The far side - the gamma-H2AX Focus Assay

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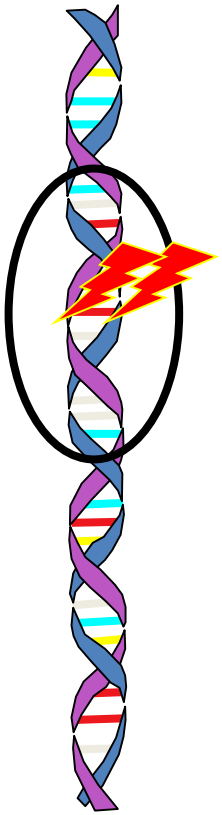
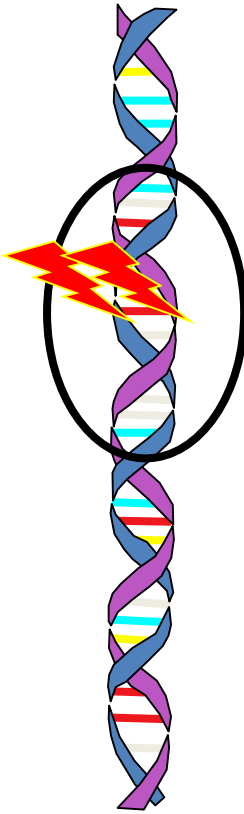
DNA double-strand breaks (DSBs) are a severe threat to genomic integrity



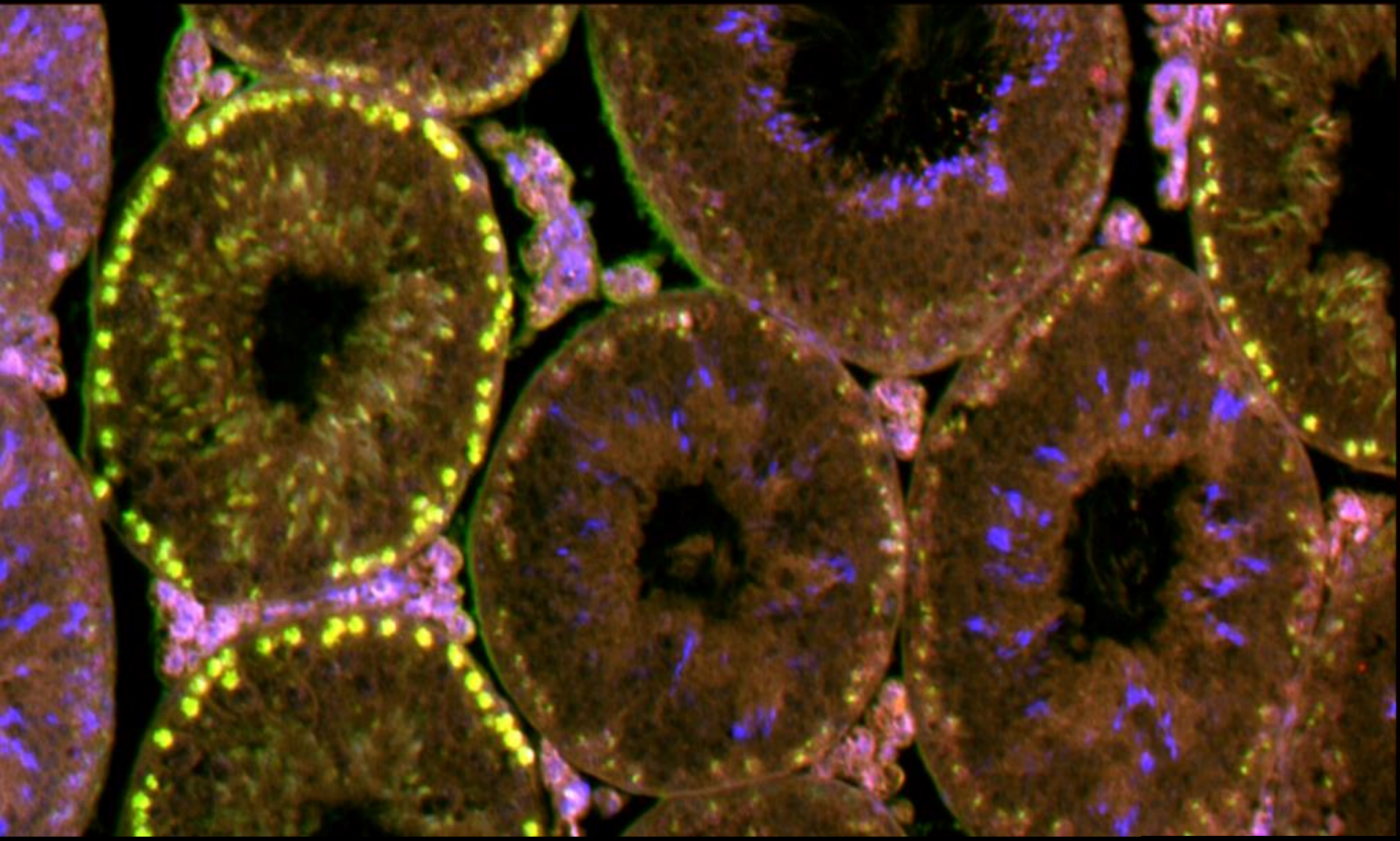


DSBs result from ionizing radiation and radiomimetic drugs

- Arise physiologically during DNA replication

- Are delivered as initiator of physiological recombination in processes like V(D)J recombination & Meiosis.

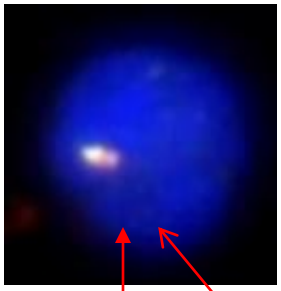
Physiological DSBs & γH2AX in mammalian meiosis



Mouse (image: H.S.)

DNA damage response - DDR

Chromatin modification ⇒ **DNA Repair**



MDC1 P 53BP1

H2A.X

H2

IR

ROS

sensing & signal transduction

M/R/NBN 53BP1

ATM (DNAPK$_{cs}$)

P

HR

NHEJ

RAD50 MRE11 NBN

RAD51 RPA RAP1 BRCA1

DNA PK Ku70/80

cABL

Apoptosis ✝
MDM2, **p53**, Bax

Cell cycle arrest
CHK1, -2; CDC25A,B

Stress response
SAPK

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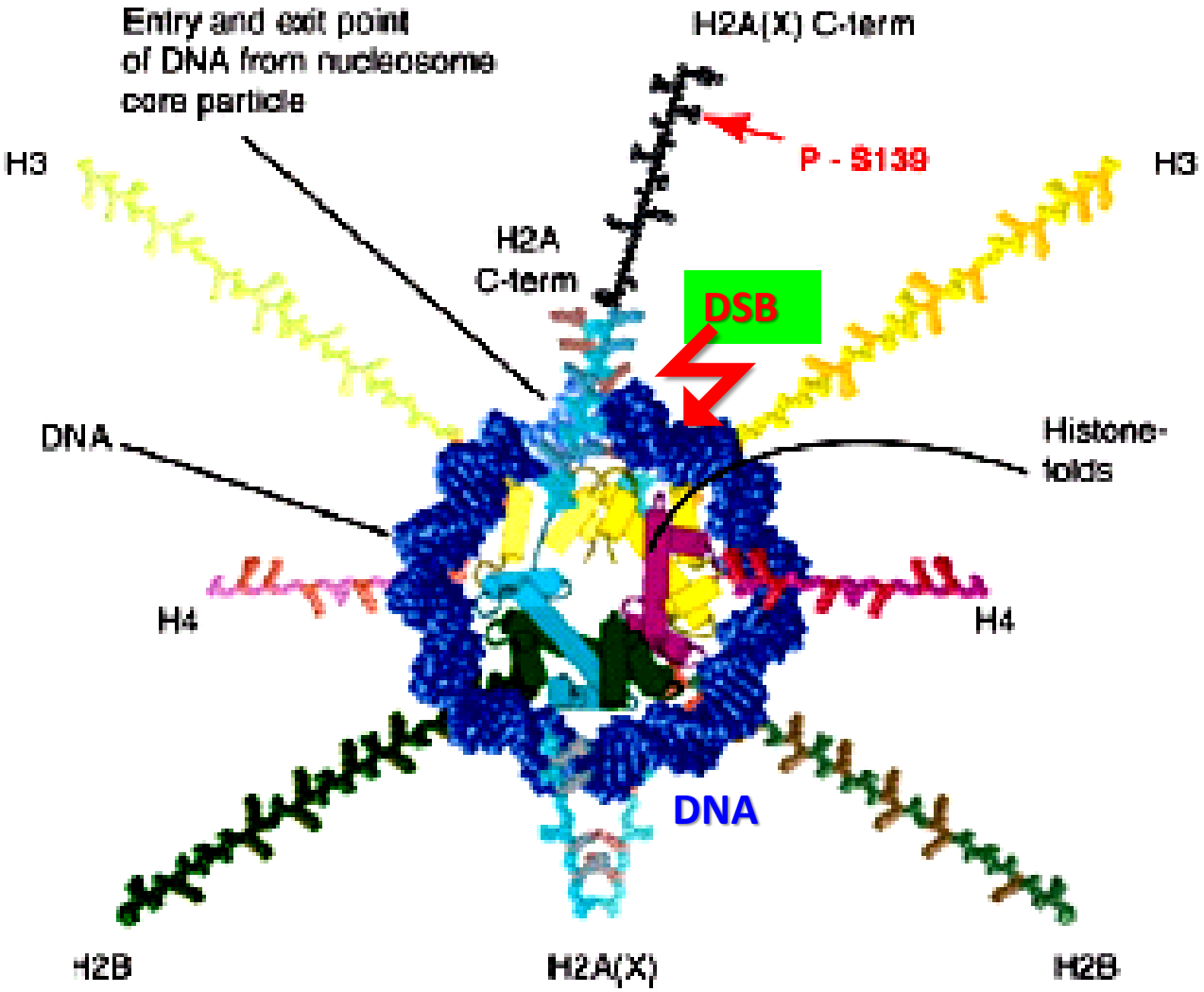
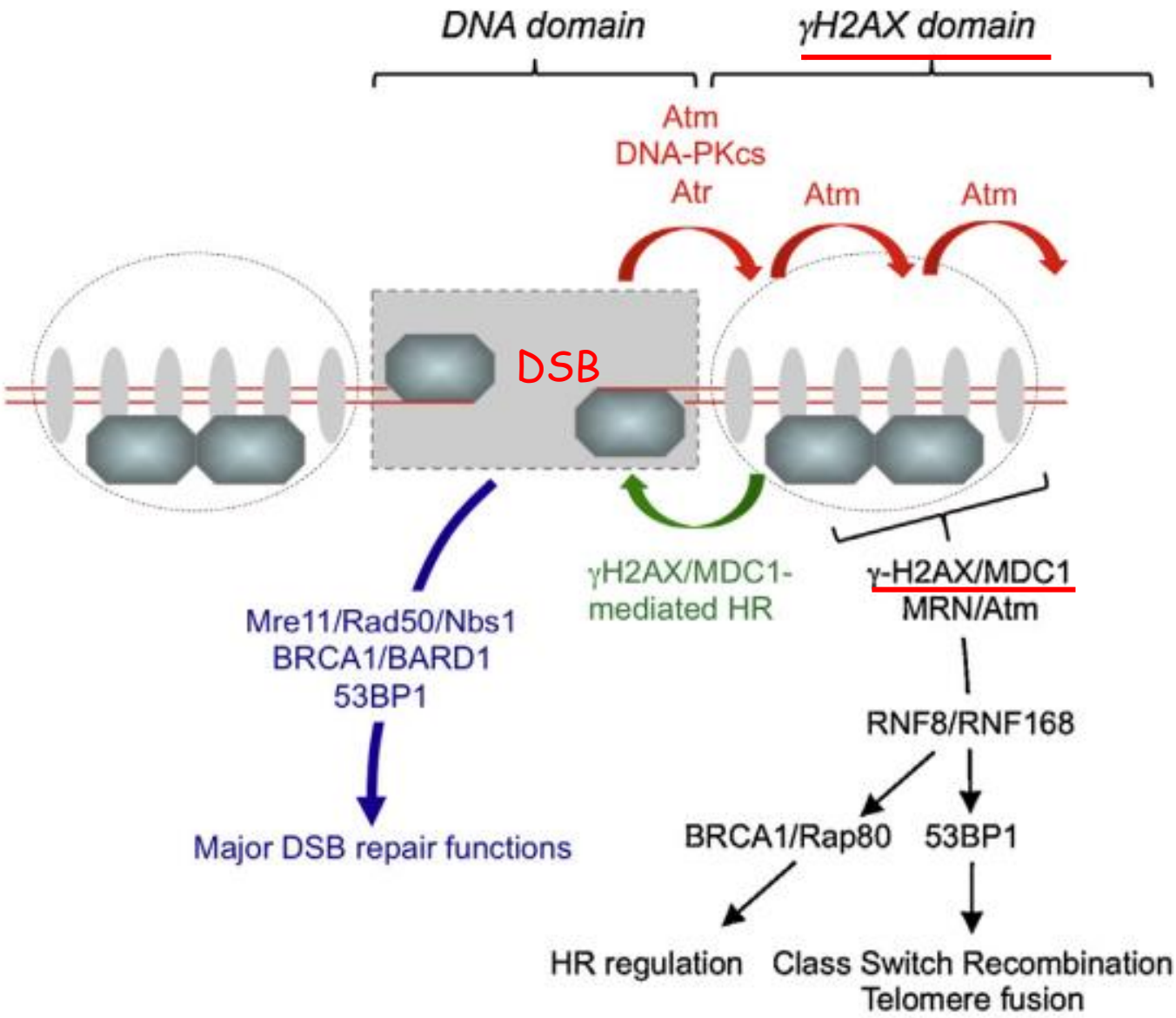
Histone H2A.X in chromatin context



Kinner et al (2008), Nucleic Acids Res. 36:5678-94

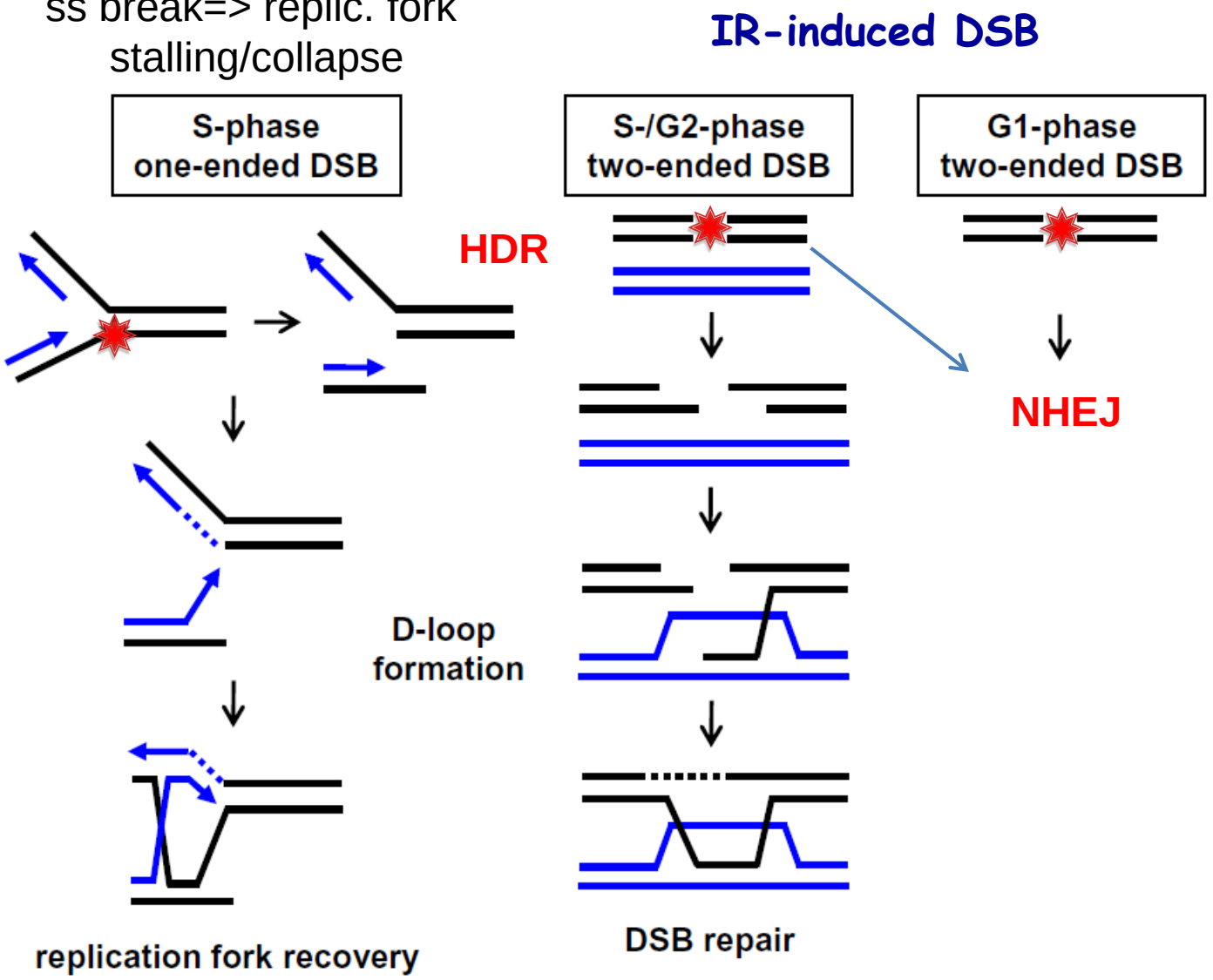
γH2AX forms a chromatin domain @ a DSB



Scully & Xie MR 2013

IR-induced DSBs & repair pathway choice

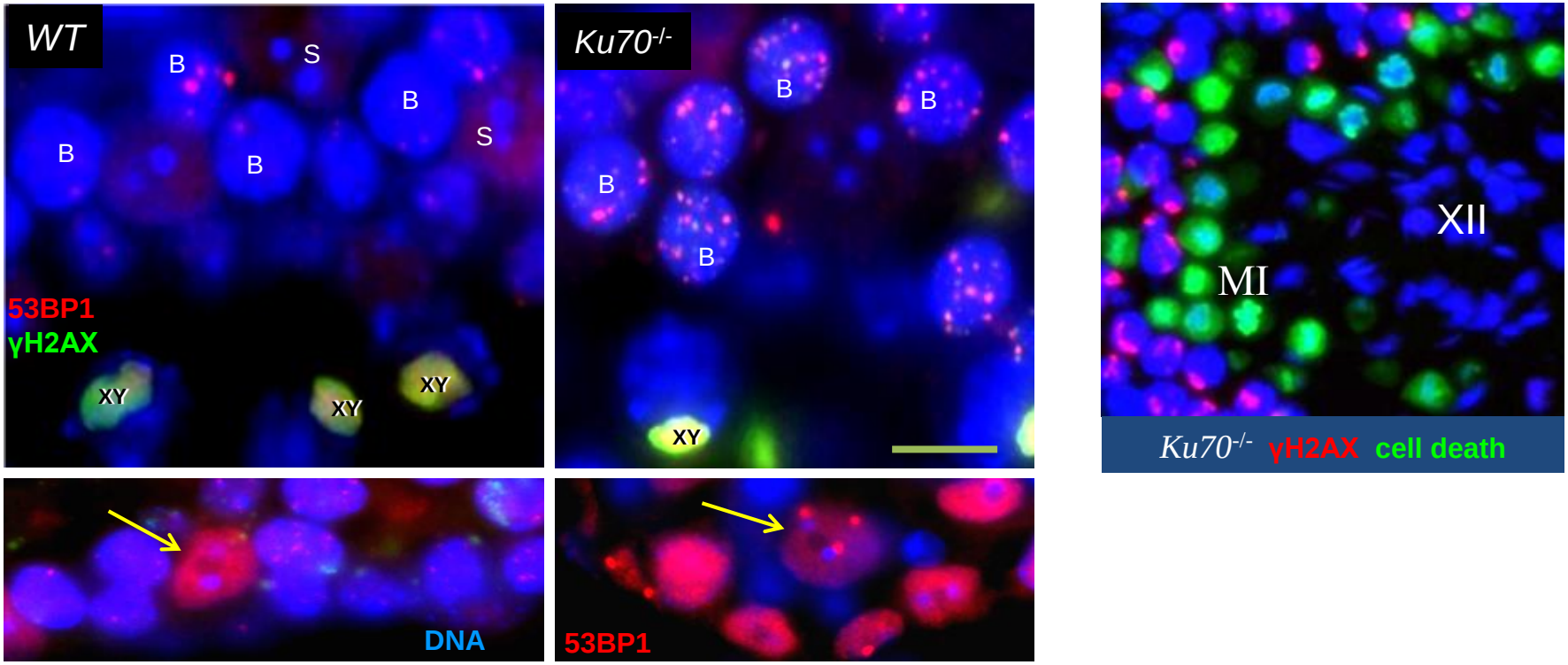
ss break=> replic. fork stalling/collapse



