, alkali labile DNA adducts

Principle

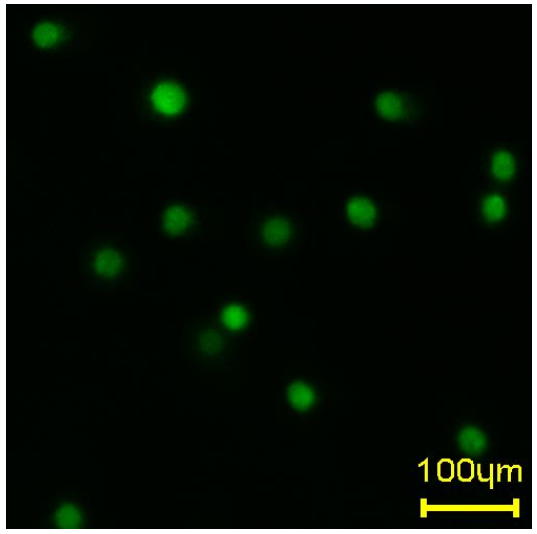
- during electrophoresis
  - intact DNA remains confined to the cell nucleus
  - fragmented DNA migrates in the electric field → forms “comet tail”

Main Assay Steps

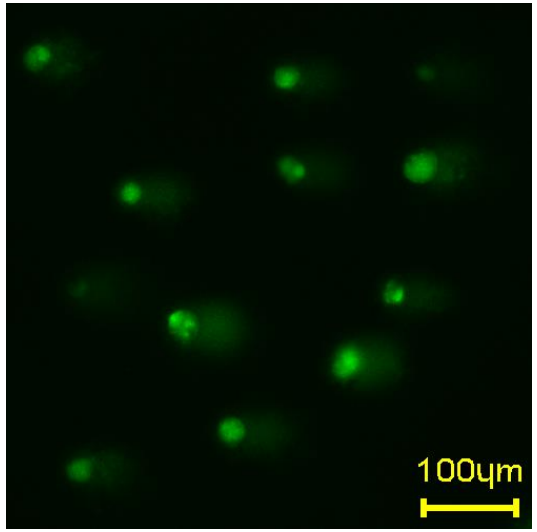
- immobilization of cells in agarose gel matrix
- incubation in lysis buffer and alkaline solution
- electrophoresis, washing and drying
- DNA staining using fluorescent dye
- image capture using a fluorescent microscope
- automated image analysis

OxiSelect 96-Well Comet Assay Kit, Cell Biolabs, Inc.