based on Jeggo et al. 2011 Rad Onc

NHEJ protects premeiotic stem cells and meiosis from accumulation of DNA damage

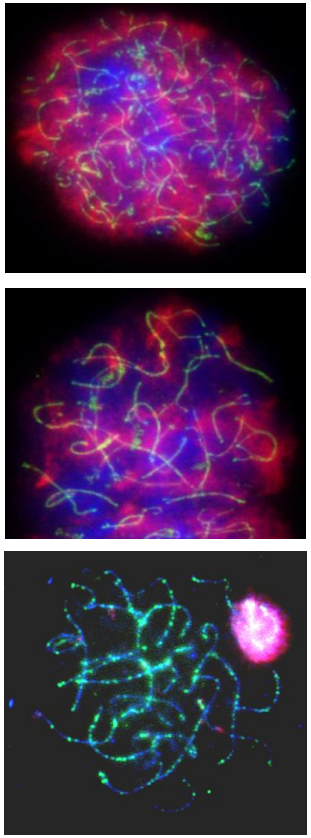
Ku70$^{-/-}$ spermatogenesis



Ahmed, Sfeir, Scherthan J Cell Sci 2013

MEIOSIS: homol. recombination DSB repair during prophase I

γ-H2AX Cytology



γ-H2AX, SCP3
♂ mouse

Liebe et al. 2006 ECR

presynapsis
synapsis
postsynapsis

double strand break — **Spo11**, MRN, Rec102,104,114

5'-3' degradation — Rad50,**Mre11**,Nbs1 (=MRN), Com1

RPA ssDNA complex — **RPA**, WRN, Tex14

ATM, *Mec1/Tel1*

γ-H2AX

Rad51 ssDNA filament — **Rad51**, **Dmc1**, **Hop2** 52, 55/57

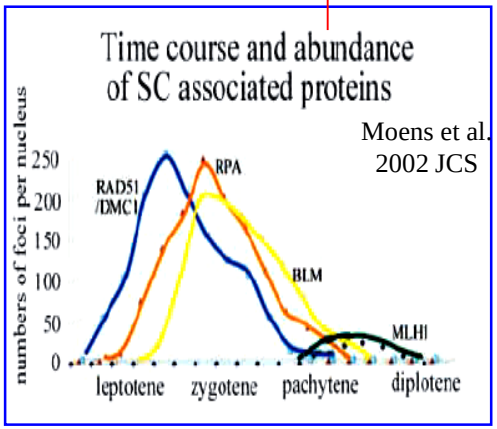
homology search, strand-invasion — BLM/Sgs, Rad51, **Rad54, Tid1** **Rap1**

repair synthesis, branch migration — Rad54

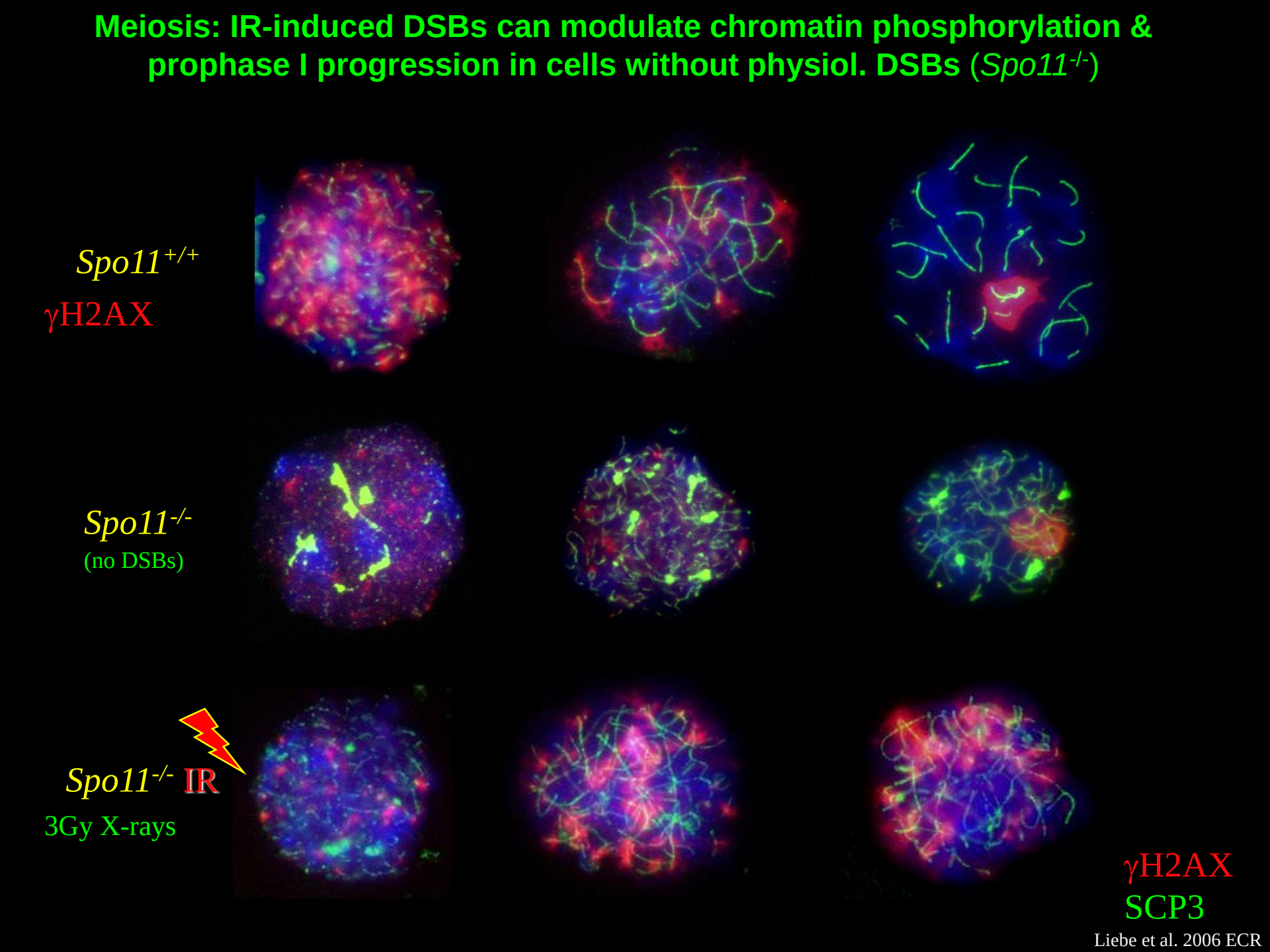
repair synthesis

branch migration — Rad54

resolution to conversion or crossover — **Zip1, Msh4,-5, Mlh1**



Meiosis: IR-induced DSBs can modulate chromatin phosphorylation & prophase I progression in cells without physiol. DSBs (*Spo11*$^{-/-}$)

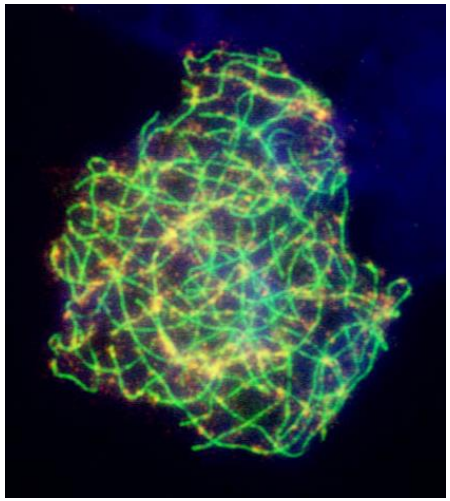


Liebe et al. 2006 ECR

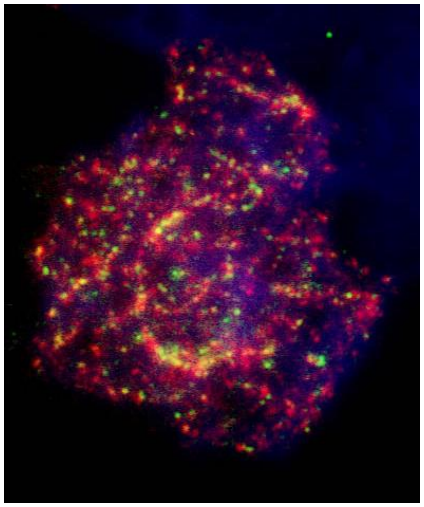
Human meiosis: DNA repair and γH2AX

♀ pachytene

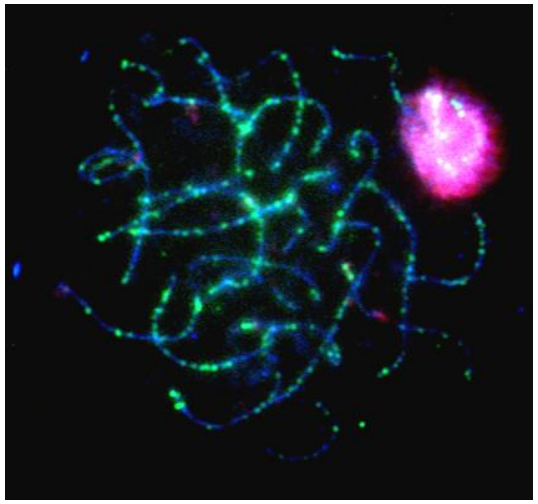
♂ pachytene



♀ SCP1, γ-H2AX, DNA

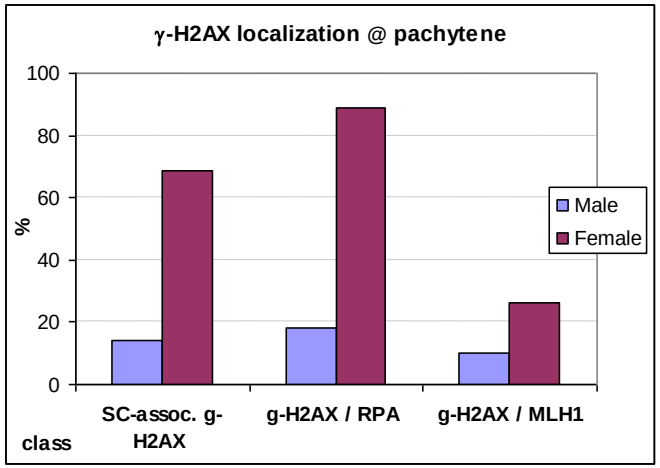


♀ γ-H2AX, RPA



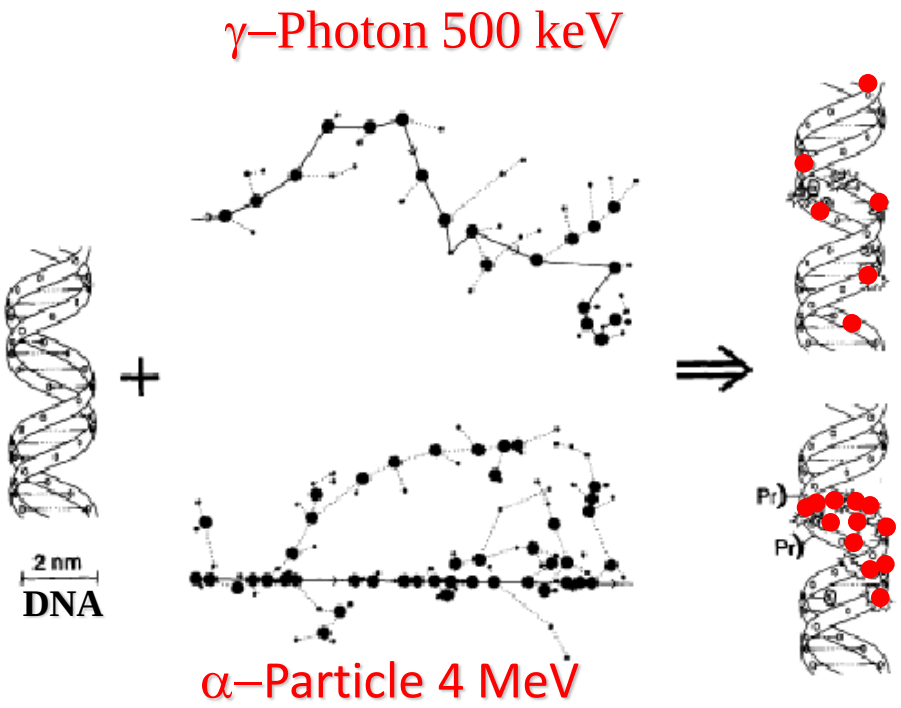
♂ γ-H2AX, RPA, SCP3

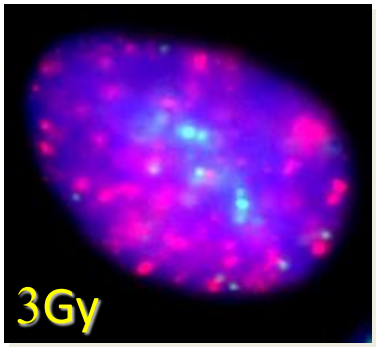
XY body



Roig et al. 2004, Chromosoma

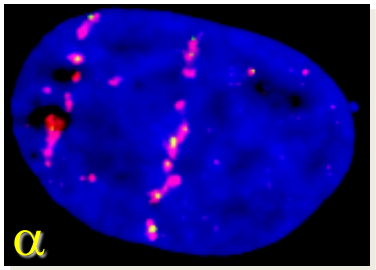
IR induces chromatin & DNA damage





γH2AX
DNA
Kinetochore

Low LET



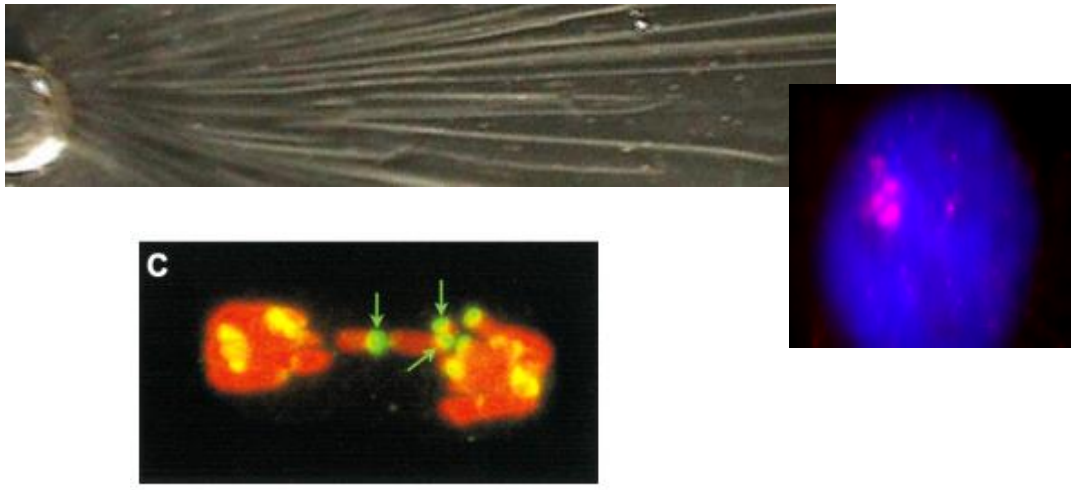
γH2AX
DNA
Rad51 (DSBs)

high LET

after Goodhead 1994, Aten 2003, Scherthan 2004

γH2AX focus enumeration for monitoring ionizing radiation exposure

⇒ Method for quick detection of radiation-induced damage: DNA-repair focus test



Rogakou et al. 1999, JCB

Sedelnikova et al. 2002 RR
Rothkamm & Löbrich 2003 PNAS

Double-stranded DNA breaks (DSBs) & γH2AX

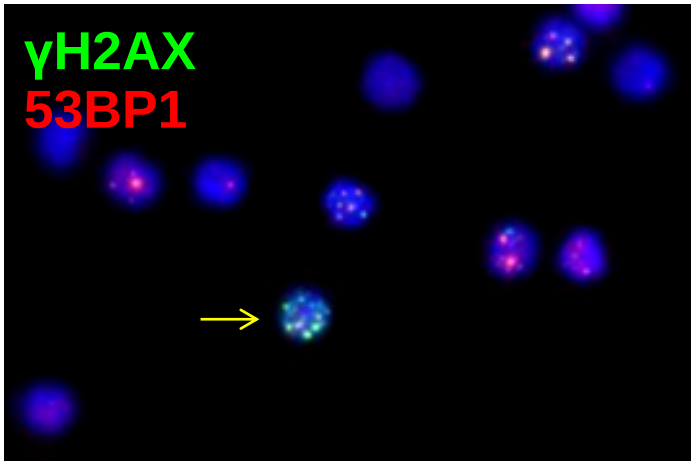
• IR-induced DSBs – ATM & DNAPKcs kinases phosphorylate the histone 2 variant H2A.X within minutes around the site of a DSB to result in γ-H2AX foci in the nucleus (Rogakou et al. 1998)

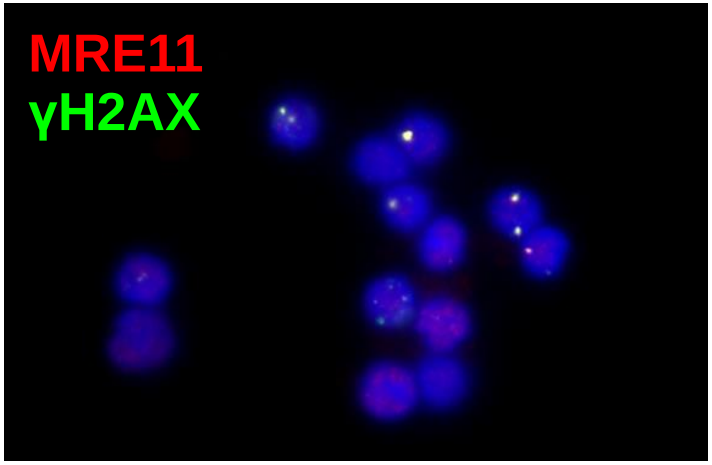
Repair half-time in the order of min –hours

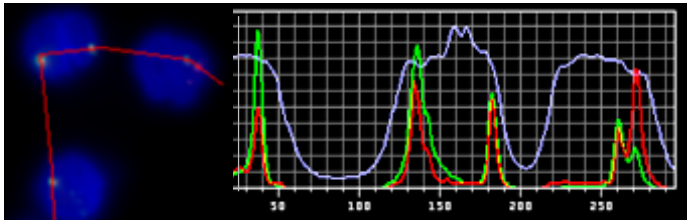
In non-cycling tissue cells (e.g. skin) some DSBs (complex DNA damage) may persist for days/weeks

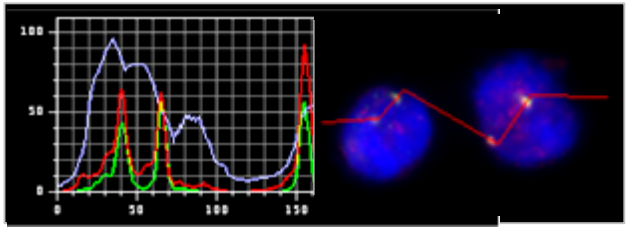
! Absence of dephosphorylation can lead to focus persistence wo DSB

Irradiation: HR repair factors colocalize at DSBs

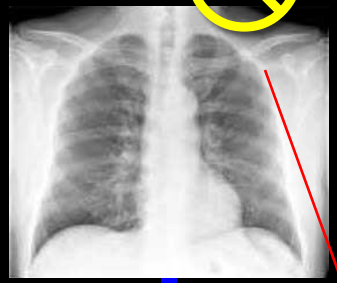




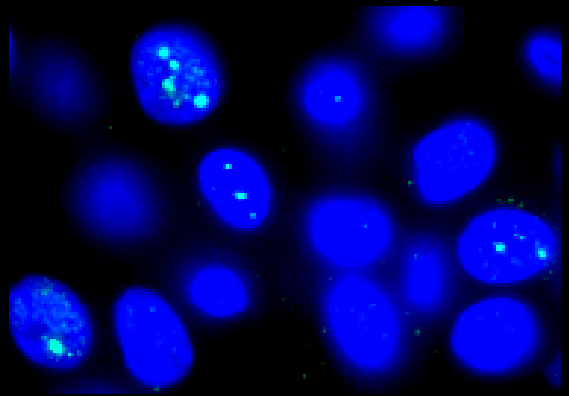




Lamkowski et al. 2014 PlosOne

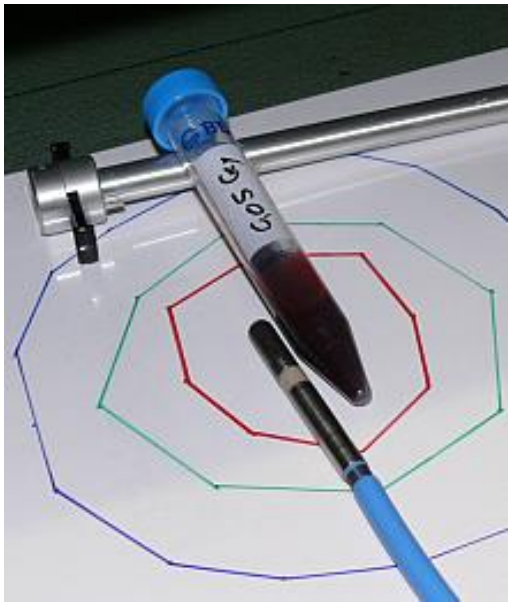


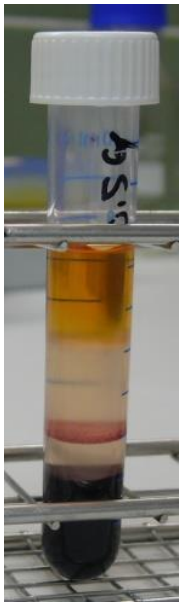
10 µGy | 100 µGy | 1 mGy | 10 mGy | 100 mGy | 1 Gy | 10 Gy | 100 Gy



after Löbrich 2003

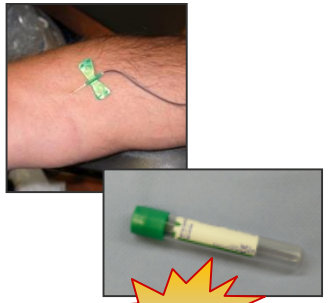
METHOD – in vitro blood irradiation

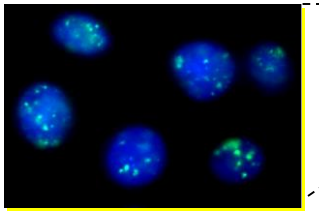


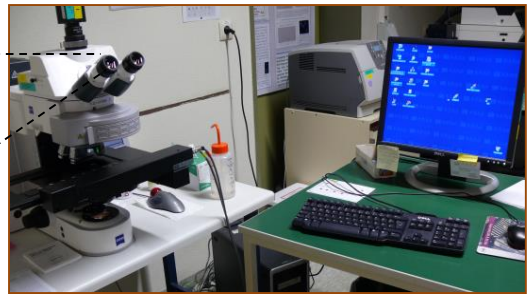


Method

1. Blood sampling
2. Leukocyte isolation
3. Fixation (store & transport [?])
4. Immunofluorescence staining
5. Analysis







Scherthan et al. 2007 Wehrm .Mschr .

Focus Assay - Staining

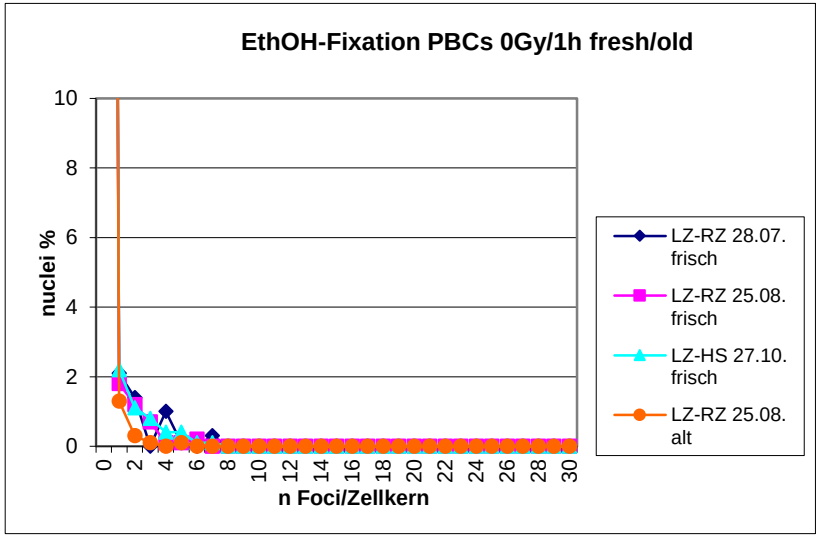
Most labs: immediate staining after IR & incubation / blood cell isolation

1) Bring the cells to a me-silane coated glass slide (cytocentrifuge; drying).
2) Fix with (1%-) 3.7% formaldehyde (!)
3) Extract with TritonX100
4) add primary antibody $\Rightarrow$ detection

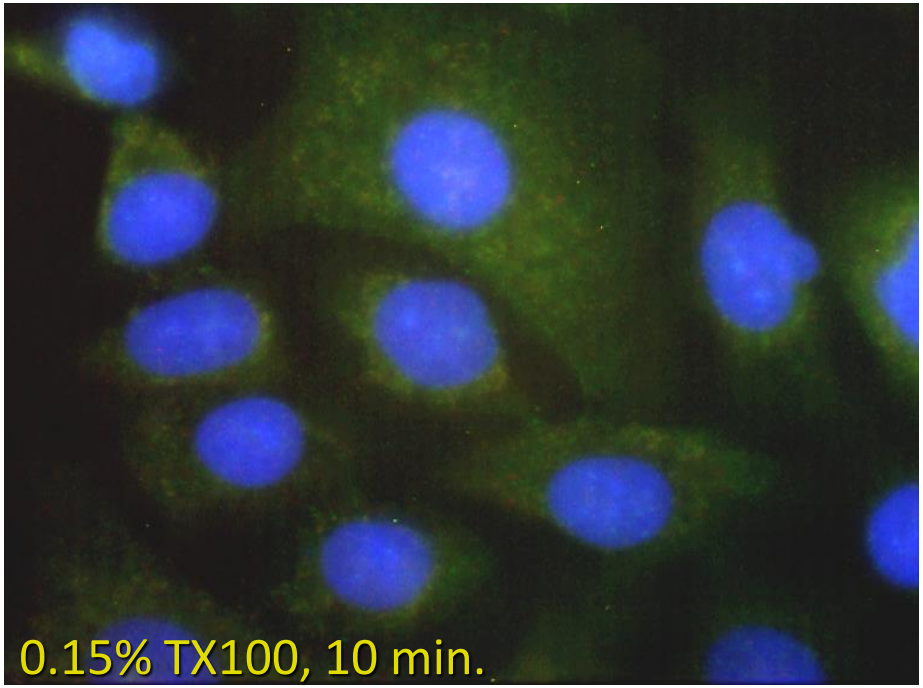
Sample storage ? – yes (but) !

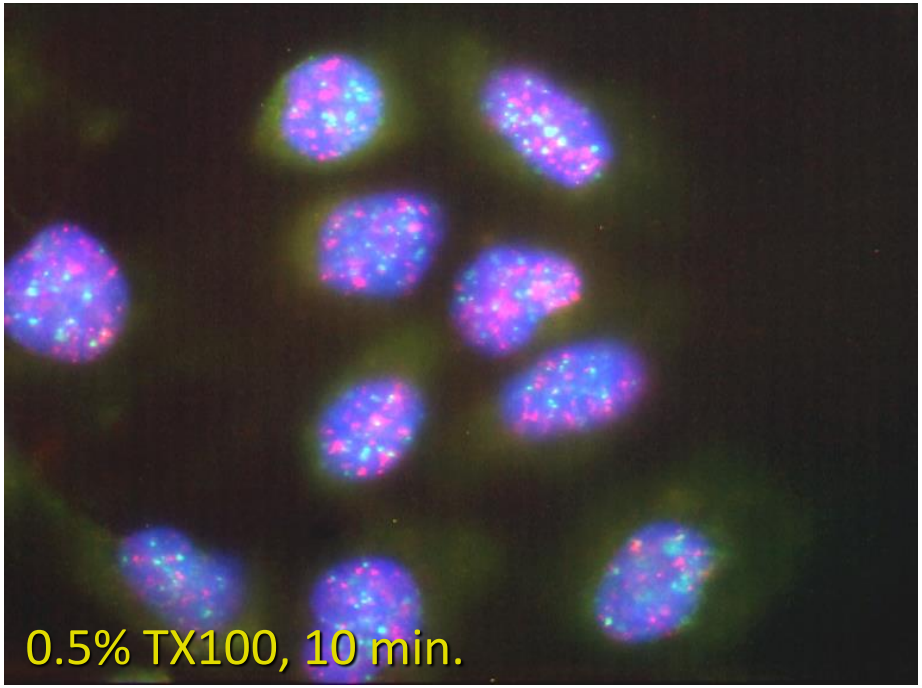
Ethanol fixed cells can be stored and shipped

- ! only compare with similarly treated (time & temp) controls.
  - We use 70% Ethanol @ -20°C (Lassman et al. 2010 Nucl.Med.)



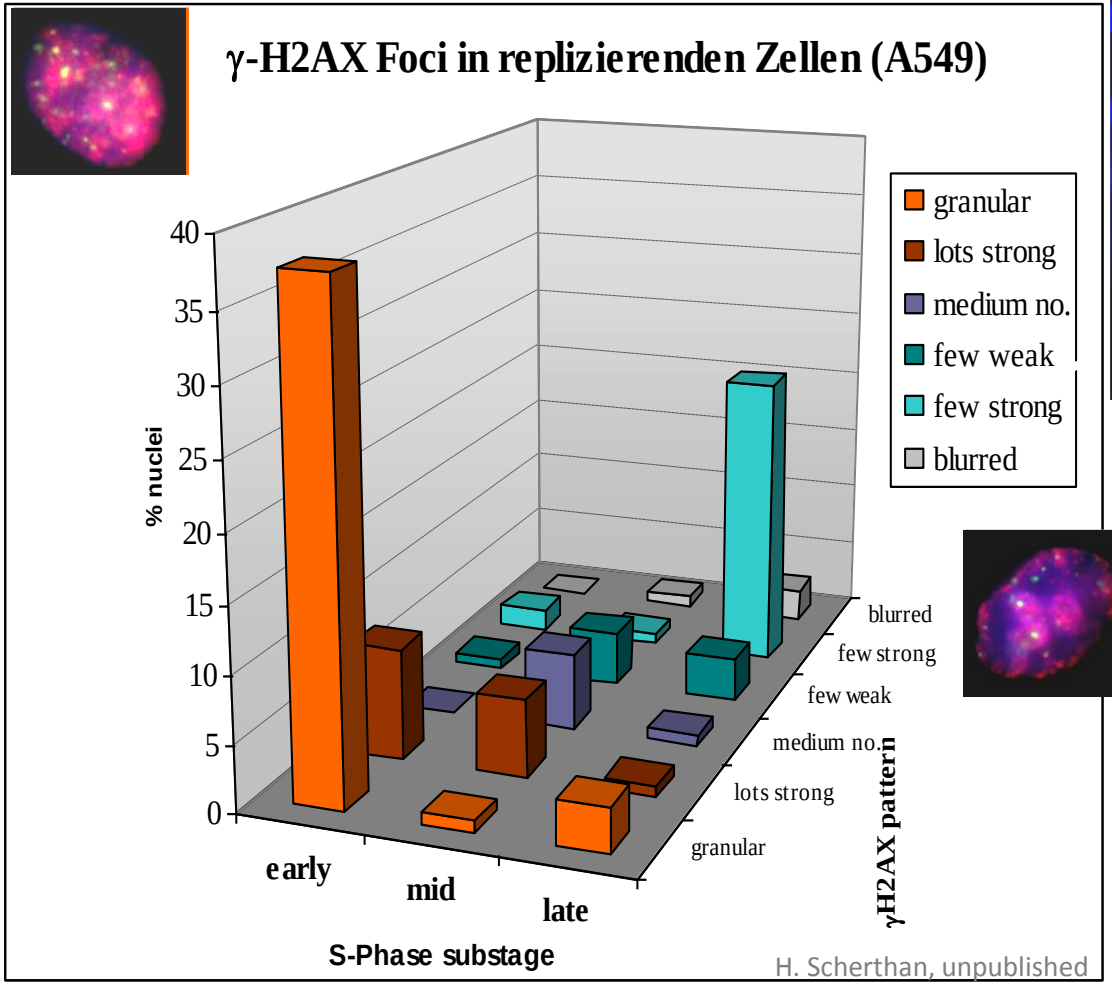
Methodological pitfalls: Fixation/extraction determines the success of γ-H2AX detection

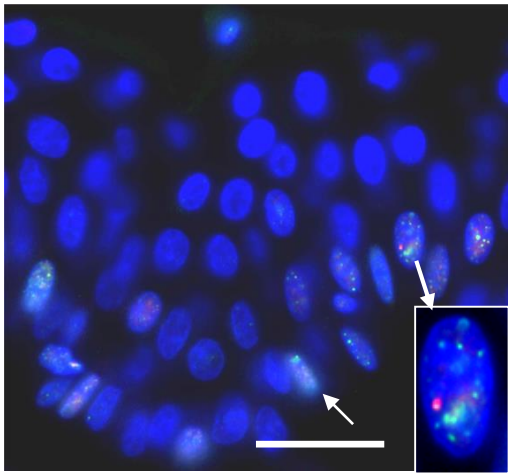




kinetochores
γ-H2AX

Replicating cells display classes of foci

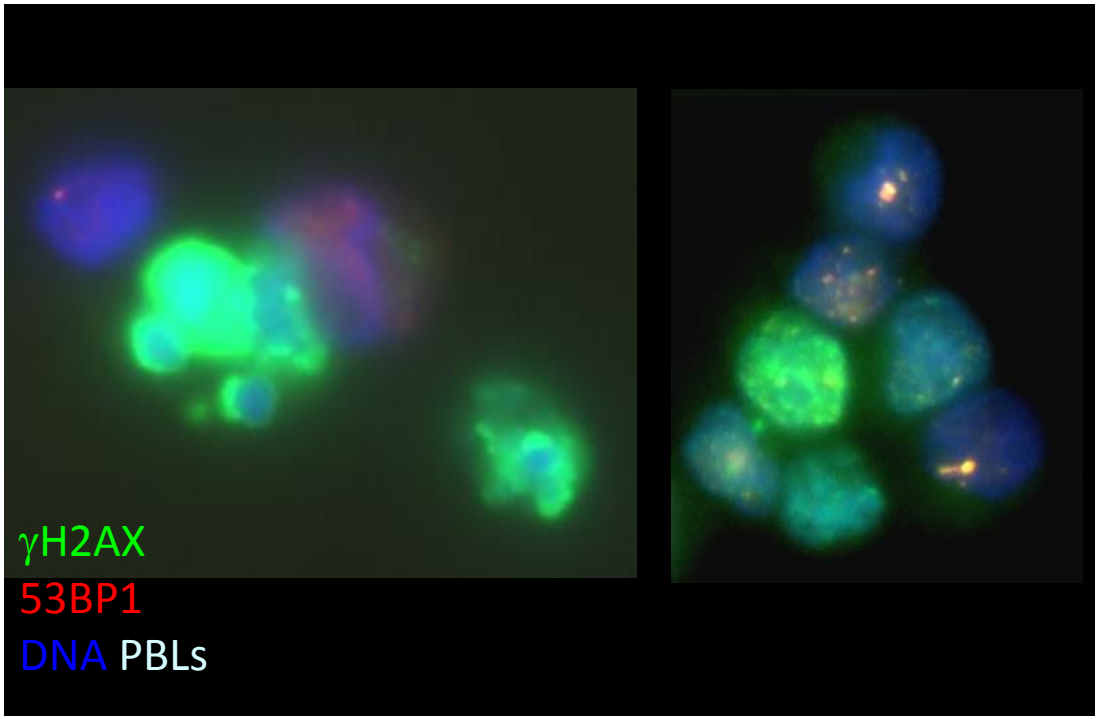


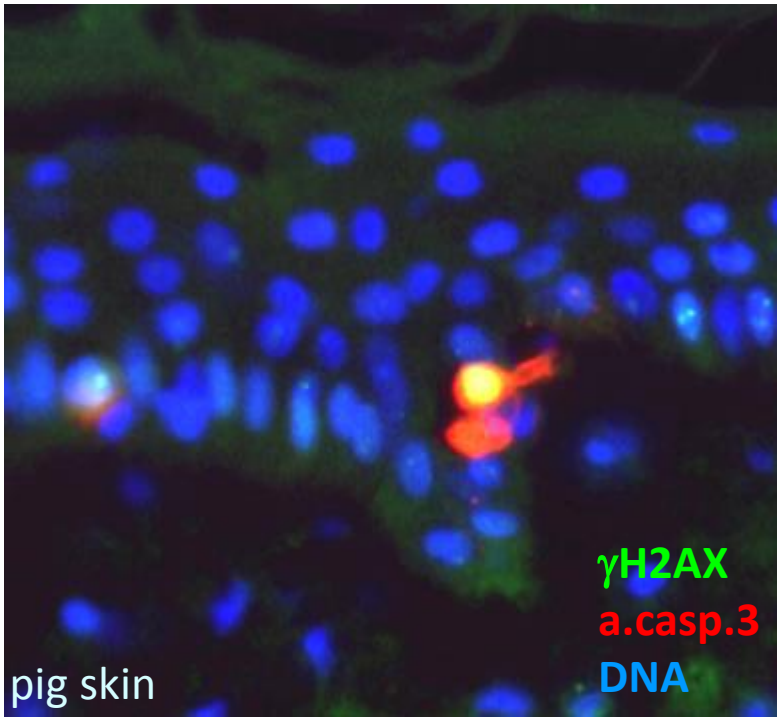


Minipig skin, Ahmed et al . 2012
PLoS One 7:e39521

BrdU
γH2AX
DNA

Apoptotic cells display strong γH2AX fluorescence



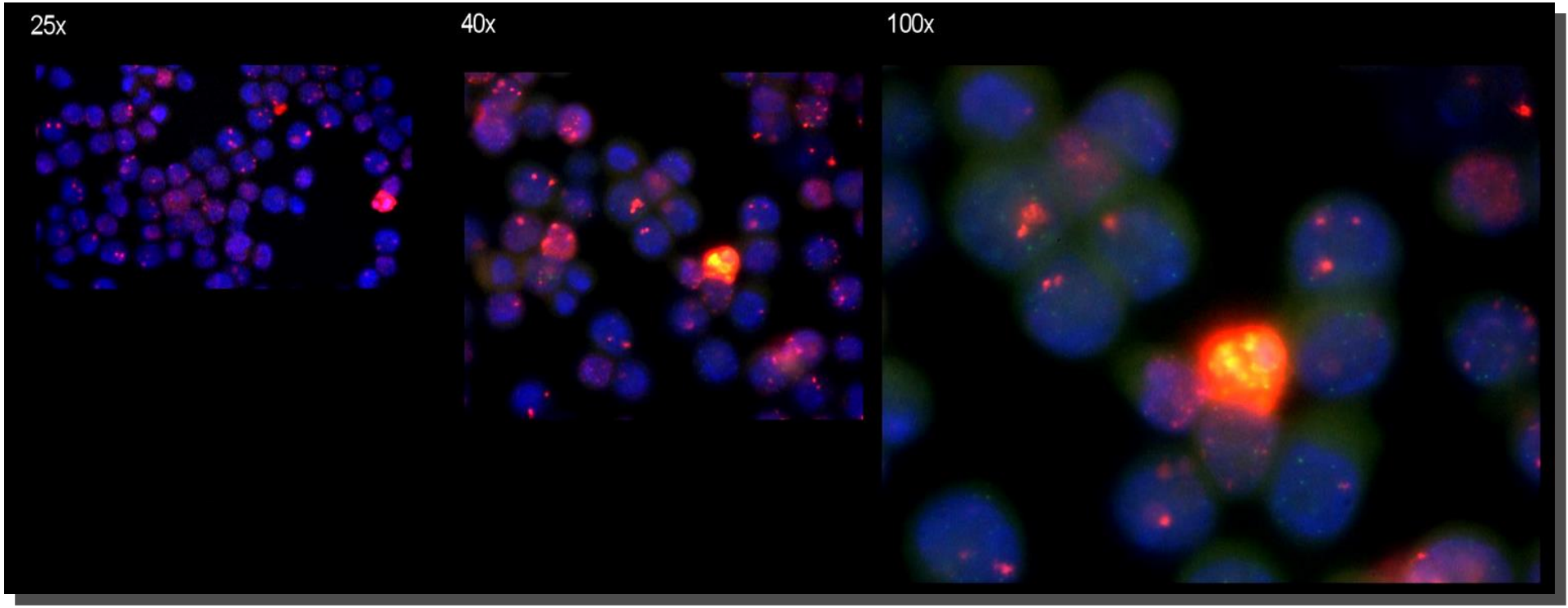


Another source of background: **granulocyte autofluorescence**

Ahmed et al . 2012 PLoS One 7:e39521

γ-H2AX Focus assay: IF

n nuclei = 68 (x4,25) **42 (x2,6)** **16**



Research: Enumerate (manually) foci numbers in 40 positive cells, or in up to 800 negative cells (Rothkamm & Löbrich 2003; & others)
Rapid diagnosis: enumerate (50) - 100 cells (Multibiodose)