intact DNA



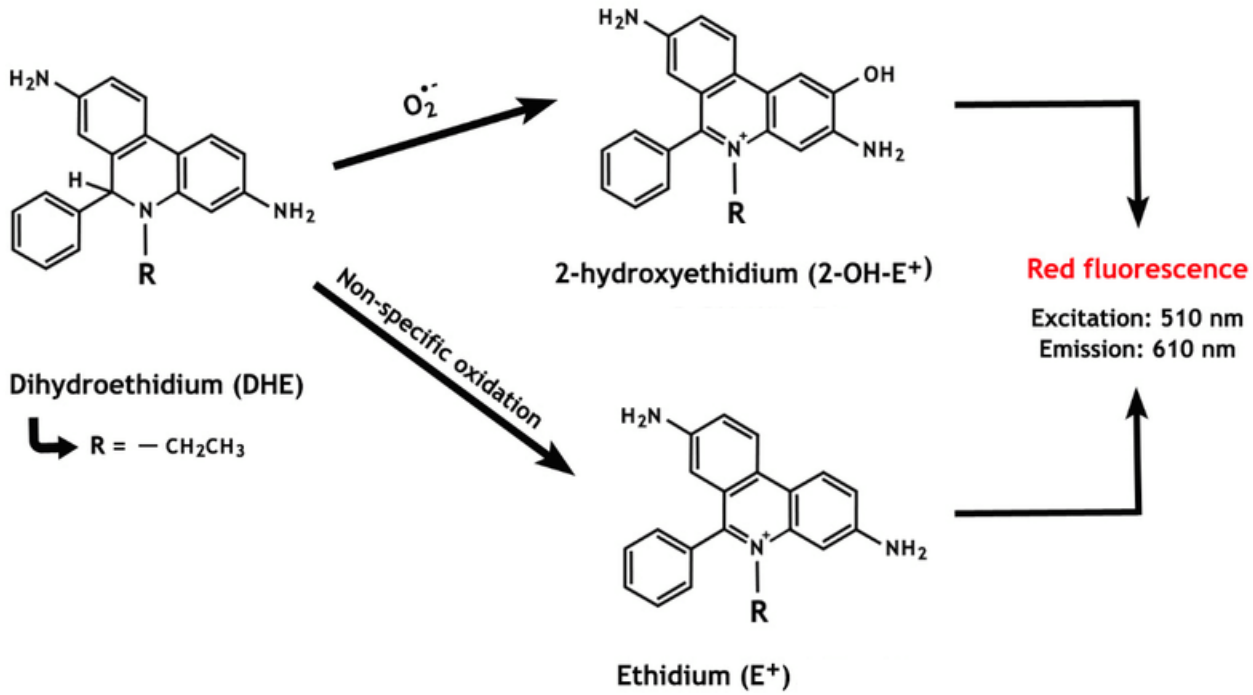
damaged DNA

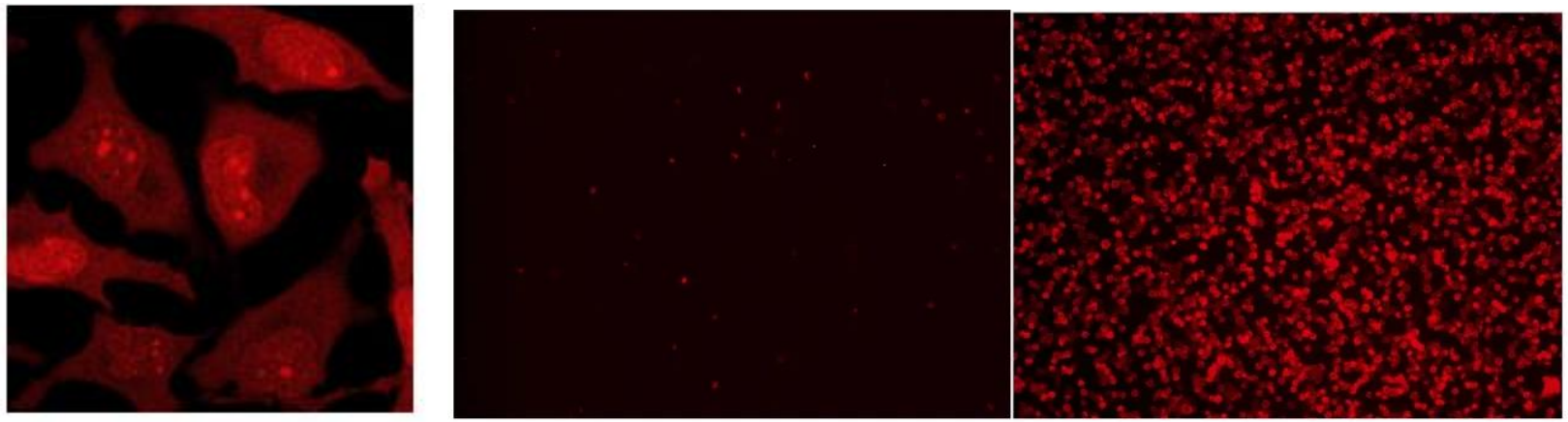


DHE Assay

Intracellular ROS level

- Dihydroethidium (DHE) – a fluorescent probe for the detection of ROS generation
- specific for superoxide and hydrogen peroxide
- measures ROS directly in live cells
- DHE is oxidized by superoxide to form 2-hydroxyethidium (2-OH-E+)
- DHE is oxidized by non-specific oxidation by other ROS sources to form ethidium (E+)





DCFH-DA Assay

Intracellular ROS level

- 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) – a fluorescent probe for the detection of ROS generation

What is measured?

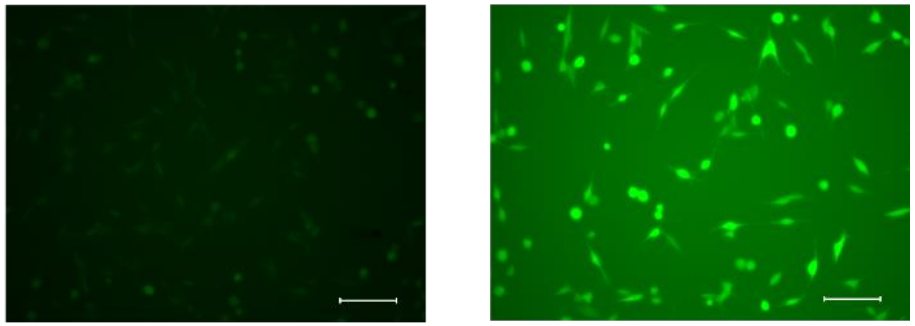
- specific for hydroxyl, peroxyl and other ROS

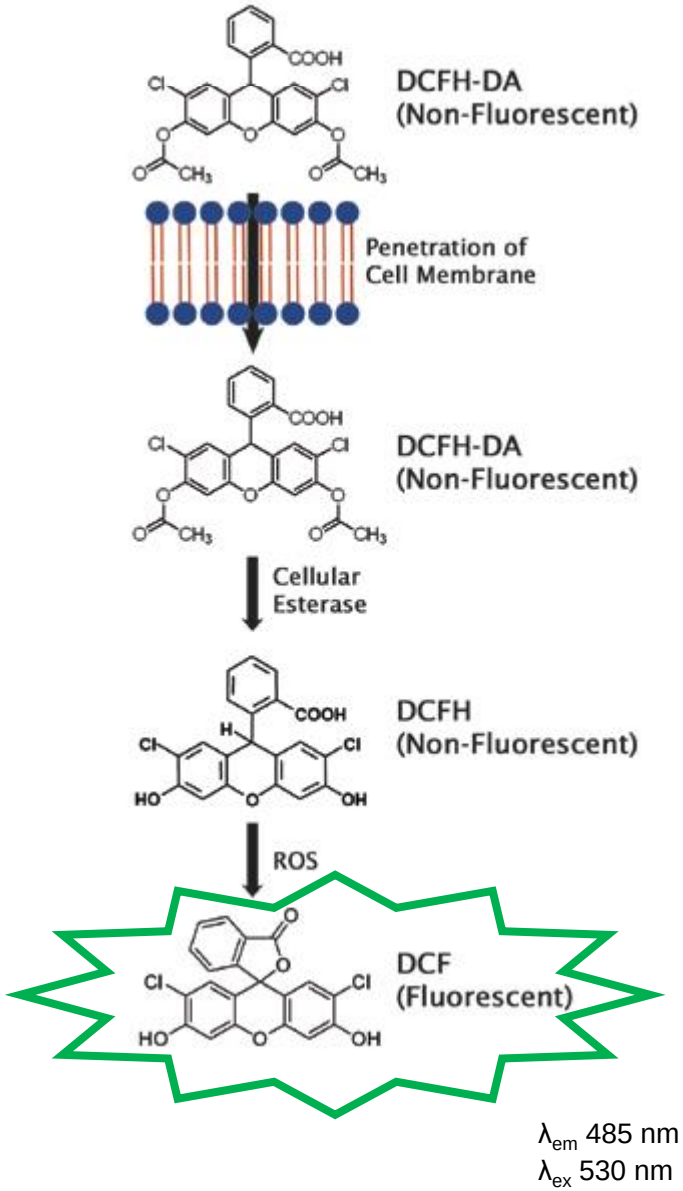
Principle

- DCFH-DA is taken up by cells where cellular esterase cleaves off the acetyl groups, resulting in DCFH
- Oxidation of DCFH by ROS converts the molecule to DCF, which emits green fluorescence