γH2AX fpc linearly correlate with doses < 2Gy (30min pIR, fibroblasts)

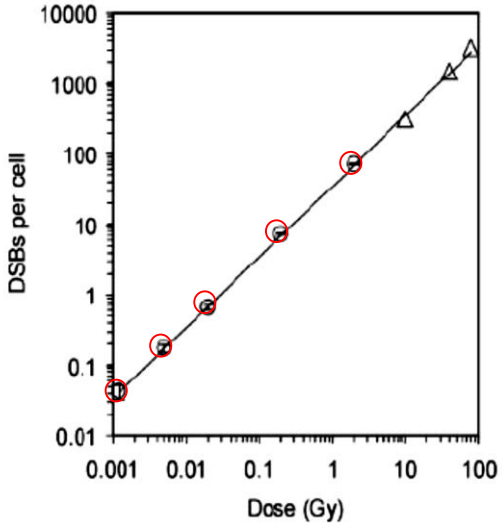
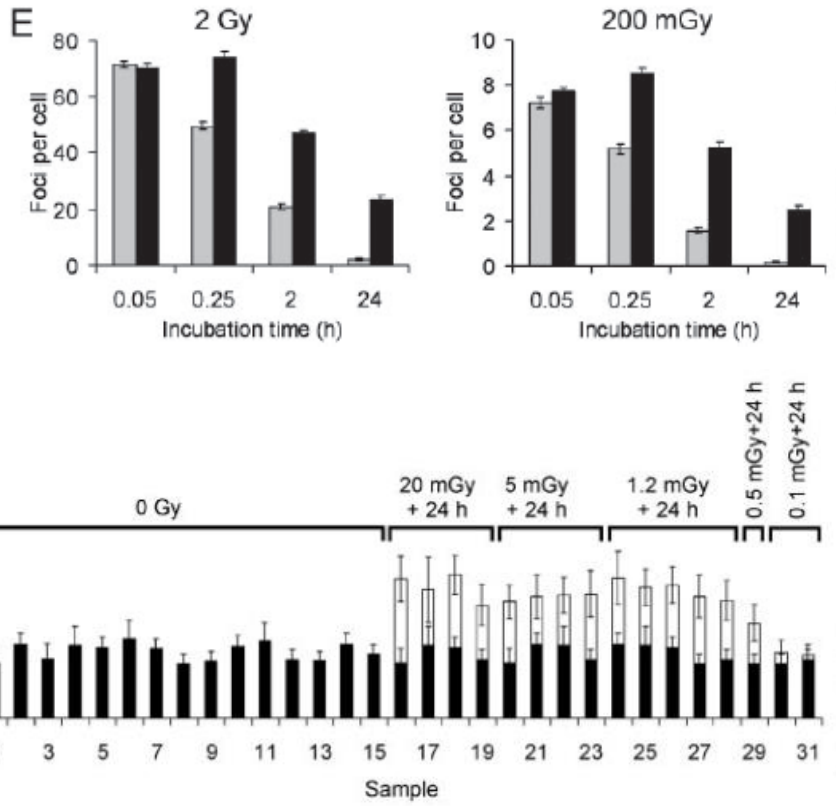


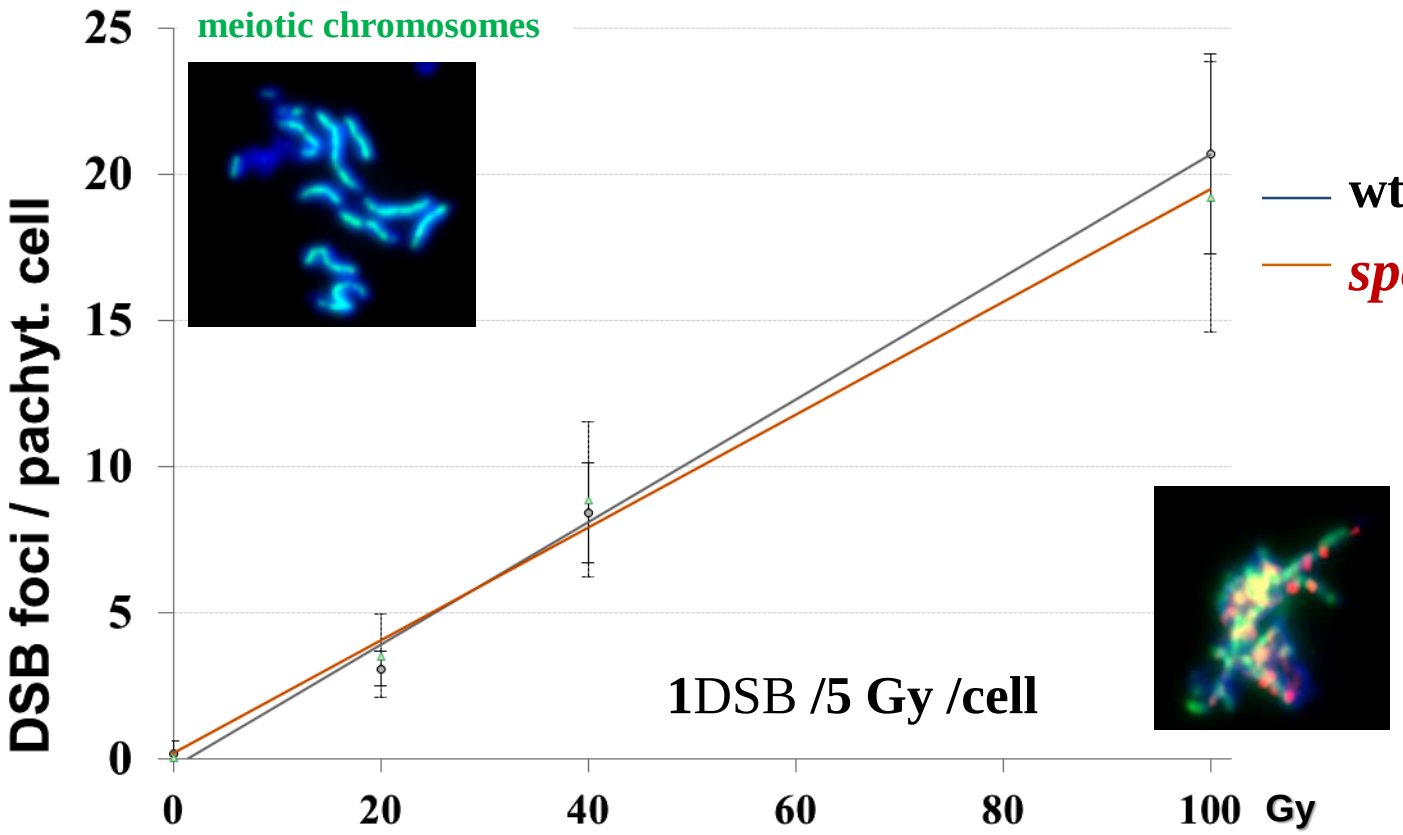
Fig. 2. DSB induction in MRC-5 cells. γ-H2AX foci were counted 3 min after irradiation, and the mean values of foci per cell are shown (circles). Triangles represent DSB induction data obtained from PFGE analysis. The line is a linear fit to the data points with a slope of 35 DSBs per cell per Gy.



- Rothkamm and Lobrich 2003 PNAS: ~40 γH2AX foci at 5‘ /1Gy; ~7foci @ 0.2Gy (35DSB/Gy) - fibroblasts
- Lobrich 2005 PNAS: ~20 γH2AX foci/lymphocyte

Direct visualization of IR-induced DSBs - lessons from yeast

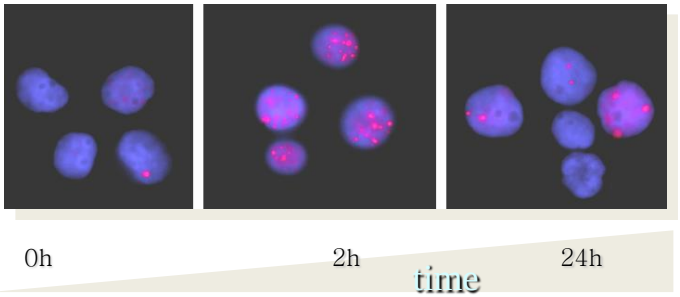
(3'-end labeling)



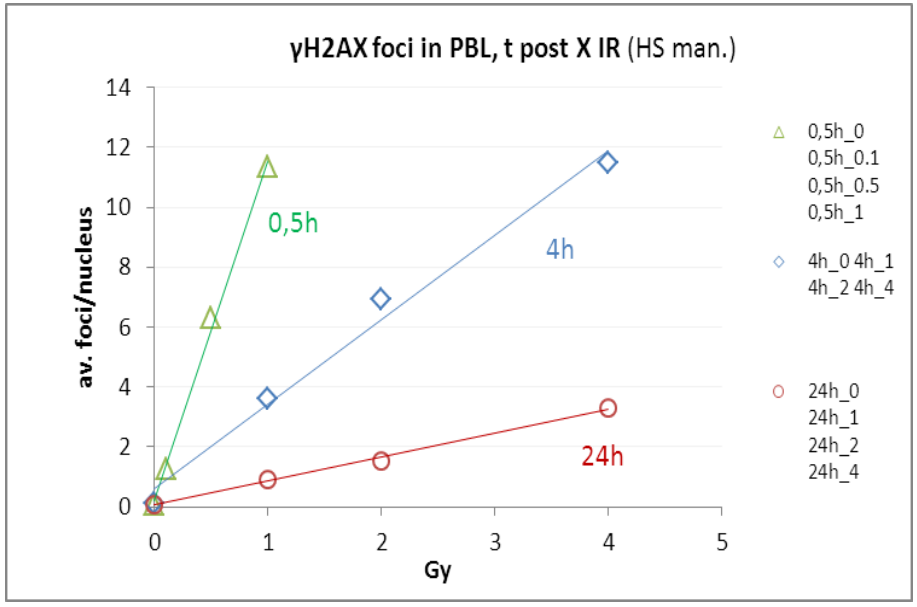
40DSBs @1Gy in human ≈ 40 DSBs @ 200Gy in yeast nuclei

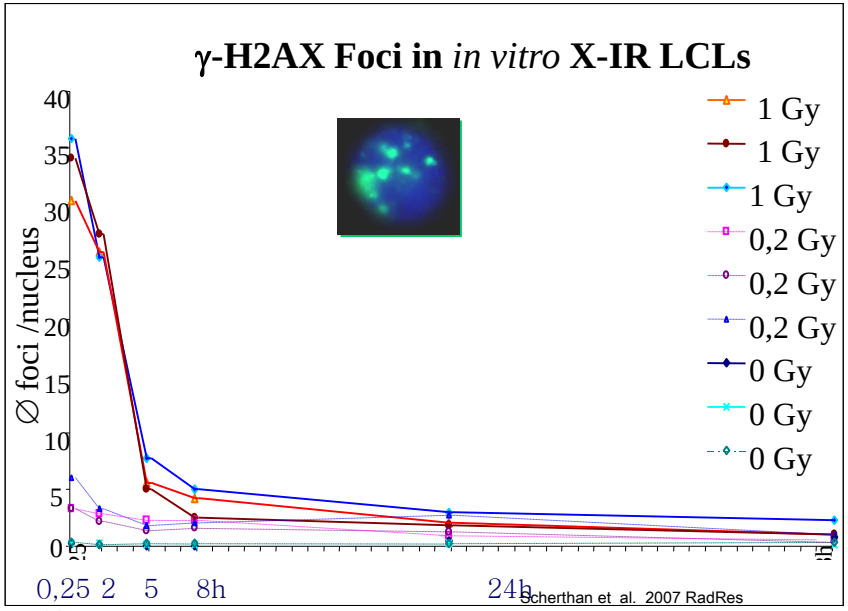
Illner & Scherthan 2013 PNAS

DNA-Repair Foci in Leukocytes: sensitive, but rapidly declining marker of IR exposure

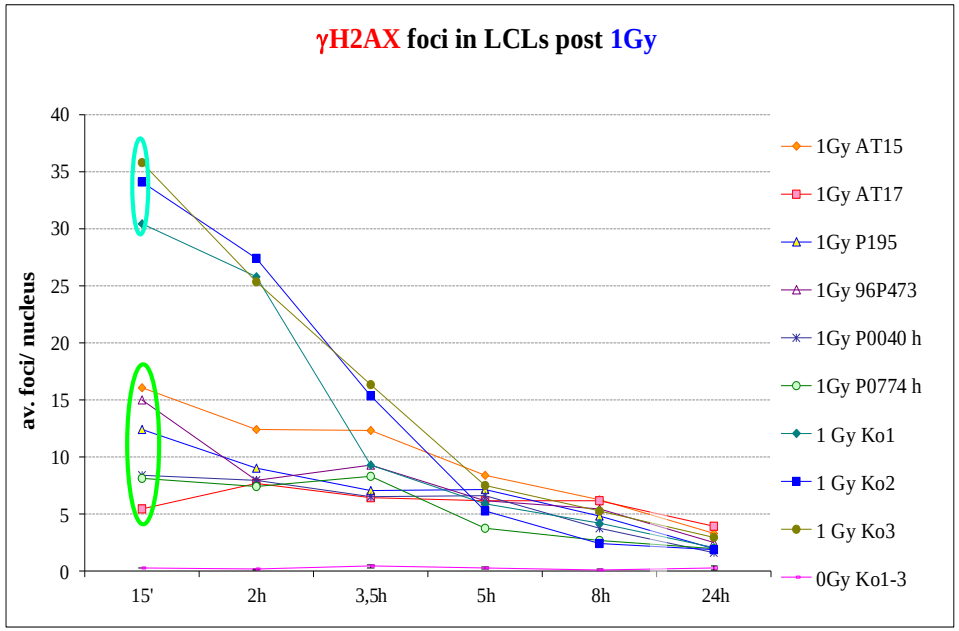


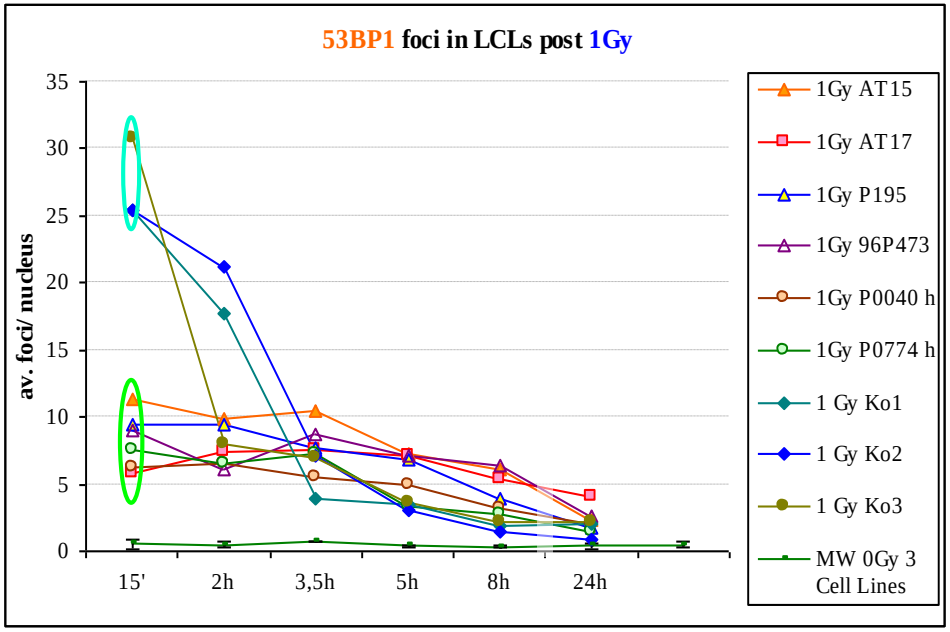
Repair foci
DNA
(nuclei)



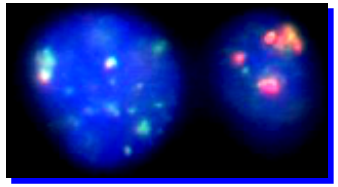


Genetic background influences RIF formation – effects of NBS1 or ATM deficiencies





All cell lines that lack ATM or NBS1 fail to induce the full level (≥30) of γH2AX or 53BP1 foci 15' after 1Gy IR. Control

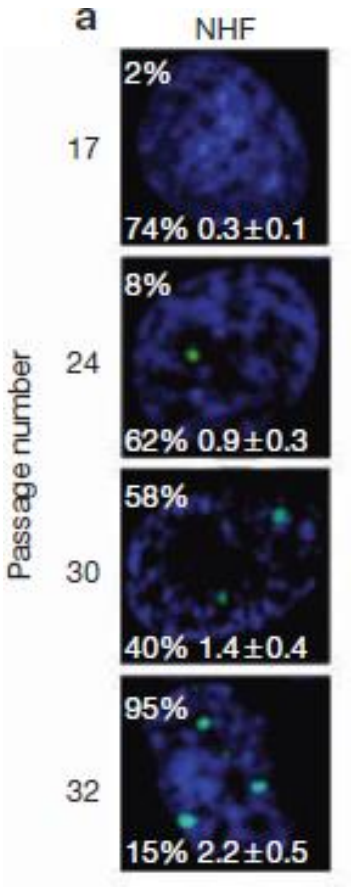


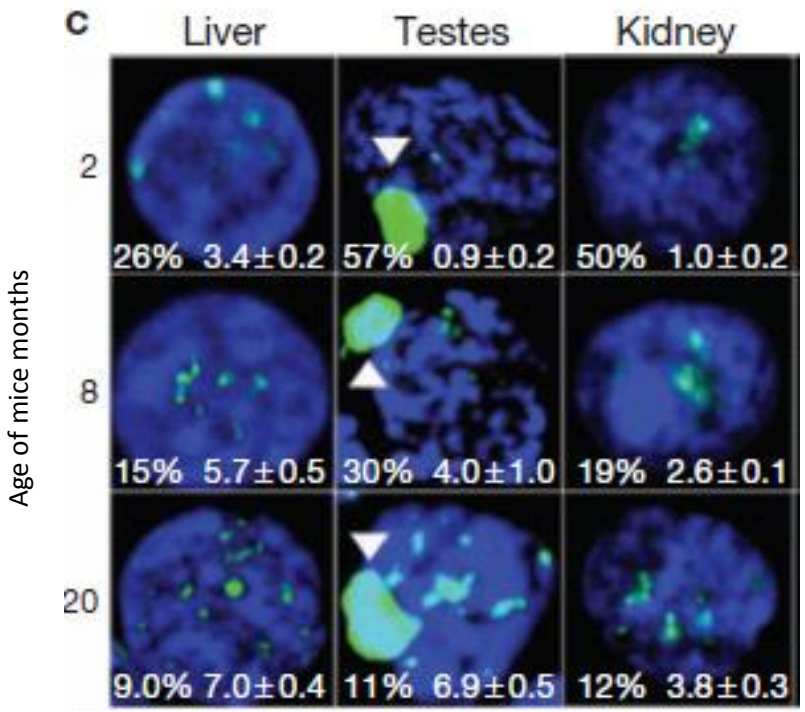
H. Scherthan, unpublished

Senescent cells contain increasing # of persistent γH2AX foci

Pittfalls:

- **Tissues with endogenous DNA damage, such as testis, lymph nodes**
- **aging cells**
- **replicating cells**