Main Assay Steps

- cells are transferred to PBS
- 5 µM DCFH-DA is added, incubated at dark
- fluorescence measured using a plate reader each 10 min up to 50 min





λ_{em} 485 nm
λ_{ex} 530 nm

H_2O_2 Assay

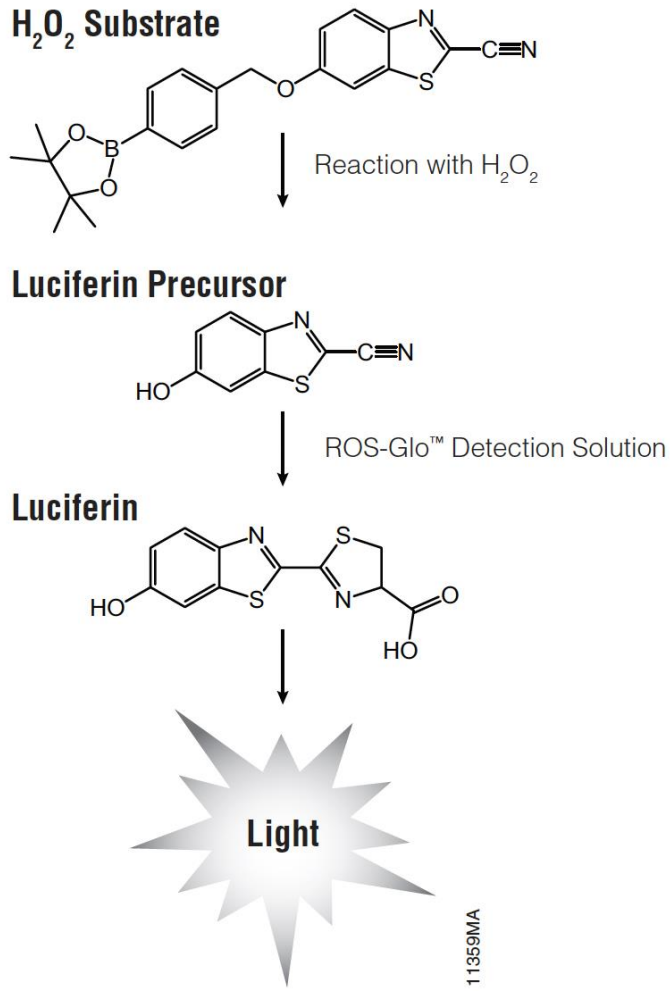
Intracellular H_2O_2 level

Principle

- substrate reacts with H_2O_2, generating a luciferin precursor
- luciferin precursor is converted by D-cysteine to luciferin
- luciferin reacts with luciferase, thereby producing a light signal proportional to H_2O_2 level

Main Assay Steps

- samples are transferred into 96-well plates
- H_2O_2 substrate solution is added & incubated for 1 h
- ROS-Glo Detection Solution added & incubated for 20 min
- luminescence is measured using a plate reader

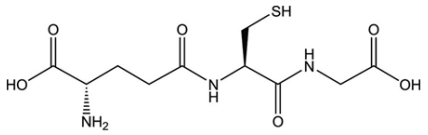


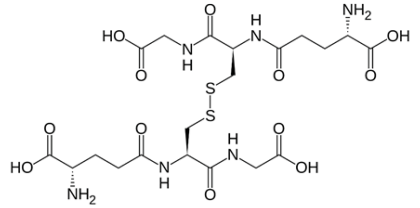
ROS-Glo H_2O_2 Assay, Promega

Glutathione Assay

Intracellular glutathione level

Courtesy of Paul Borchert



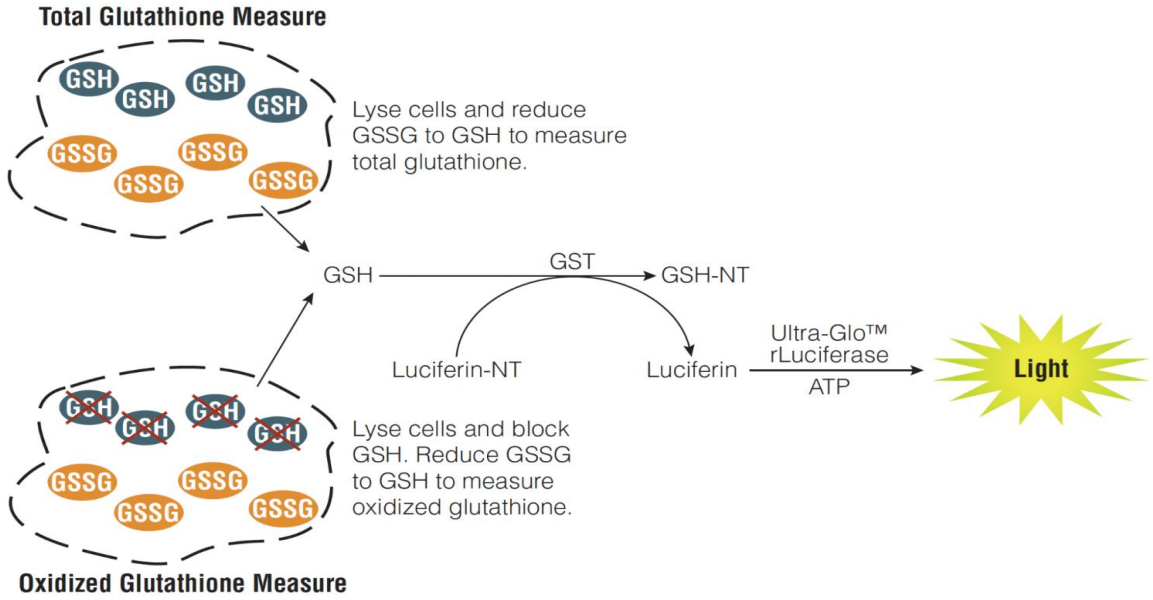


What is measured?

- total (TG) or oxidized (OG) glutathione level

Principle

- GSSG (oxidized glutathione) is reduced to GSH
- cells are lysed, releasing glutathione
  - for OG: GSH is blocked
- under the presence of GSH, glutathione S-transferase catalyzes reaction of luciferin-NT to luciferin
- luciferin reacts with luciferase, thereby producing a light signal proportional to GSSG+GSH / GSSG level



Main Assay Steps

- samples are transferred into 96-well plates
- TG / OG reagent added & incubated for 5 min
- luciferin generation reagent is added & incubated for 30 min
- luciferin detection reagent is added & incubated for 15 min
- luminescence is measured using a plate reader

GSH/GSSG-Glo Assay, Promega

Lipid Peroxidation (LPO) Assay

LPO Assay Kit, BQC Redox Technologies

What is measured?

- lipid peroxides in cells

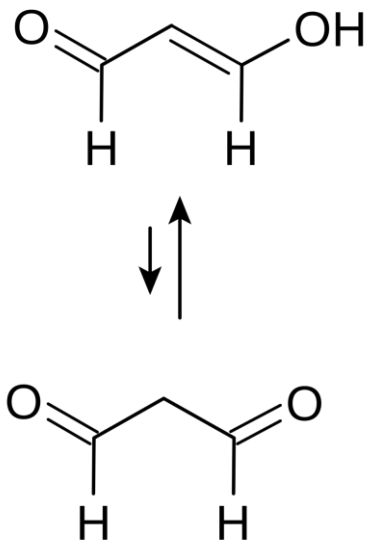
Principle

- lipid peroxides quickly decompose into reactive aldehydes, which are used as biomarkers for LPO
- 4-HNE and MDA react with an indole compound to produce a chromophore

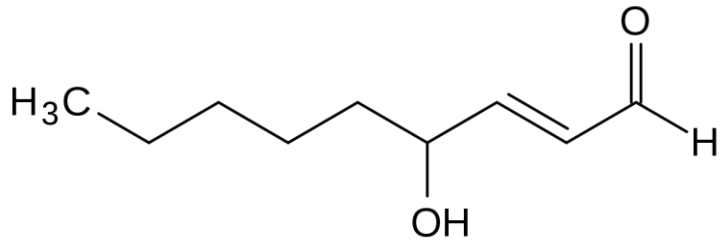
Main Assay Steps

- samples are transferred into 1.5 ml centrifuge tubes
- solution A and reagent B is added
- mixture is incubated for 40 min
- transferred into a 96-well plate
- absorbance at 586 nm using a plate reader

malondialdehyde (4-MDA)



4-hydroxynonenal (4-HNE)



Week 50, FLASH bio: ongoing report

…more at next PPS, 21. Dec

Physics:

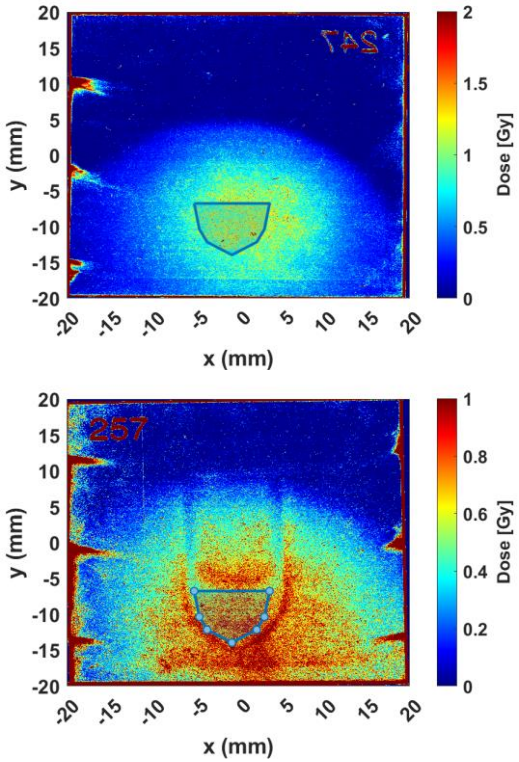
- HDR & LDR irradiation of HEL299 & A549 cells
- High is ok for LDR, but not HDR → ?
- 9 film pairs are scanned → 10th pair not analysed? (last control each set)
- High background/noise (especially on the films after tube → backscattering?) during beam prep
- Beam jitter high & double dose irradiation with HDR
- Work timing failed

Biology:

- Cell transfer at 37°C
- 4 cell culture compatible assays are under analysis:
  - DNA damage with comet assay
  - Cell proliferation with clonogenic assay
  - Cell viability with MTT
  - Cell death type with Caspase 3/7
- 5 redox status assays tested → cells should be irradiated in PBS for:
  - ROS with DCFH-DA, DHE and Promega
  - Antioxidant system with glutathione ratio
  - Biomolecules oxidation with lipid peroxidation (MDA)

Cell transfer at 37°C in the CellBox Go Incubator

(PITZ is beta-tester of the device)