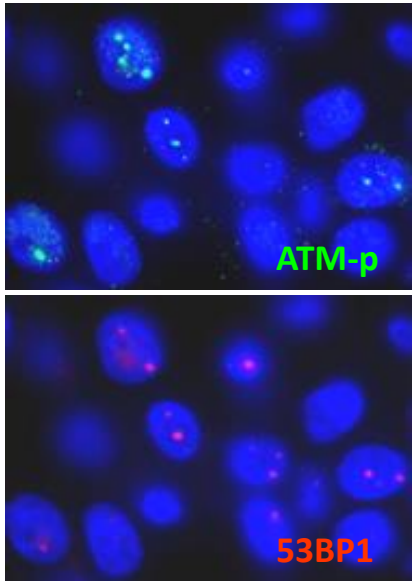


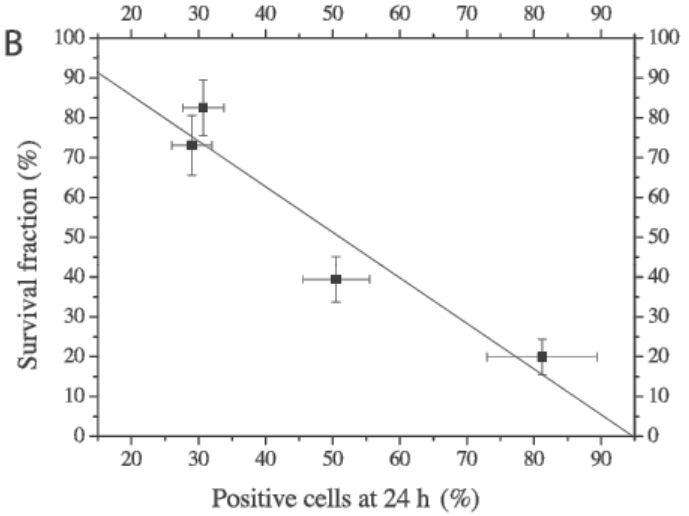


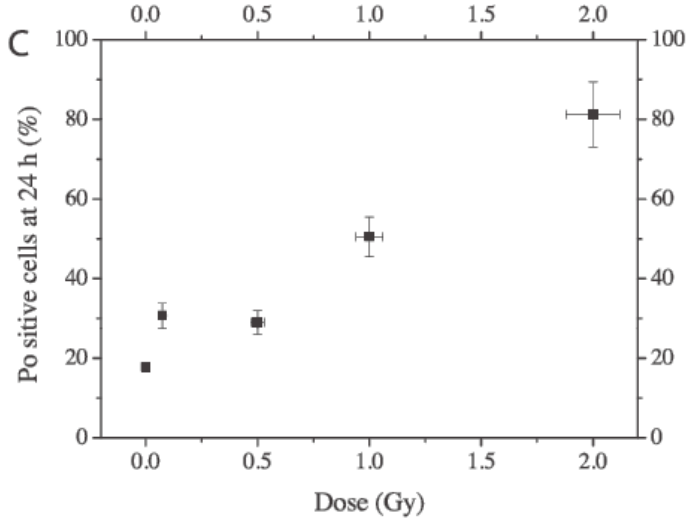
% focus-free cells
av. focus # in +cells

Sedelnikova et al. NCB 2004

The fraction of cells with γH2AX foci 24h after IR correlates with clonogenic survival

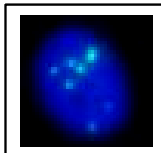




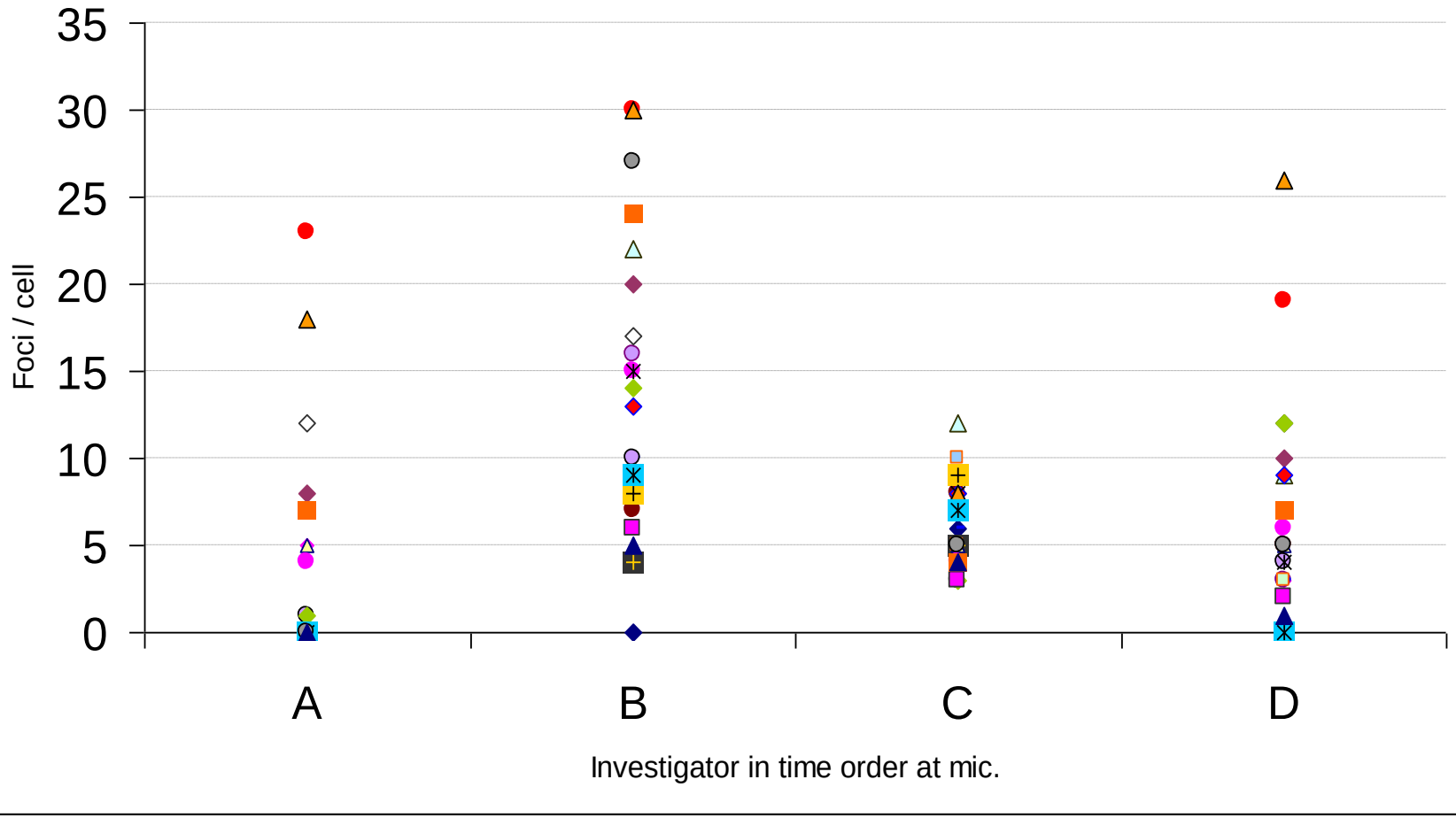


Wéra et al. Rad Res. 2013
Menegakis et al. IJRB 2009

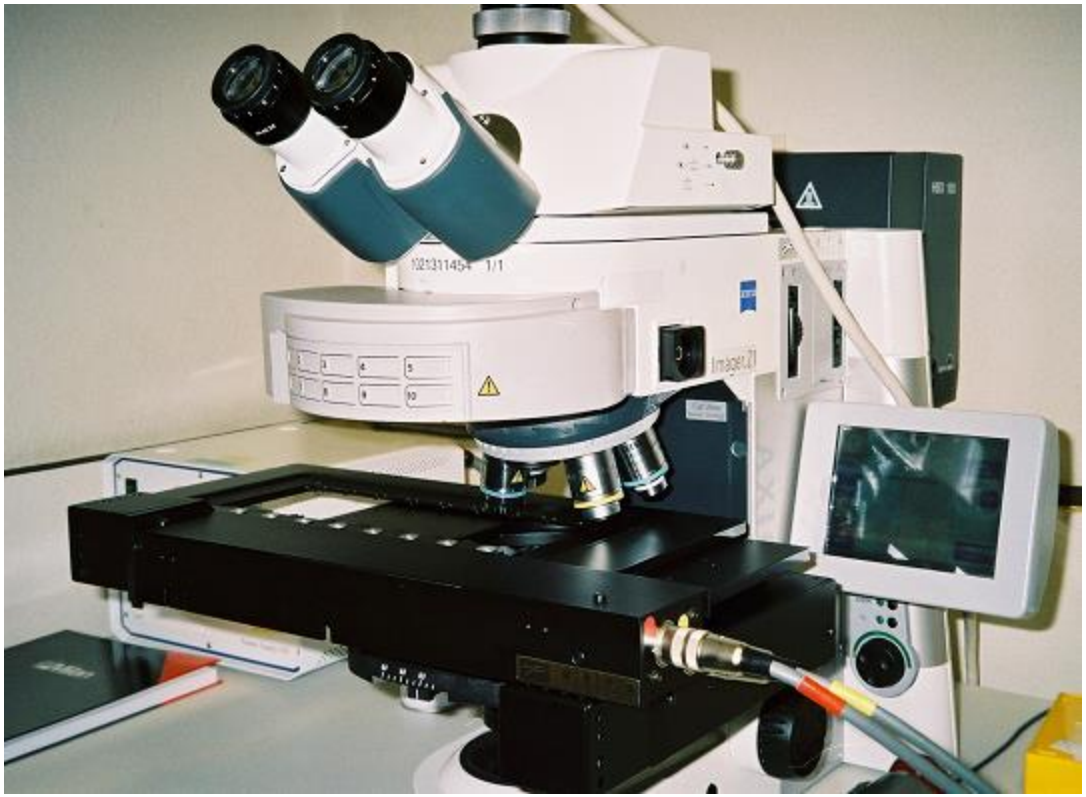
Variability in manual analysis



Focus assay: variation among investigators => automation

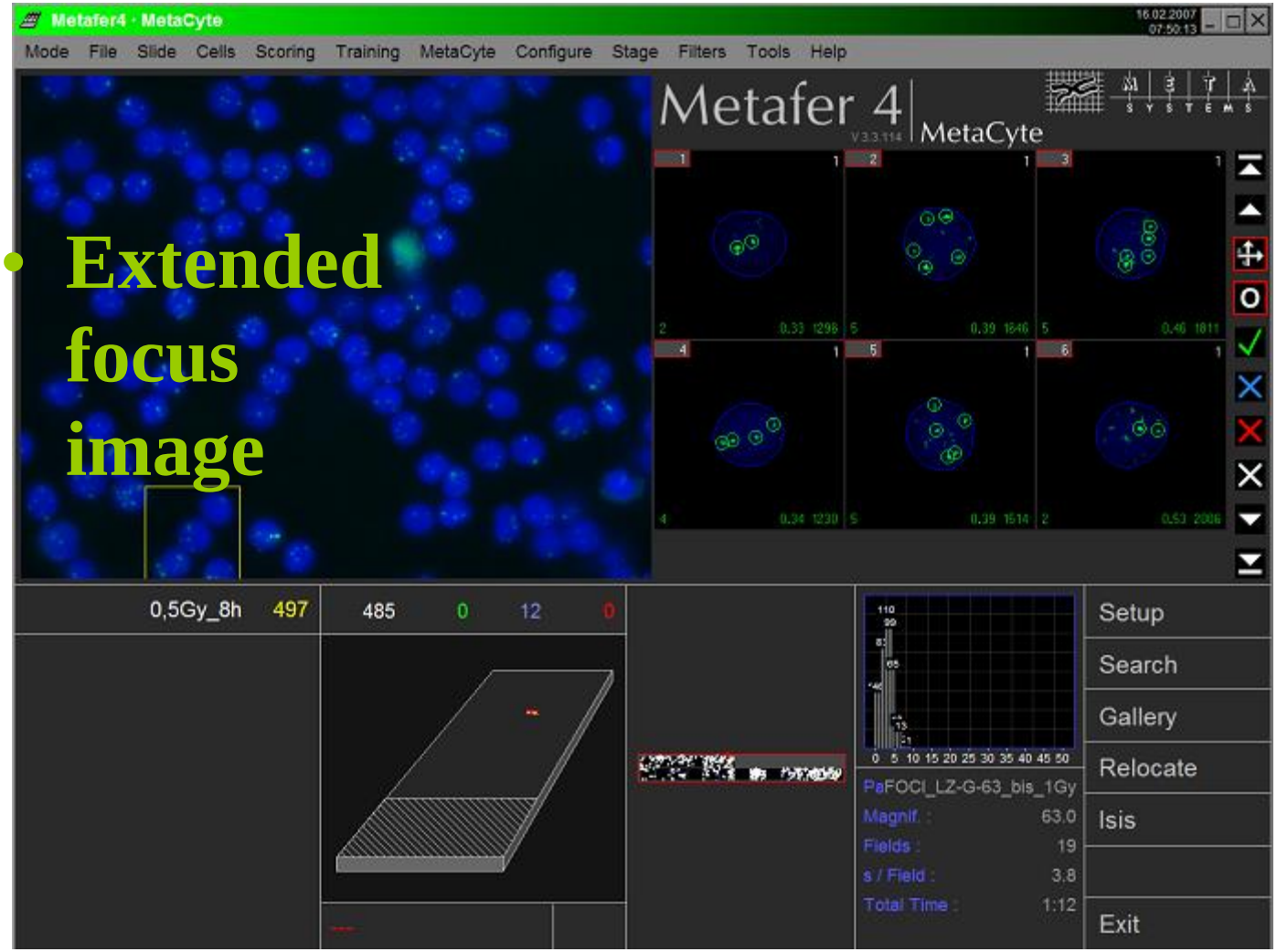


Analysis / semi-automated image capture and processing



- **motorized mic**
- **motorized slide table**
- **e.g., MetaSystems fluor. imaging sys.**

Computer aided focus analysis



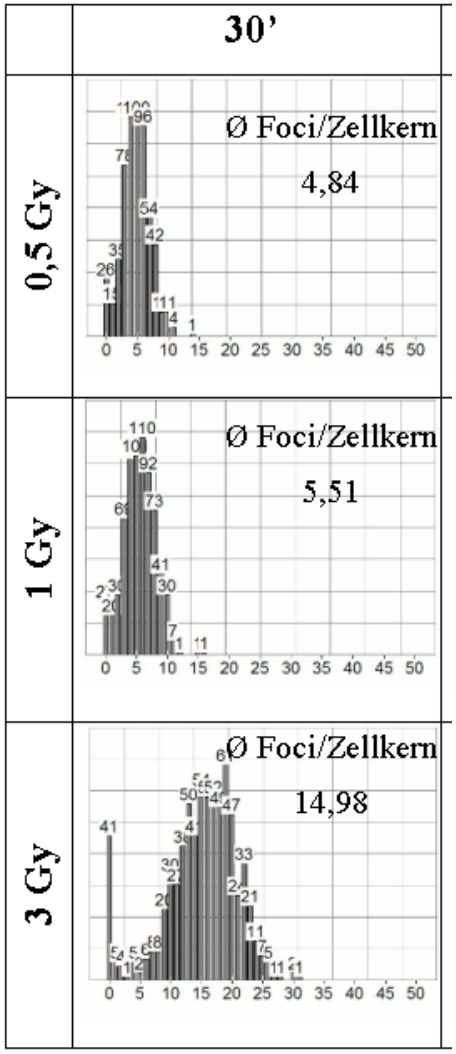
- **Extended focus image**

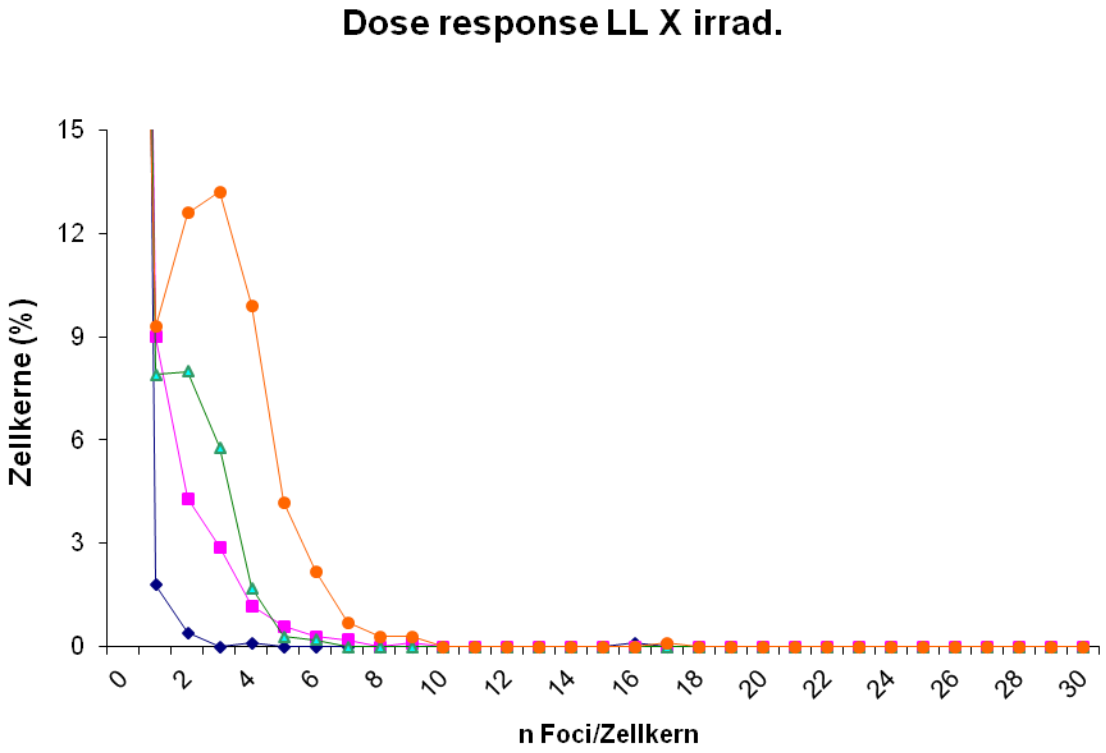
- **Single cell analysis**

- **Data output**

Automated scanning & image analysis – not so variable?

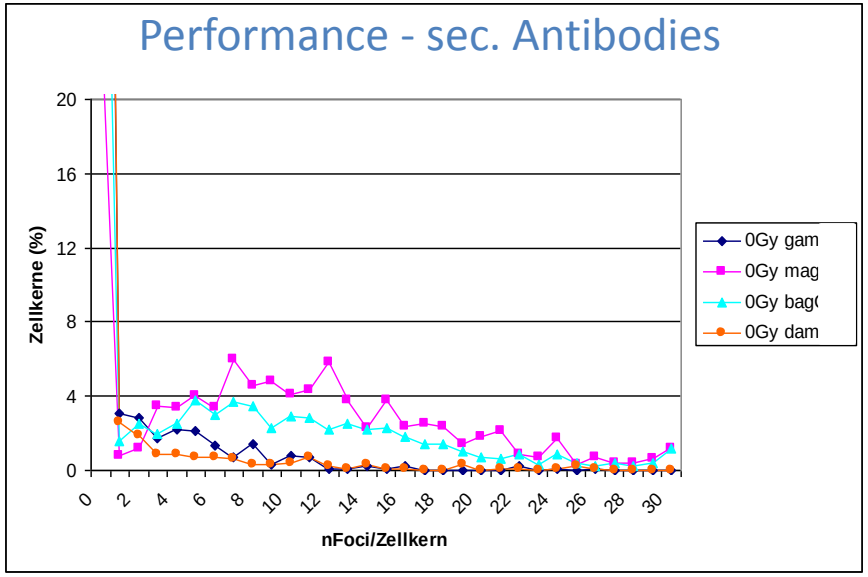
Dosisbereich 0,5 bis 3 Gy:

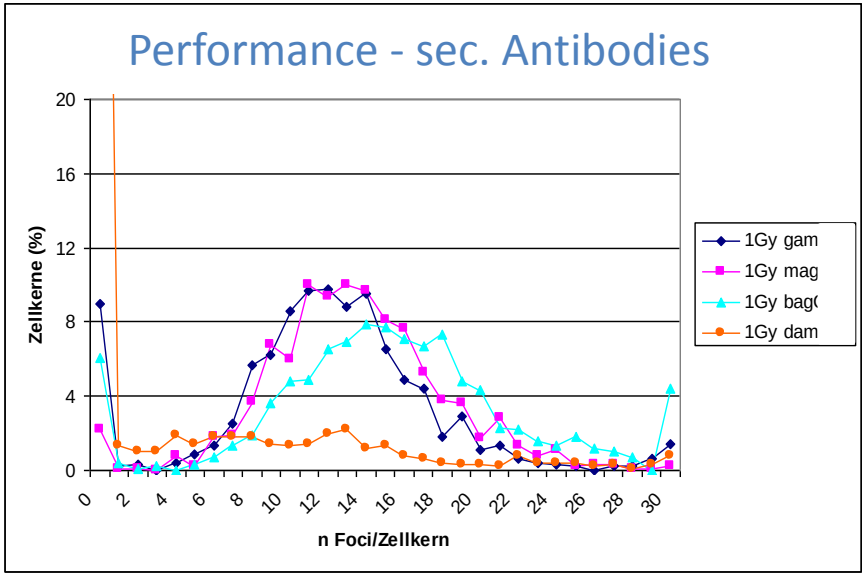




Reagent-induced variation

different secondary Abs –> variation

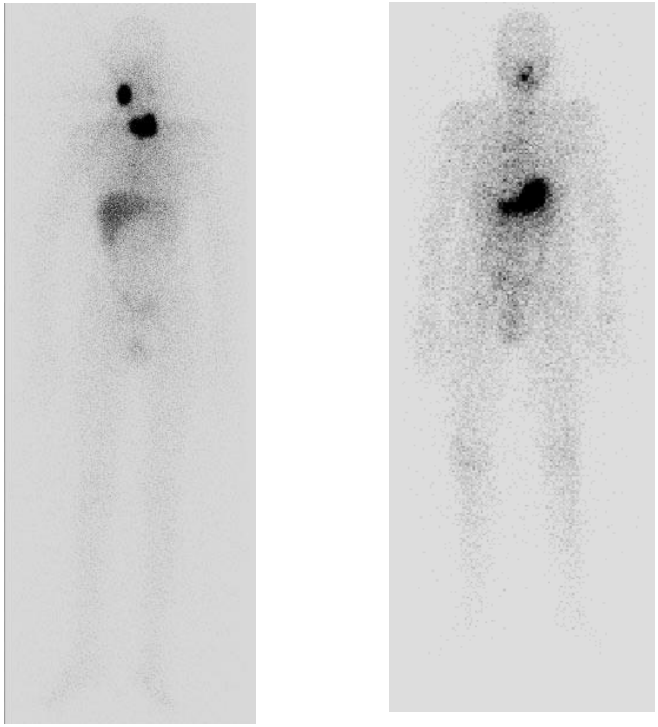




Consequence: standardize your staining protocol,
run positive & negative controls

In vivo: **repair focus formation in PBL after thyroid cancer therapy with I-131**

- M. Lassmann, Clinic of Nuclear Medicine, Univ. of Würzburg, GER



DTC: Lymph node & thyroid rest treatment by ablation w 3,7 GBq I-131

DTC treatment scheme

differentiated thyroid tumor

Tumor stage: pT1, pT2, pN0, cN0

Low-risk

OP

T4 withdrawal or rhTSH

3 – 4 wk

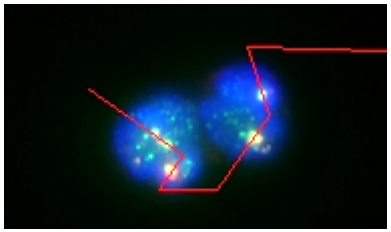
I-131 Ablation (3,7 GBq)

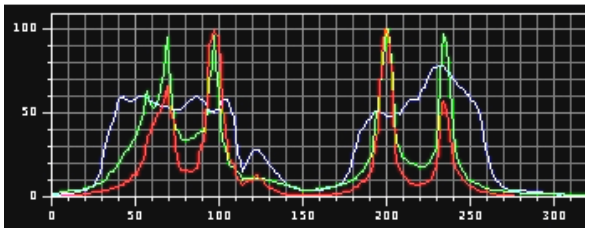
pT3, pT4, N1, M1 (TNM 6. Aufl.)