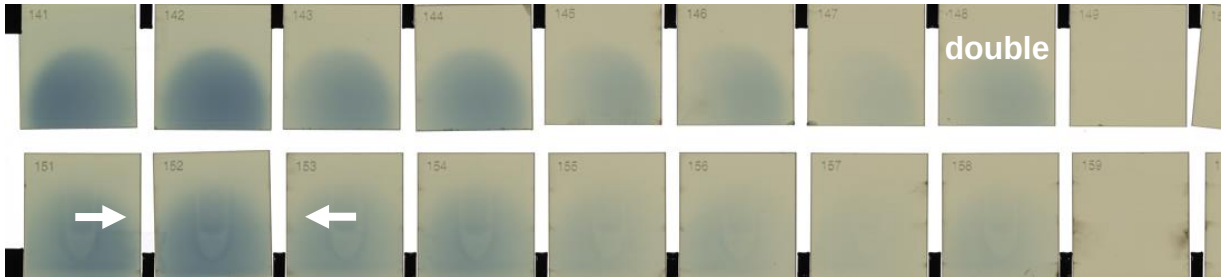


0.3 x 10^6 cells in 300 µL, irradiated with 1 Gy

13. Dec – HDR

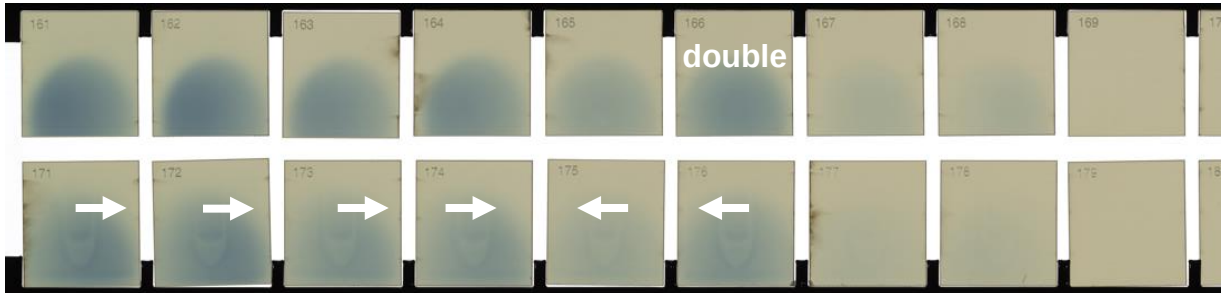
- HEL299 and A549 cells

HDR set 1 – irradiated ok



delivered dose	difference, Gy	difference,%	Av	Planned
9.186556	0.8	8	9.7437072	10
10.30086	-0.3	-3		
5.135505	-0.1	-3	5.0737866	5
5.012068	0.0	0		
2.146575	0.4	14	2.2209337	2.5
2.295293	0.2	8		
0.707399	0.3	29	1.2922142	1
~~1.87703~~	~~-0.9~~	~~-88~~		
0.047461	0		0.0474605	0

HDR set 2 – irradiated not ok



delivered dose	difference, Gy	difference,%	Av	Planned
7.669781	2.3	23	7.4916807	10
7.31358	2.7	27		
4.337412	0.7	13	4.5148029	5
4.692194	0.3	6		
2.356069	0.1	6	2.3560692	2.5
~~4.950754~~	~~-2.5~~	~~-98~~		
0.638004	0.4	36	0.6147545	1
0.591505	0.4	41		
0.040306	0		0.0403058	0

HDR set 3 - cancelled

- Booster and gun interlocks

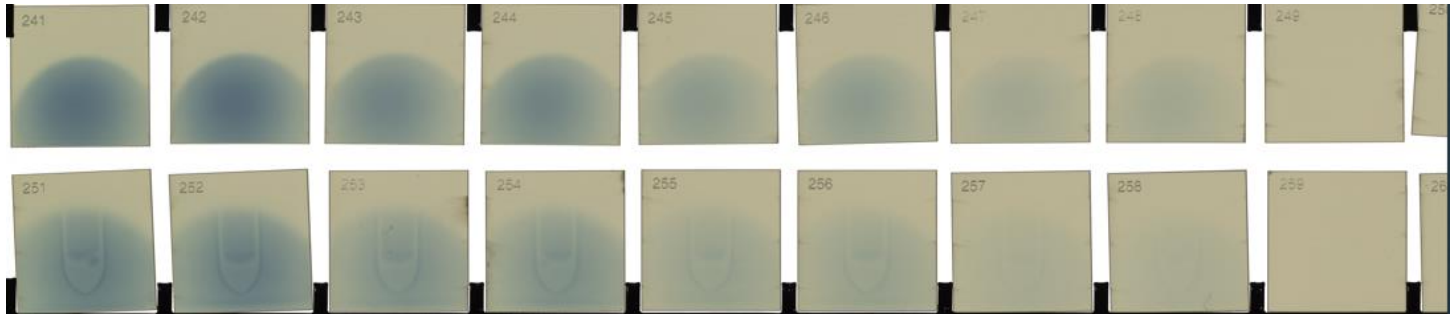
HDR extra

- Theoretical maximum 15 Gy
- Practical maximum 13 Gy with the given beam
- 11.56 Gy irradiated 6 samples
- 2 samples double irradiated

14. Dec – LDR

- Only A549 cells

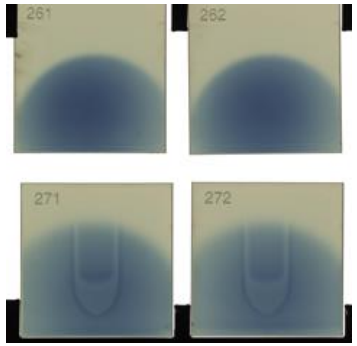
LDR set 1



delivered dose	difference, Gy	difference,%	Av	Planned
9.986122	0.9	-11	10.061882	9
10.13764	0.9	-13		
5.514225	0.5	-10	5.5098011	5
5.505377	0.5	-10		
2.437332	0.2	-6	2.3958251	2.3
2.354318	0.2	-2		
0.944101	0.1	6	0.9934942	1
1.042888	0.1	-4		
0.03476	0.035		0.0347596	0

LDR extra

- 22 Gy irradiated



delivered dose	difference, Gy	difference,%
23.42928	1.4	-6
21.81675	-0.2	1
0.041779	0.0	

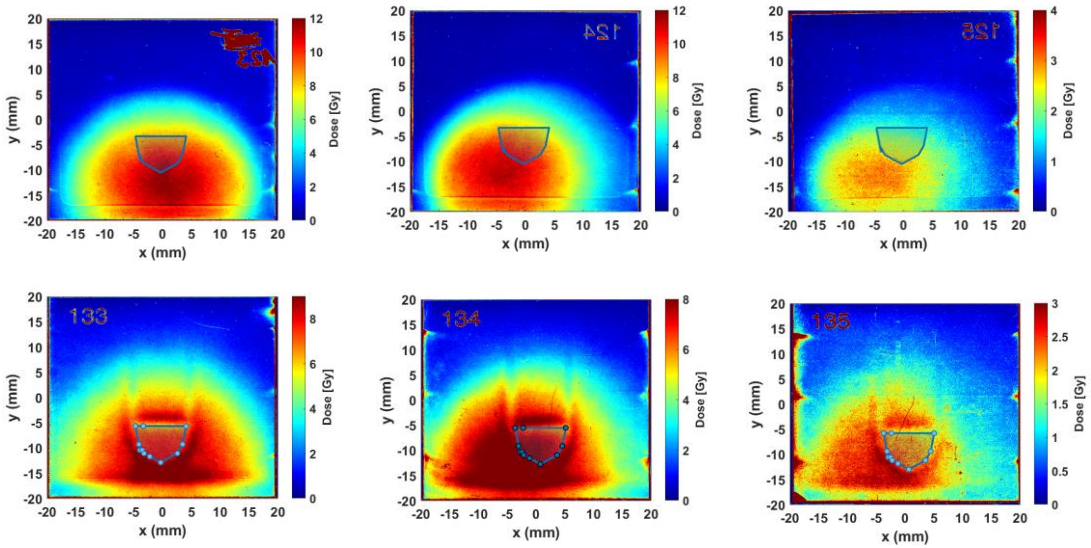
Why with same time (8 min 4 sec)
1.6 Gy difference?

Observed difficulties

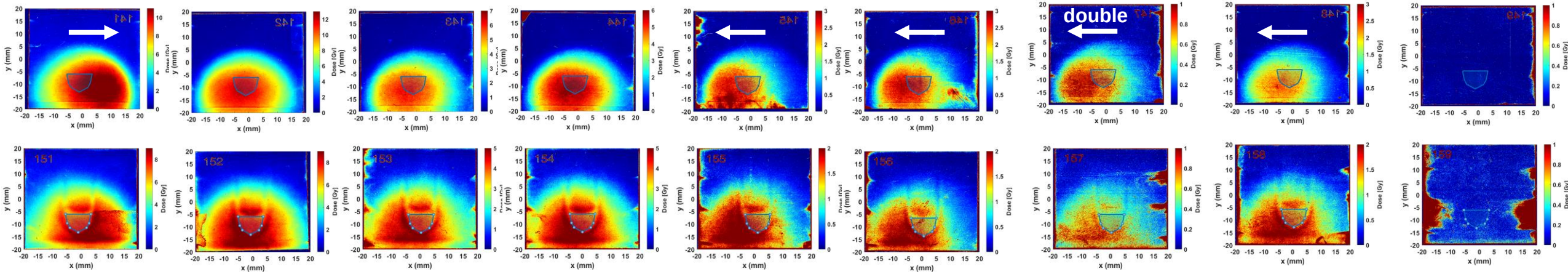
DELIVERED DOSE ESTIMATION

HDR dosimetry

- Cells were already delivered
- Dosimetry scanned & analysed
- Dose + 15% in NoP based on the scanned films
- Hight is adjusted with beam



HDR set 1 – irradiated ok

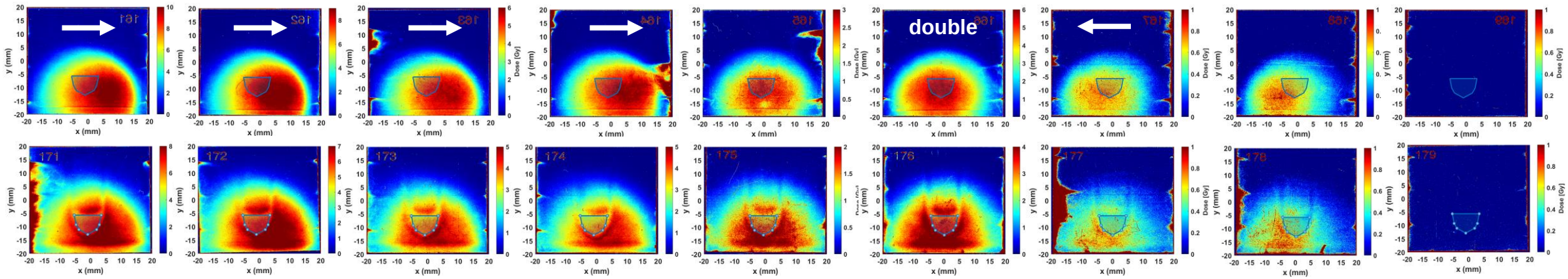


- Delivered doses ± 8 % (one 14%)
- Dose duplication 12.5 % - for all sets 18 % (4 out of 22)
- Beam jitter
- Samples position should be set lower / Beam position should be set higher

Observed difficulties

BEAM JITTER

HDR set 2 – irradiated not ok



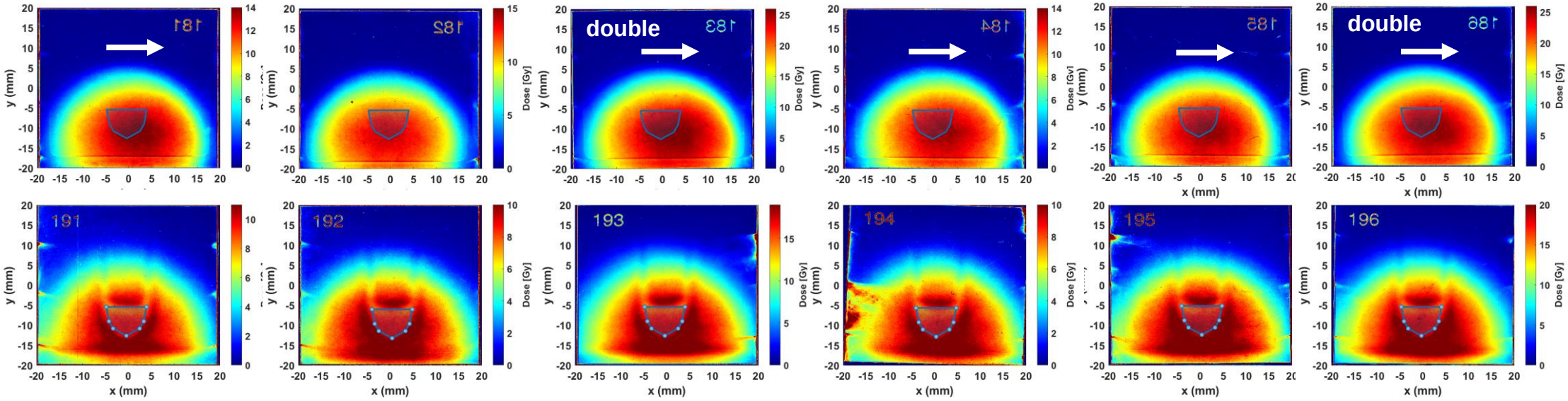
- Delivered doses ± 6 - 41 %
- Dose duplication 1 out of 8
- Beam jittering huge
- Samples position should be set lower / Beam position should be set higher