High-risk

Physical dosimetry

Repair foci estimation



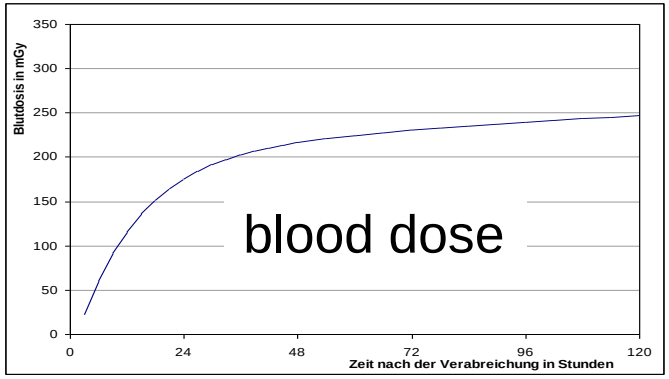


Lassmann et al. 2010 J.Nucl. Med.
Eberlein, Lassmann & Scherthan, in prep.

Dosimetry – absorbed dose to the blood

Mean specific absorbed dose to the blood: 0.105±0.067 Gy/GBq (25 Pat.)

Mean **absorbed dose to the blood**: **0.39**±0.40 **Gy**, range 0.2 – 2 Gy



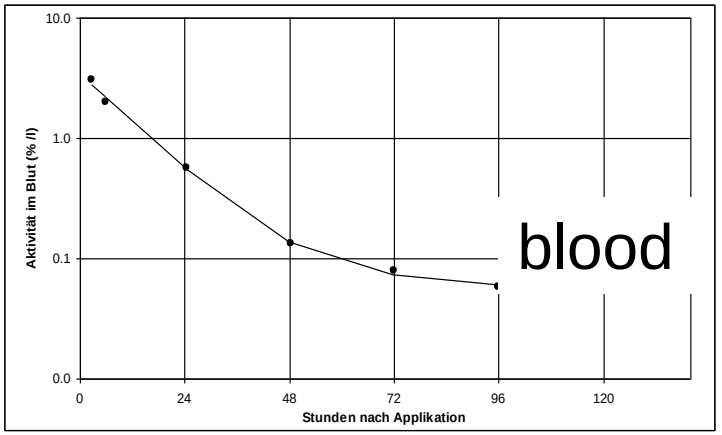
Mean **Dose Rate**: @ 2h 15.4±3.2 mGy/h

@ 24h 3.4±0.9 mGy/h

@ 48h 1.1±0.4 mGy/h

@ 72h 0.8±0.3 mGy/h

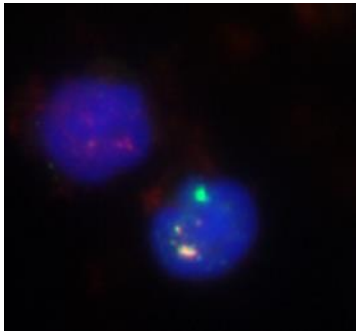
@≥96h <0.5 mGy/h

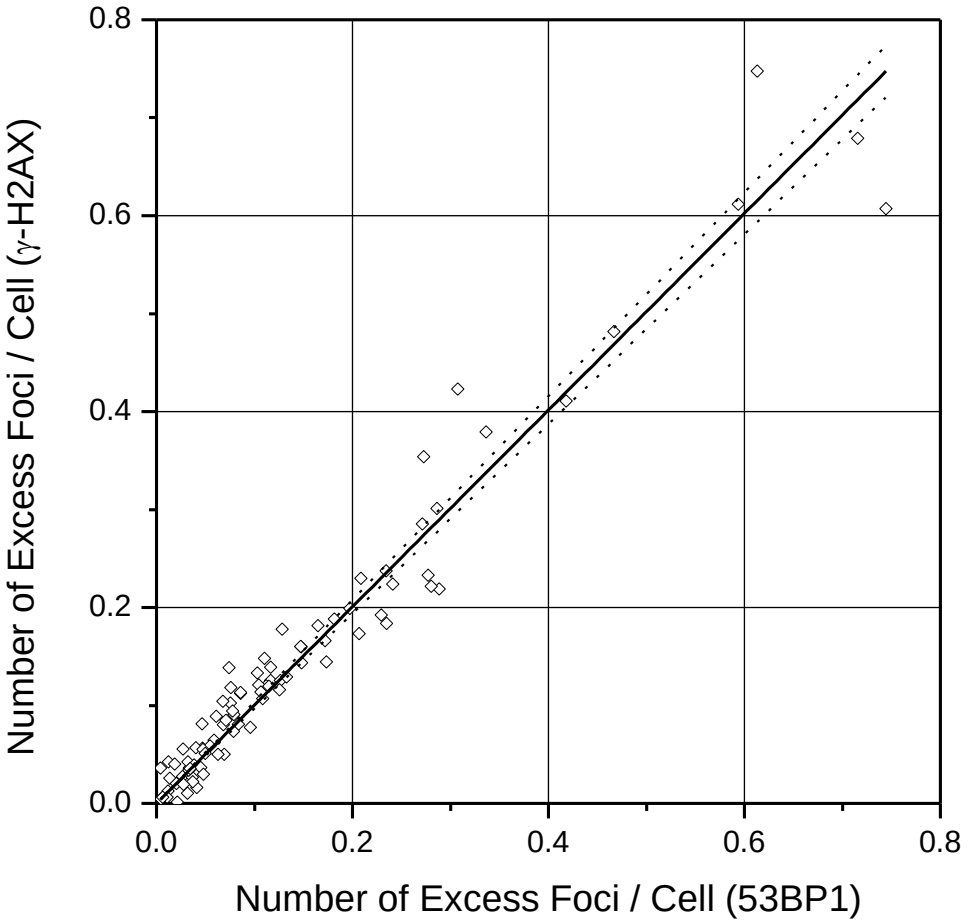


whole body activity
(% appl. act.)

Lassmann et al. J Nuc Med. 2010

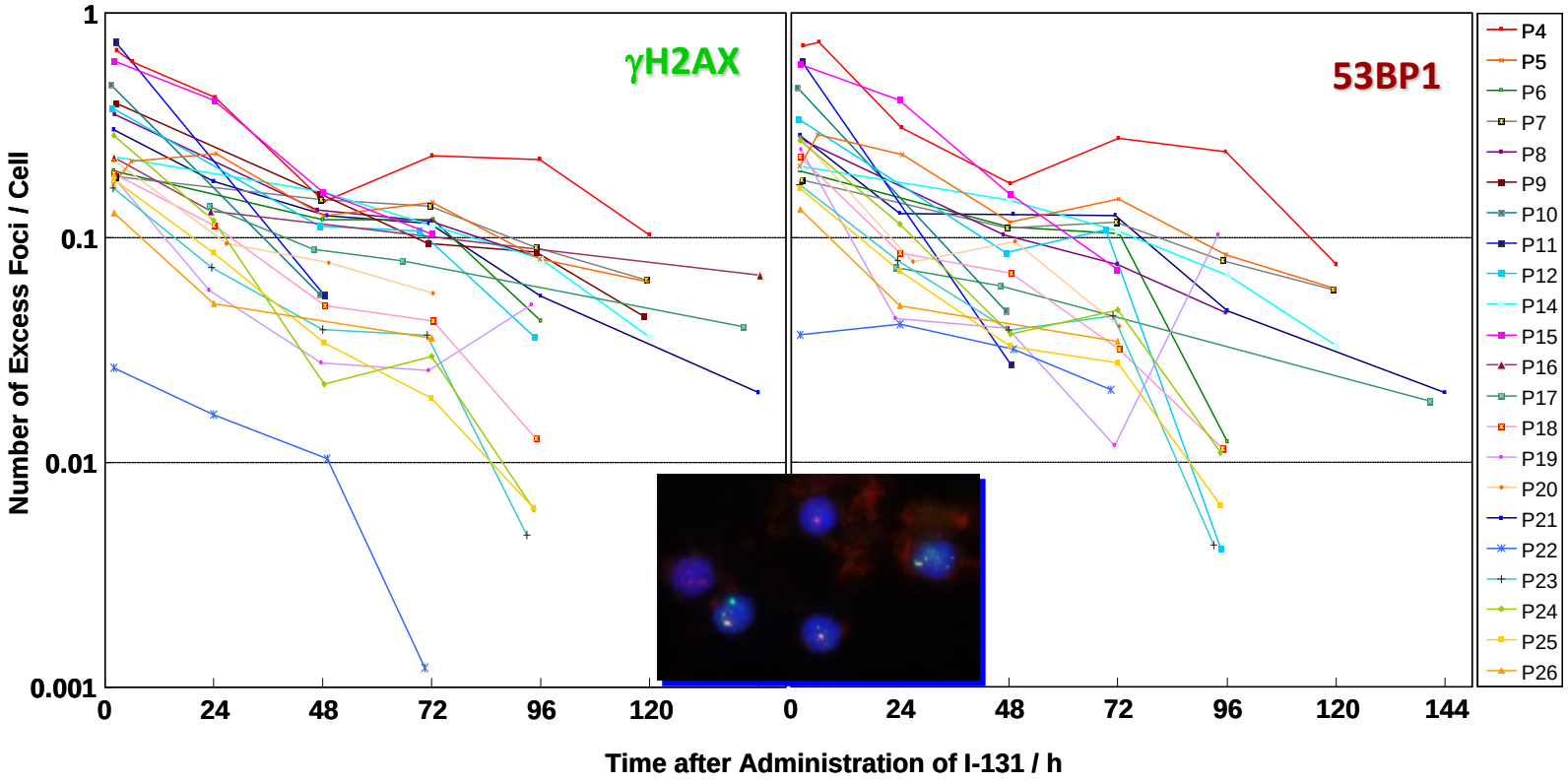
Good correlation of γH2AX & 53BP1 repair foci





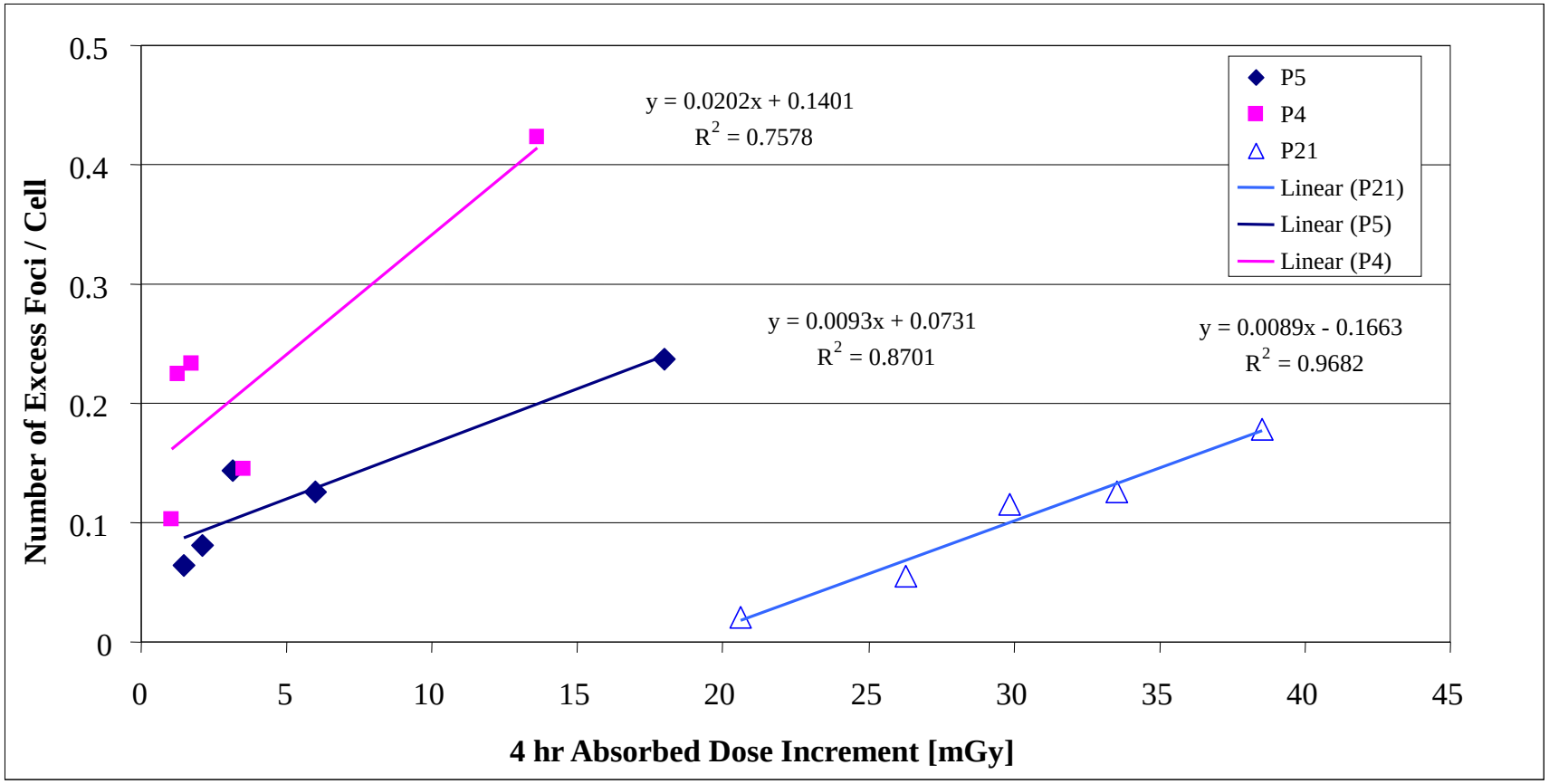
Lassmann et al. 2010, J Nucl Med

Focus analysis among DTC patients treated with ~3.5 GBq I-131: high inter-individual variability



Lassmann et al. 2010, JNM

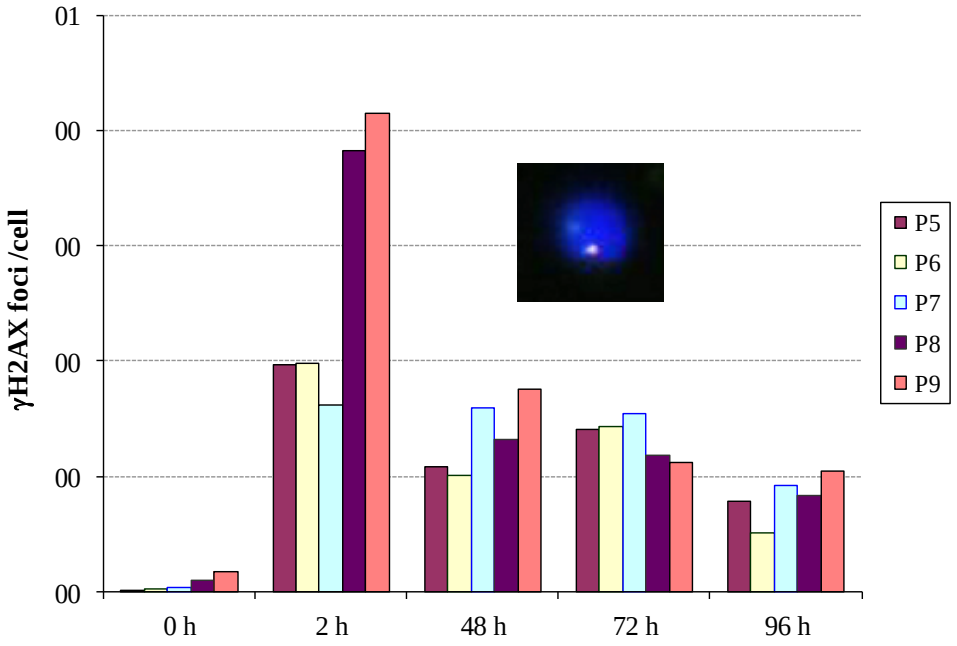
Correlation foci / physical dosimetry

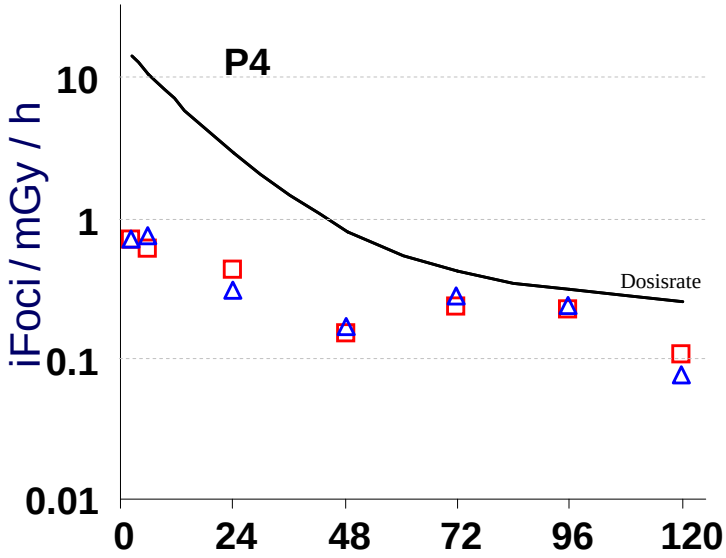


Lassmann et al. 2010 NucMed

RIT: increased foci numbers at low dose rate

γH2AX Foci post RIT (~3,7MBq 131-I)





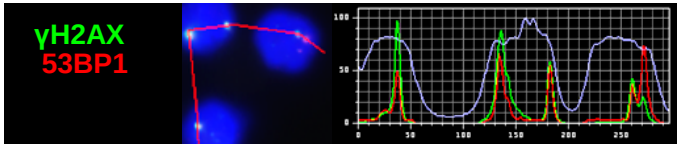
Induced foci relative to absorbed dose rate increases with w decreasing dose rate

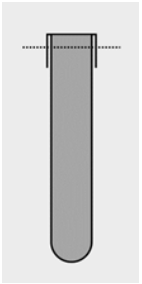
Lassmann, Scherthan, et al. 2010; J Nuc Med

Calibration of the Focus assay for radionuclide incorporation

- Monte Carlo simulation of decay and dose built up according to the volume & geometry of the vials to realize doses to the blood (0-95mGy)