Observed difficulties

DOSE DUPLICATION

HDR set extra

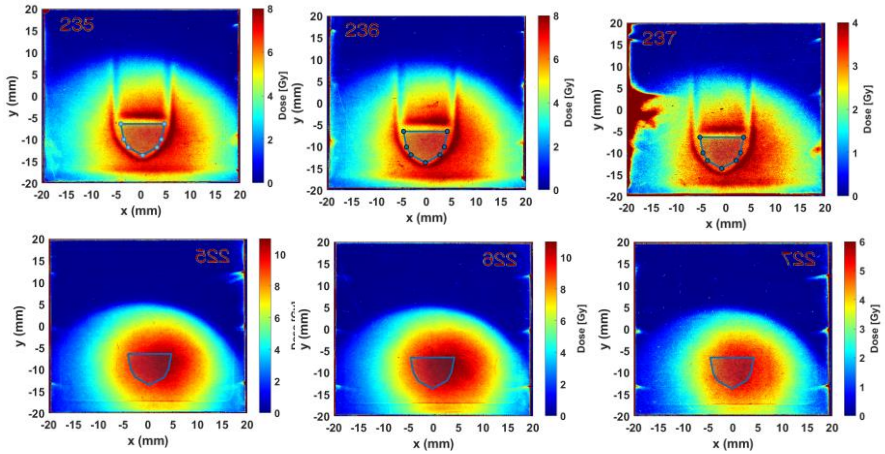
- For testing bioassays & higher differences higher doses are needed
- 15 Gy planned as max possible
- 13 Gy calculated as max
- 11.56 Gy delivered



- Delivered doses ± 12 %
- Dose duplication 2 out of 6

Observed difficulties

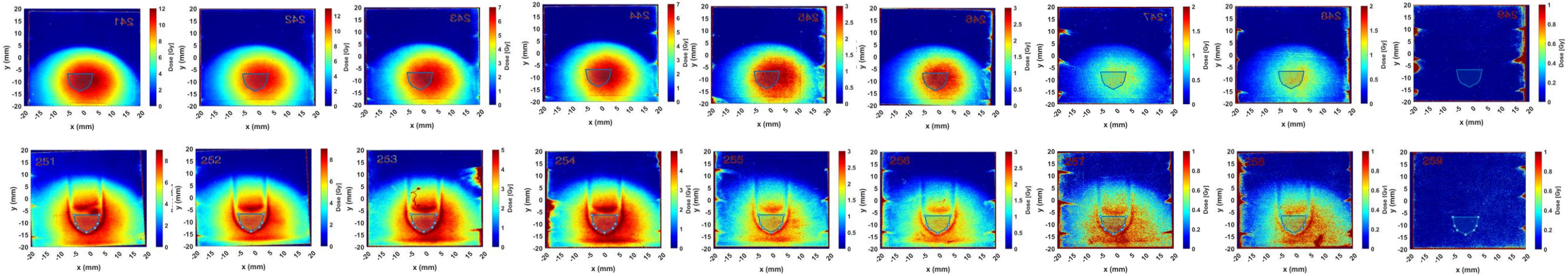
DELIVERED DOSE ESTIMATION



LDR dosimetry

- Planned dose corrected with delivered HDR dose
- Cells were already delivered
- Dosimetry scanned & analysed
- Dose + 10% in NoT based on the scanned films

LDR set 1 – irradiated ok



- Delivered doses ± 13%
- No dose duplication
- Hight is ok
- Horizontal position could be improved

Observed difficulties

HIGH BACKGROUND

- especially on the films after tube
- w/o eppie shadow
- with specific shadow pattern

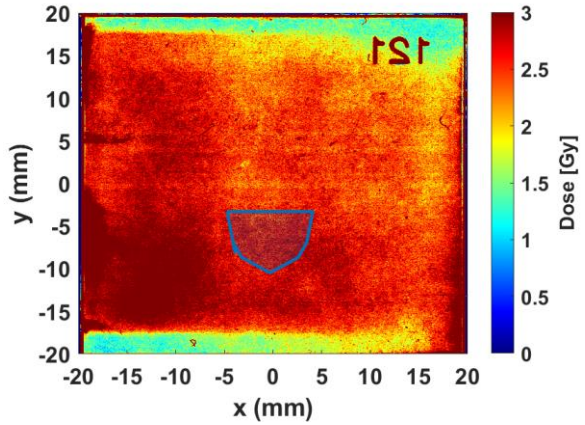
→ backscattering?