- 3 healthy individuals; 7 experiments: with I-131 (4) and with Lu-177 (2+1)
- Activity in I-131/Lu-177 aliquot measured by germanium detector
- Blood samples (3,5ml) partitioned to different tubes + 1ml NaCl diluted radioactive solution
- Incubation for 1h at 37°C under mixing
- Sample preparation as published (Lassmann et al. 2010, NucMed)
- Mic analysis of γH2AX+53BP1 FPC



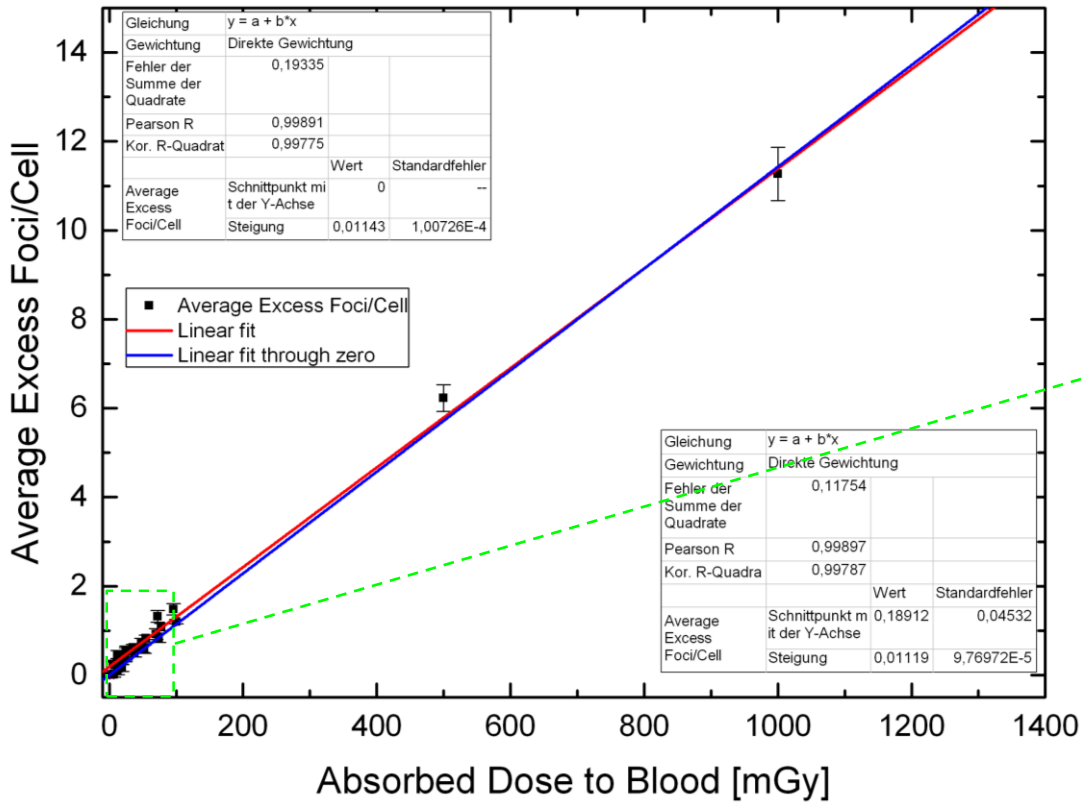


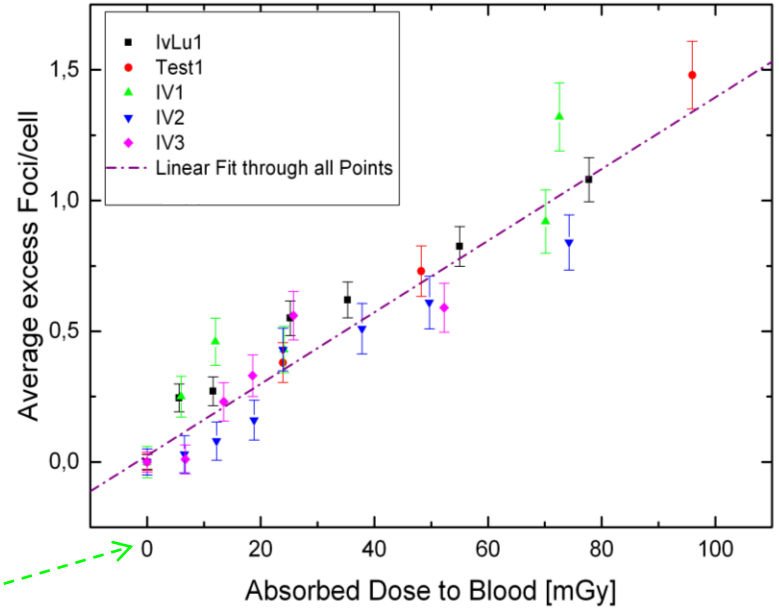
Eberlein et al. 2015, PloSOne

In vitro calibration of fpc yield and absorbed dose to the blood after in solution exposure to ^{177}Lu und ^{131}I radionuclides

(Cooperation w M. Lassmann, Nuclear Medicine WÜ University, DE)

Fit inklusive Punkte aus externer Bestrahlung (100mGy-1Gy)

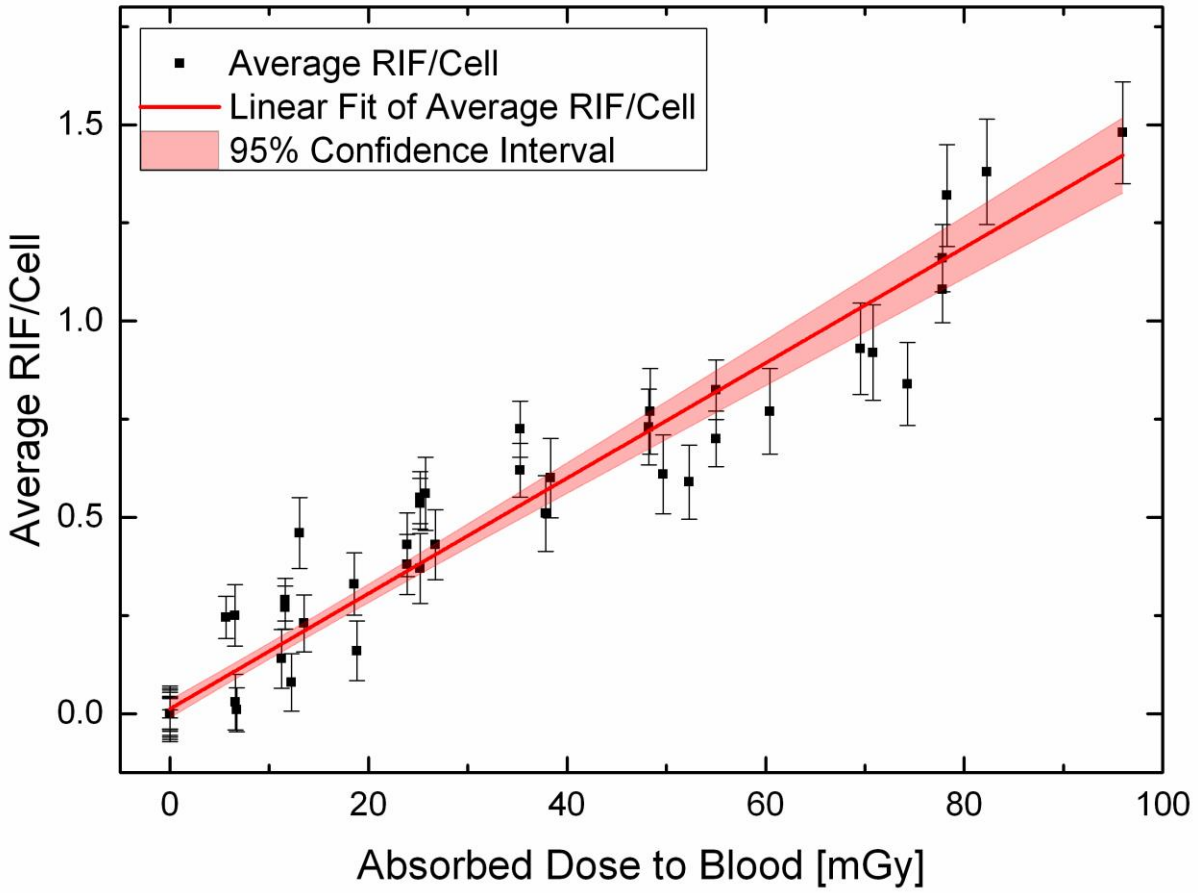




- RIF (radiation induced foci): γ-H2AX-foci colocalized w 53BP1 (manual count w DBP-filter)
- No nuclide and test person specific behaviour

Eberlein et al. 2015, PloSOne

FPC calibration curve of 1h ^{131}I, ^{177}Lu radionuclide-treated PBL samples



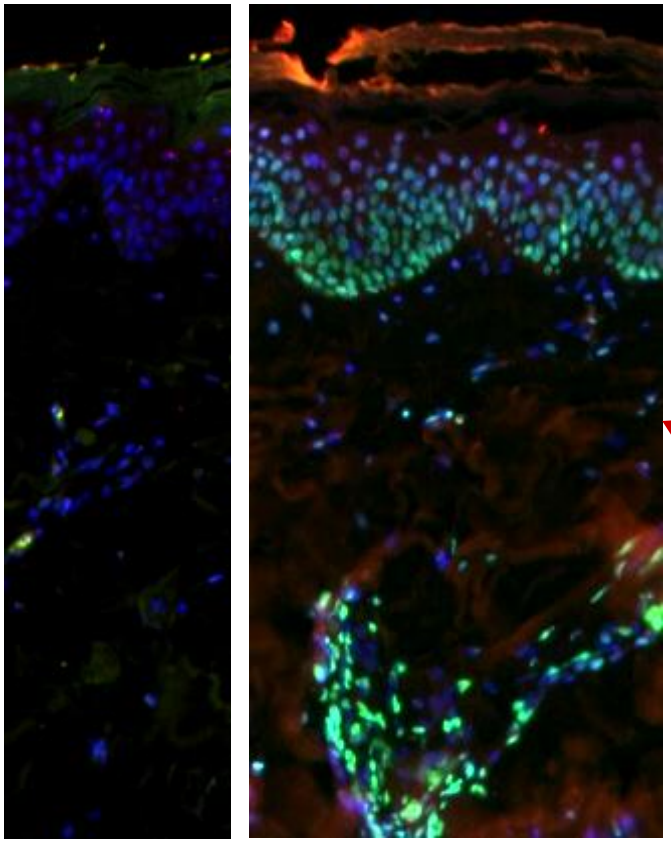
$$y=0.012\cdot RIF/cell+0.015\cdot RIF/cell\cdot mGy^{-1}\cdot x$$

Eberlein et al. 2015, PloSOne

Partial body, acute high dose γ-irradiation: Focus yield in skin & blood cells

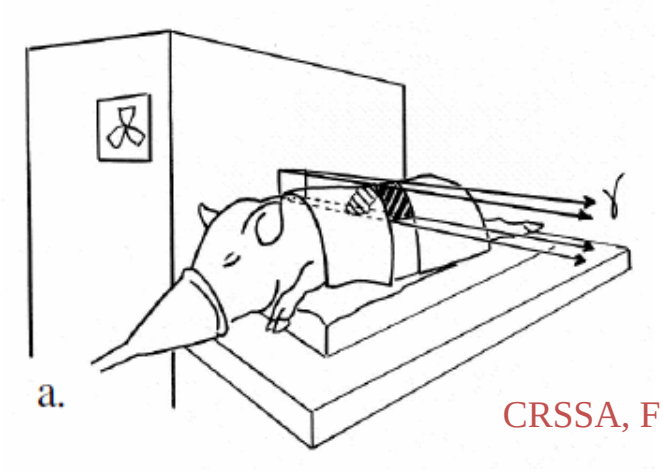
Pig-skin

non-IR | 4h post 50Gy

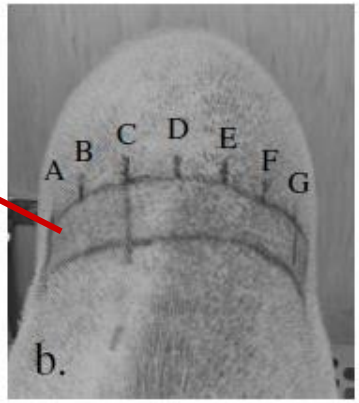


InstRadBioBw

Pig model

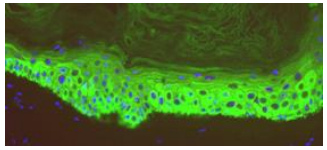


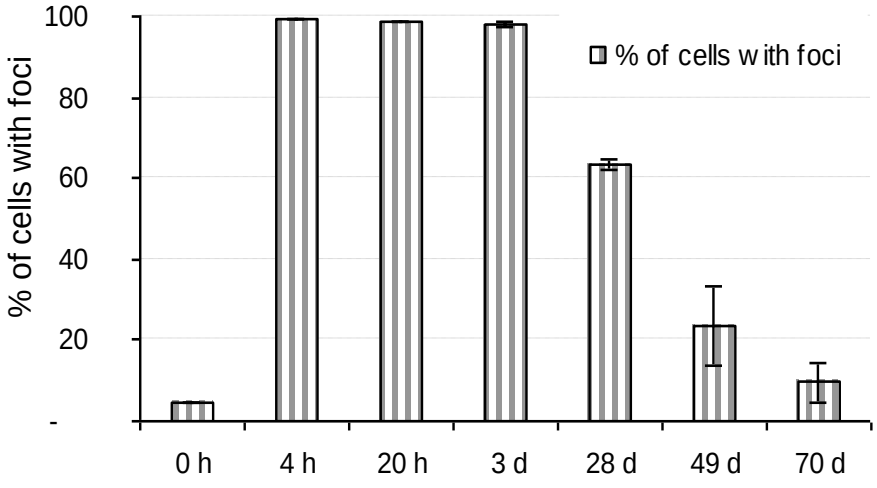
CRSSA, F

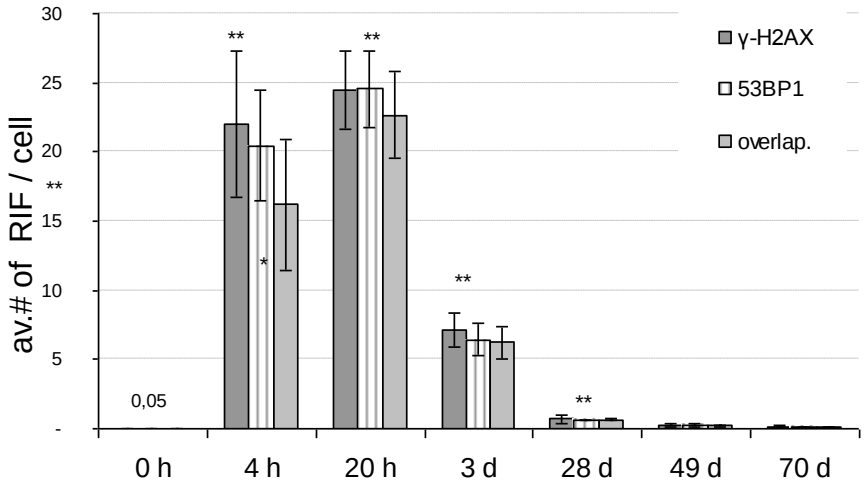


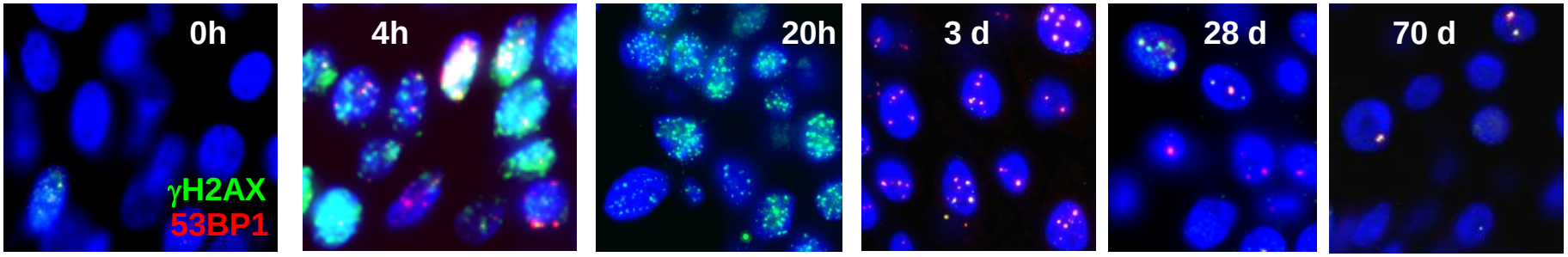
Agay, et al. Exp. Hemat. 2010

High dose rate IR - 50Gy γ-IR doesn't saturate the DDR







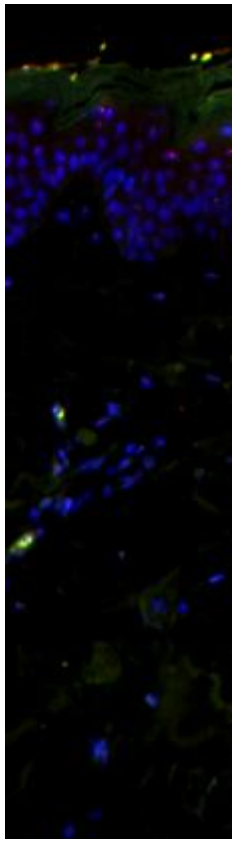


Ahmed et al. PloS One. 2012

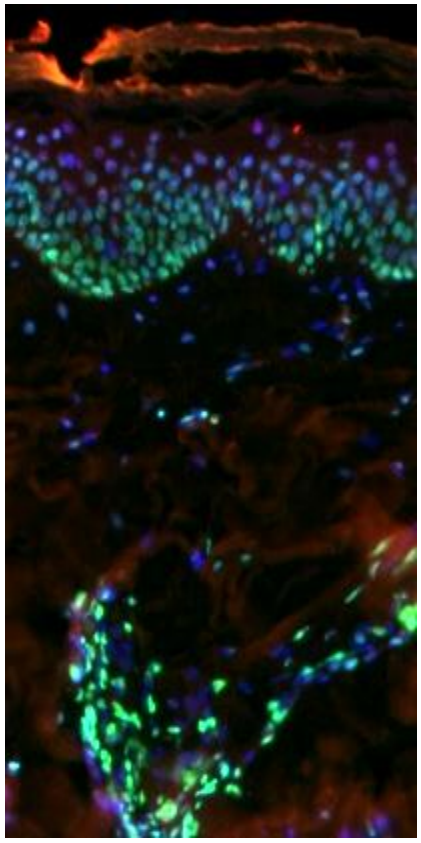
No correlation of foci # in skin & blood after 50 Gy partial body γ-irradiation