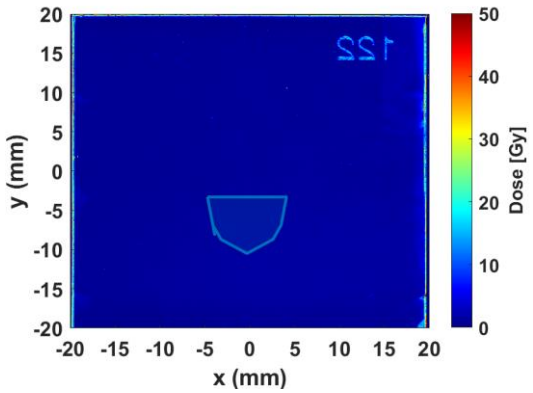
Position 1

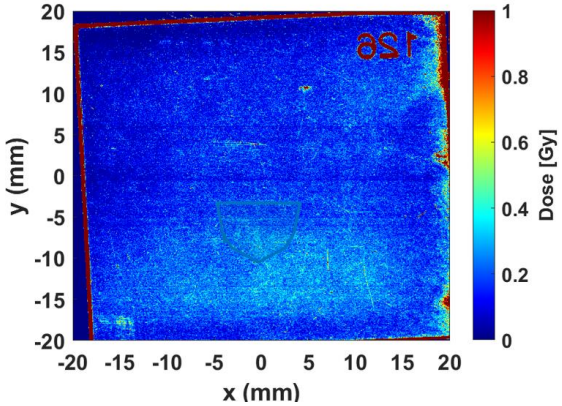
Position 2

Position 6

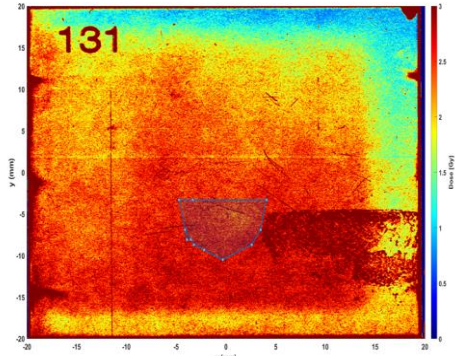
before

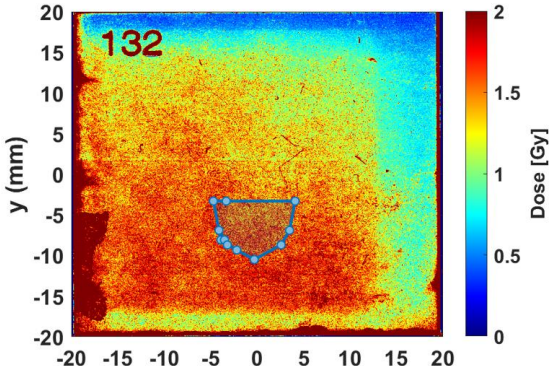


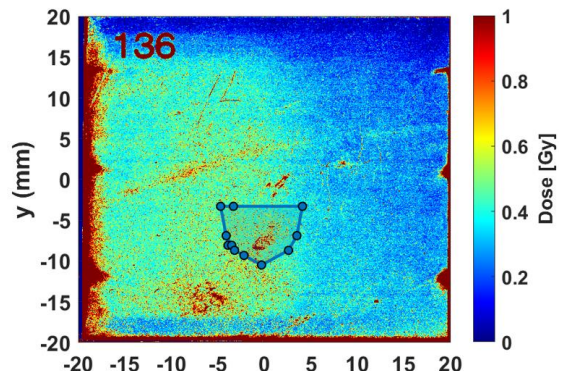




after







0 Gy

dark current 60 sec

0 Gy

Observed difficulties

SCATERRING PLATE DEMAGE

- not only melted, but vaporized

→ more radiation hard material?

Courtesy of Felix Riemer

Time challenge failed

planned

to do:	Operation						
Week 50	Mon Dec-11	Tue Dec-12	Wed Dec-13	Thu Dec-14	Fri Dec-15	Sat Dec-16	Sun Dec-17
Morn. 07:00 to 15:30	Stephan Zeeshan	Gross Kalantaryan	dosimetry, HDR Krasilnikov Kalantaryan	dosimetry LDR Stephan Riemer	Stephan Riemer	Vashchenko Zeeshan	Vashchenko Amirkhanyan
Late 15:00 to 23:30	Vashchenko Kelisani	Vashchenko Zhang	Cells, HDR Gross Zhang	Cells, LDR Gross Lotfi		Boonpornpras Lotfi	Boonpornpras Riemer
Night 23:00 to 07:30	Richard Dmitriiev	Beam transport HDR Richard	Beam transport LDR Hoffmann Zeeshan	Hoffmann Zeeshan		Gross Kalantaryan	Gross Kalantaryan

- Inform Anna 3 h before sample installation into tunnel
- Irradiate cells as soon as possible

done

to do:	Operation						
Week 50	Mon Dec-11	Tue Dec-12	Wed Dec-13	Thu Dec-14	Fri Dec-15	Sat Dec-16	Sun Dec-17
Morn. 07:00 to 15:30	Stephan Zeeshan	Gross Kalantaryan	Beam transport HDR Krasilnikov Kalantaryan	dosimetry LDR Stephan Riemer	Stephan Riemer	Vashchenko Zeeshan	Vashchenko Amirkhanyan
Late 15:00 to 23:30	Vashchenko Kelisani	Vashchenko Zhang	dosimetry, HDR Gross Zhang	Cells, LDR Gross Lotfi		Boonpornpras Lotfi	Boonpornpras Riemer
Night 23:00 to 07:30	Richard Dmitriiev	Beam transport HDR Richard	Cells, HDR Hoffmann Beam transport	Hoffmann Zeeshan		Gross Kalantaryan	Gross Kalantaryan

- Cell delivered as informed with 2 extra sets each time
- Each time + 1.5-2 h waiting time (ongoing dosimetry analysis) before irradiation
- HDR set over
- On 13. Dec cell irradaítion finished at 10:20 PM → working time for biology group until 4:40 AM !!!

So, what?

Physics:

- High is ok for LDR → to be measured & used for next experiments, HDR beam to position as LDR
- 9 film pairs are scanned → 10th pair not analysed? (last control each set)
- High background/noise (especially on the films after tube → backscattering?) during beam prep → to be improved / separeted with dosimetry
- Beam jitter high & double dose irradiation with HDR → to be improved?
- Scaterring plate to be replaced
- Work timing should be much improved → **cells are to be irradiated before 15:00 (better earlier)**:
    - The beam quality should be tested → „green light“ for bio experiment
    - Dosimetry prep should be done at night → plan biowork when SSB on night shift
    - Anna participation in shifts?
    - Someone else responsible for dosimetry?
    - Do 1 set per day?
- Some bio assays need higher doses ≤ 30 (≤ 50) Gy to 300 µL sample → ?

Biology:

- 4 cell culture compatible assays are under analysis
- For 5 redox status assays cells should be irradiated in PBS → less time for irradiation → to be tested