Pig-skin

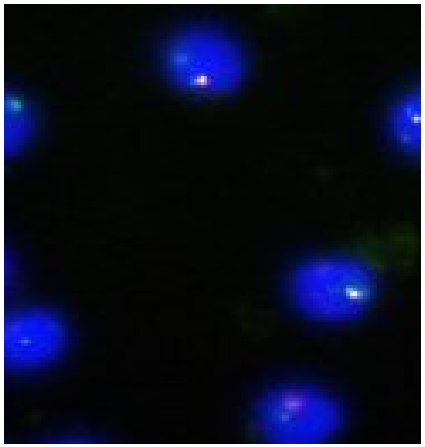
non-IR

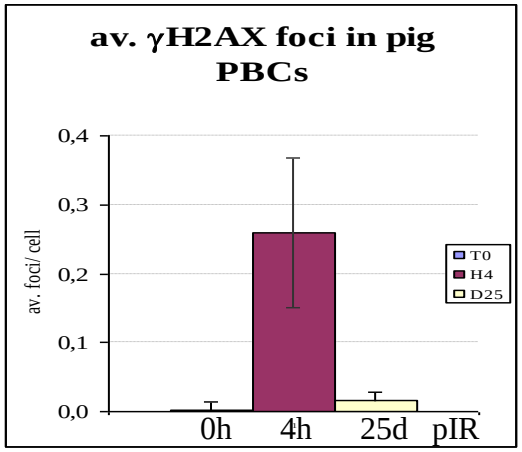


4h post 50Gy

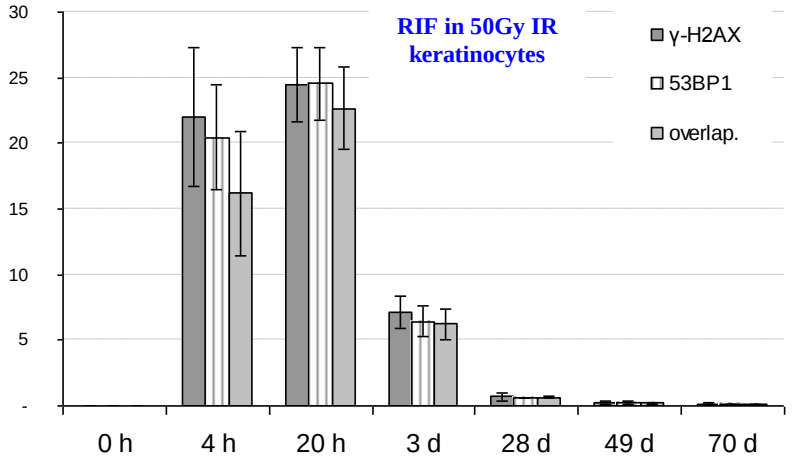


PB Leukocytes



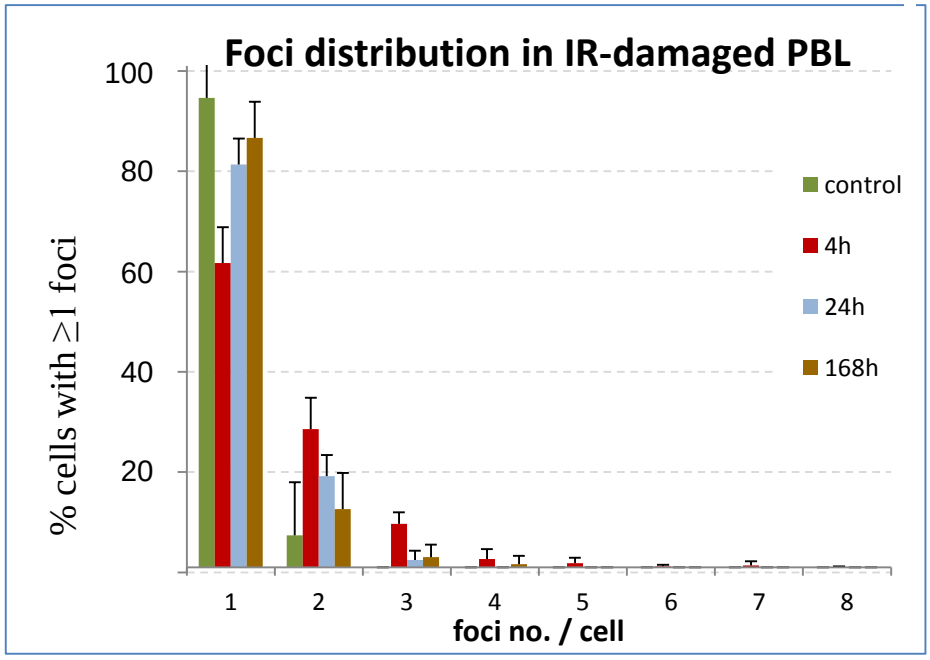


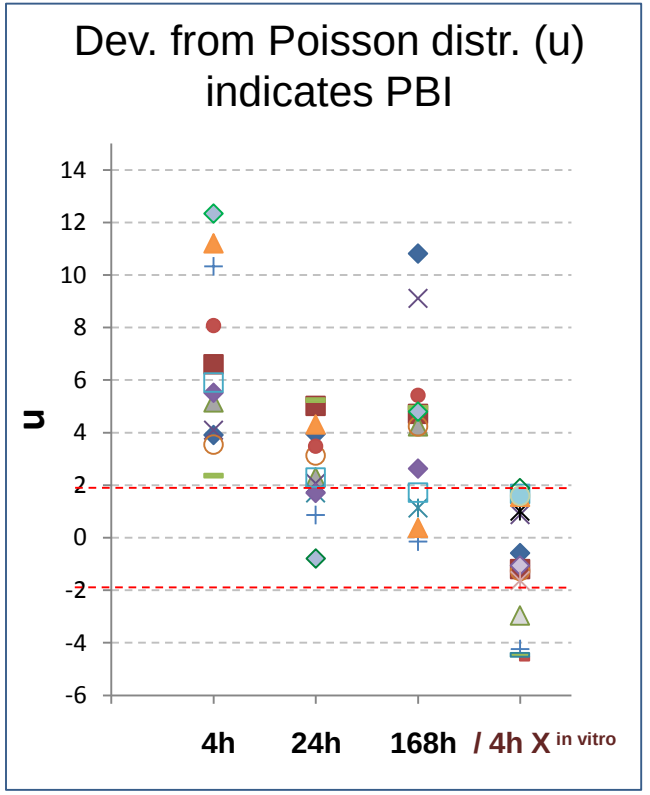
Range 1-3 1-8 1-4 foci/+cell



Ahmed et al. 2012; Lamkowski et al. 2014, PloSOne.

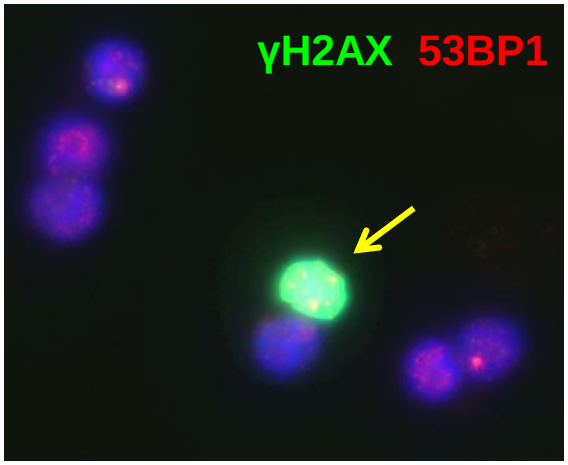
High dose rate partial body γ-IR doesn't saturate the DDR

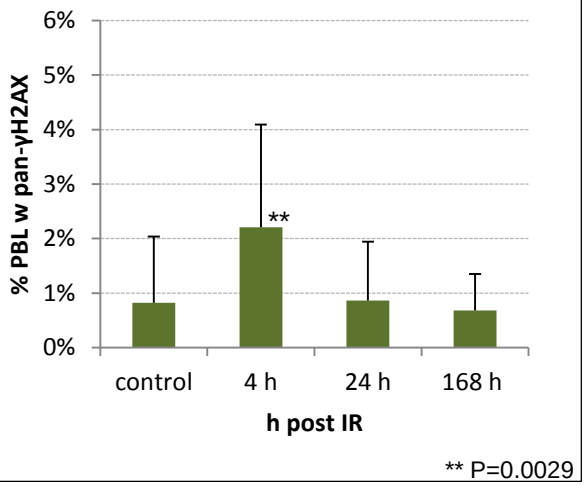




Lamkowski et al. 2014 PlosOne

High dose & dose rate partial body γ-IR: Appearance of pan-γH2AX nuclei indicates PBI early after the exposure





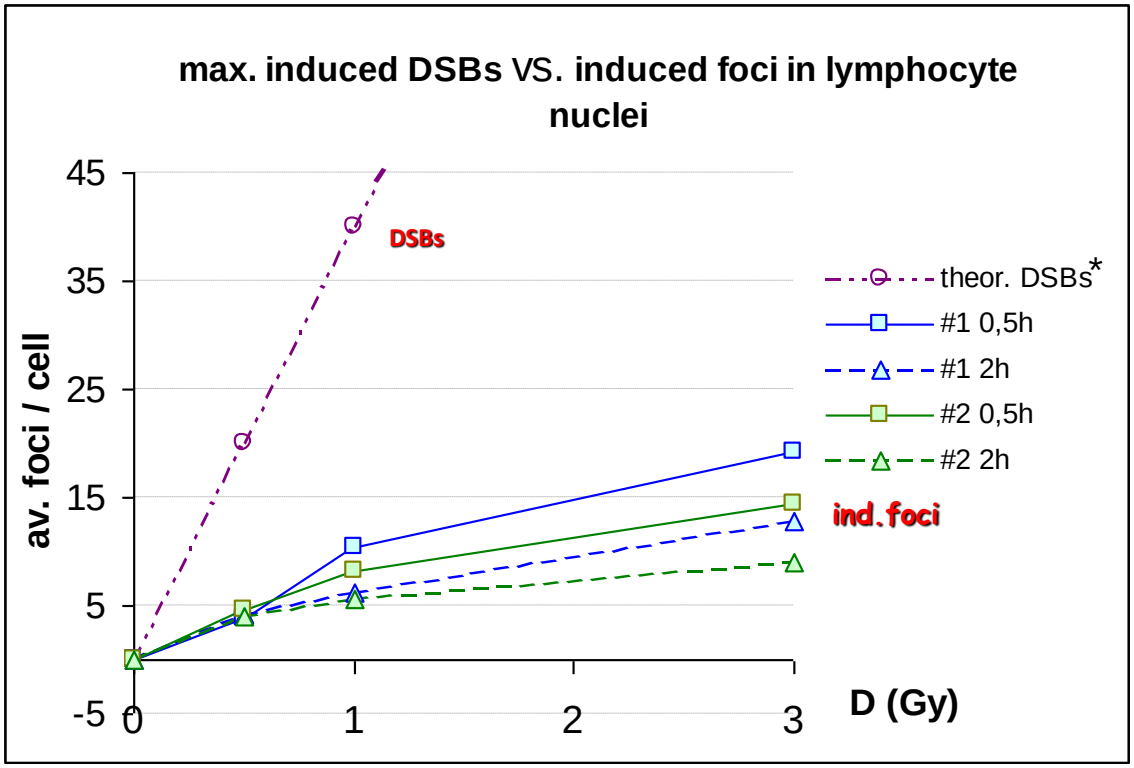
Lamkowski et al. 2014 PloSOne

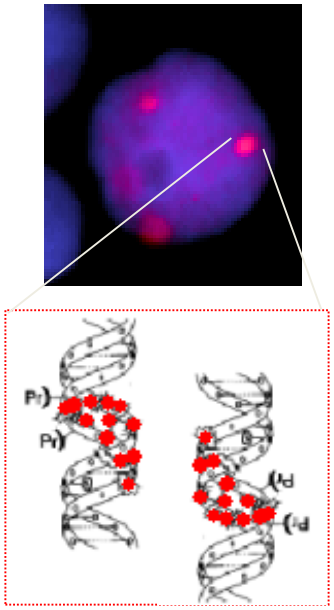
Question

How many breaks/focus?

Error prone repair in foci
@ high doses?

High doses of IR induce more DSBs than Foci



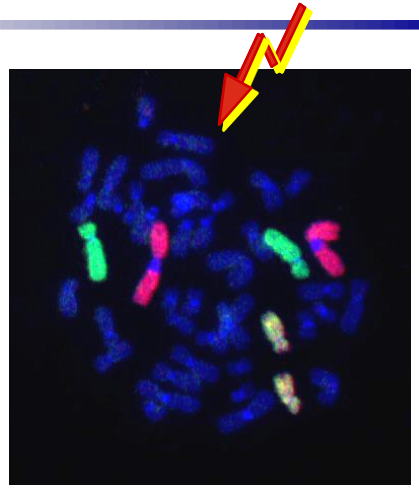


Growing # of DSBs / Repair Foci with ↗ Dose

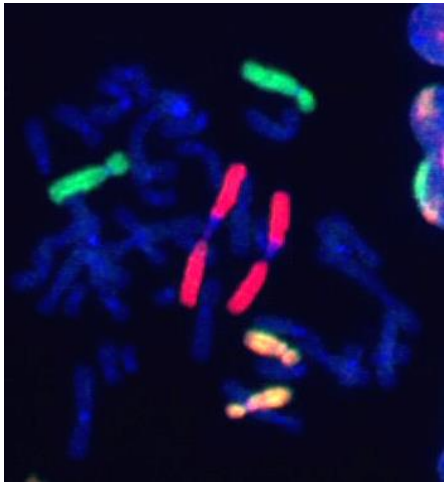
⇒ probability of misrepair ↗

*Ward 1991
Scherthan et al. 2008

IR w high doses /dose rates

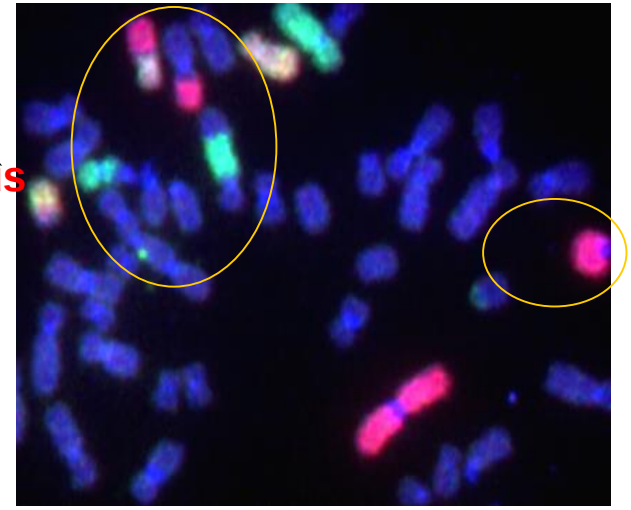


Repair



correct

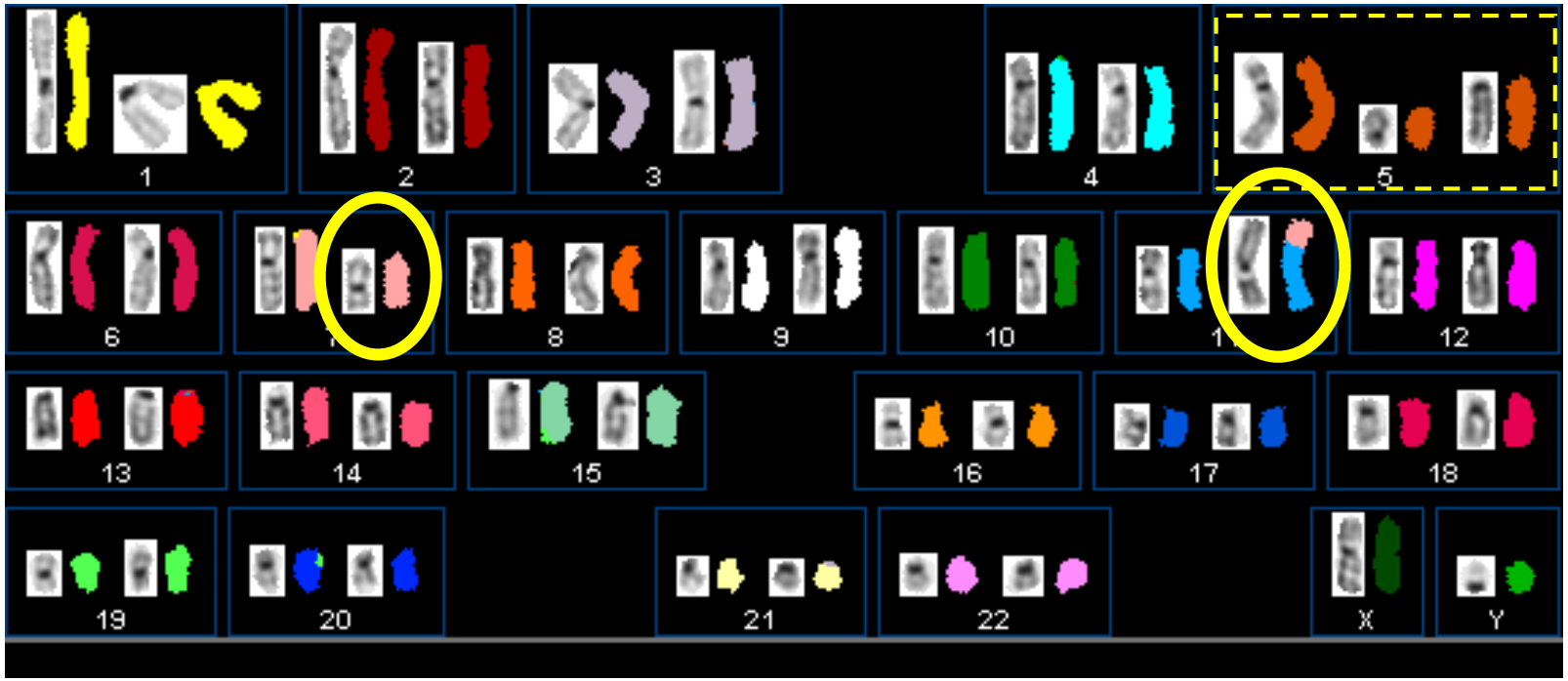
erroneous



# of Chromosome aberrations

⟶ Measure for misrepair

mFISH analysis: translocation yield in lymphocytes

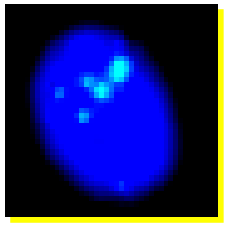


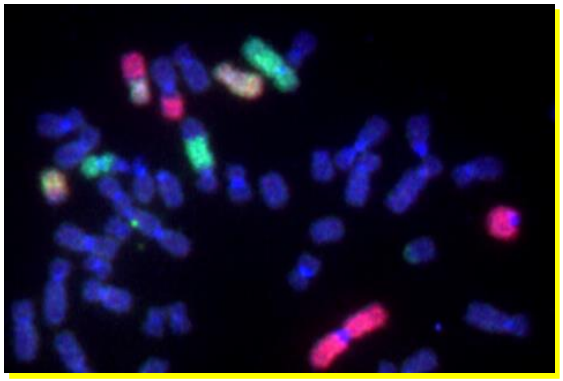
24 human chromosomes in different colors ⇒ translocations rates as a measure of misrepair

Minimal Number of (misrepaired) Breaks (MNB) for this cell: 3

Scherthan et al. 2008, BBRC

High doses of X irradiation cause accumulation of DSBs in repair foci ⇒ increased translocation yield (misrepair)





Dose (Gy)	Foci # 0,5 h post IR	2 h	MNB / induced focus[a]	maxim. break # / focus
0.5	**4.15** ±0.4	4.07 ±0.4	0.072	**4,8**
1	**9.31** ±1.1	5.84 ±1.1	0.070	**4,3**
3	**16.8** ±2.3	10.8 ±1.9	0.26	**7,1**

(±): standard error of the mean. [a] based on the 30 min value; MNB from Sky data.

Scherthan et al. 2008, BBRC

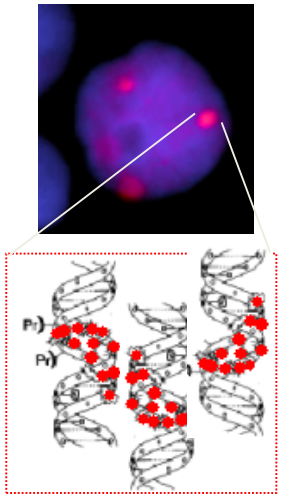
Conclusions

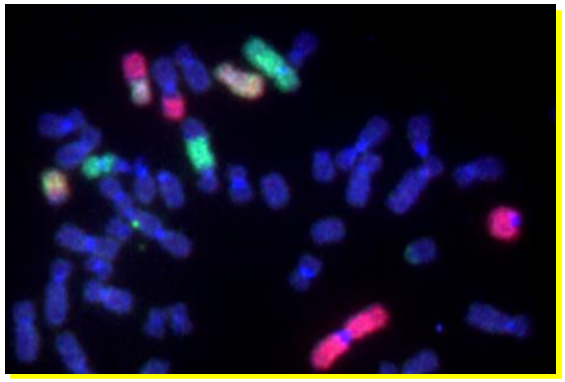
DNA Repair occurs in foci ("factories")

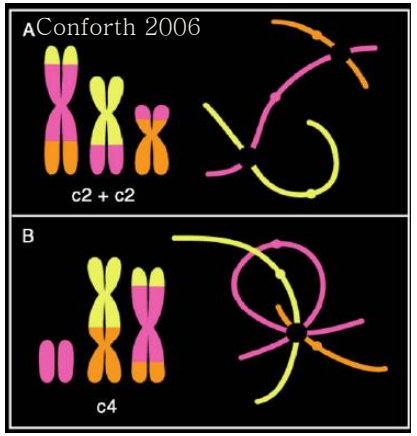
- Not always a linear dose relationship
  - high inter-individual variation
- DSB No. / focus increases with dose

⇒ source of mutation and complex exchanges.

Low dose effects



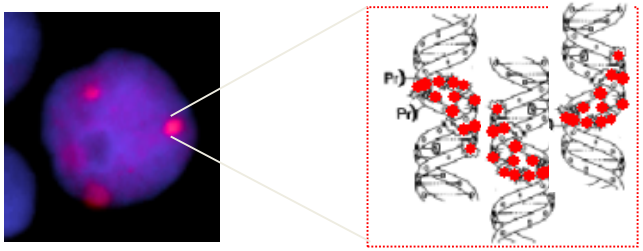




Conclusions

DSB Repair (γH2AX) Foci

- High sensitivity
  - Good indicator of WB exposure, dose reconstruction difficult
    - High inter-individual variability. Rapid decline
      - Residual damage (>24h) can correlate with radiation sensitivity. In skin indicates IR for weeks
        - Problematic for dose reconstruction after partial body exposure



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Thank you

"... and stay away from scientists - they cause cancer"