



# The far side: the gamma-H2AX Focus Assay

Harry Scherthan

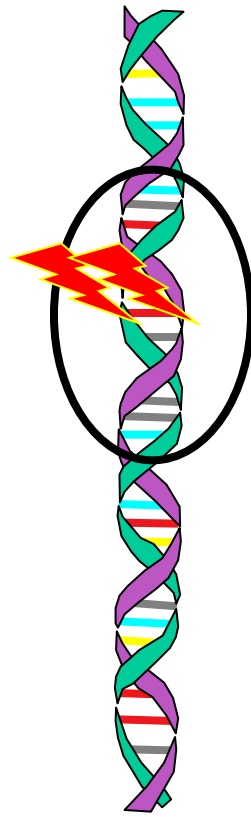
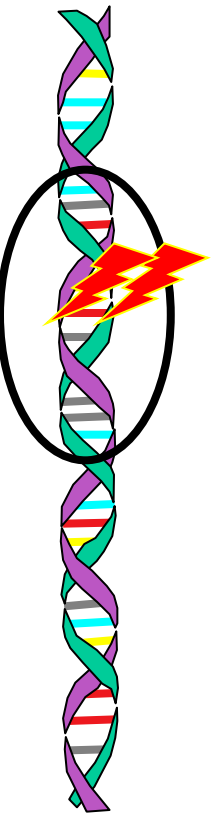
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# DNA double-strand breaks (DSBs) are a severe threat to genomic integrity



They result from ionizing radiation and radiomimetic drugs

- Arise endogenously during DNA replication
- Are delivered as initiator of physiological recombination processes like V(D)J recombination and meiosis.

# **Ionizing radiation: DNA - the critical target molecule**

**1 Gy X Rays =>**

**4-5000 Base modifications /cell**

*quick repair (min)*

**~ 1.000 ssDNA breaks/cell:**

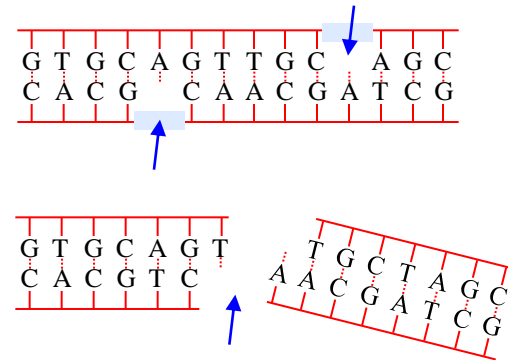
*quick repair (min)*

**~40 DSBs /cell =>**

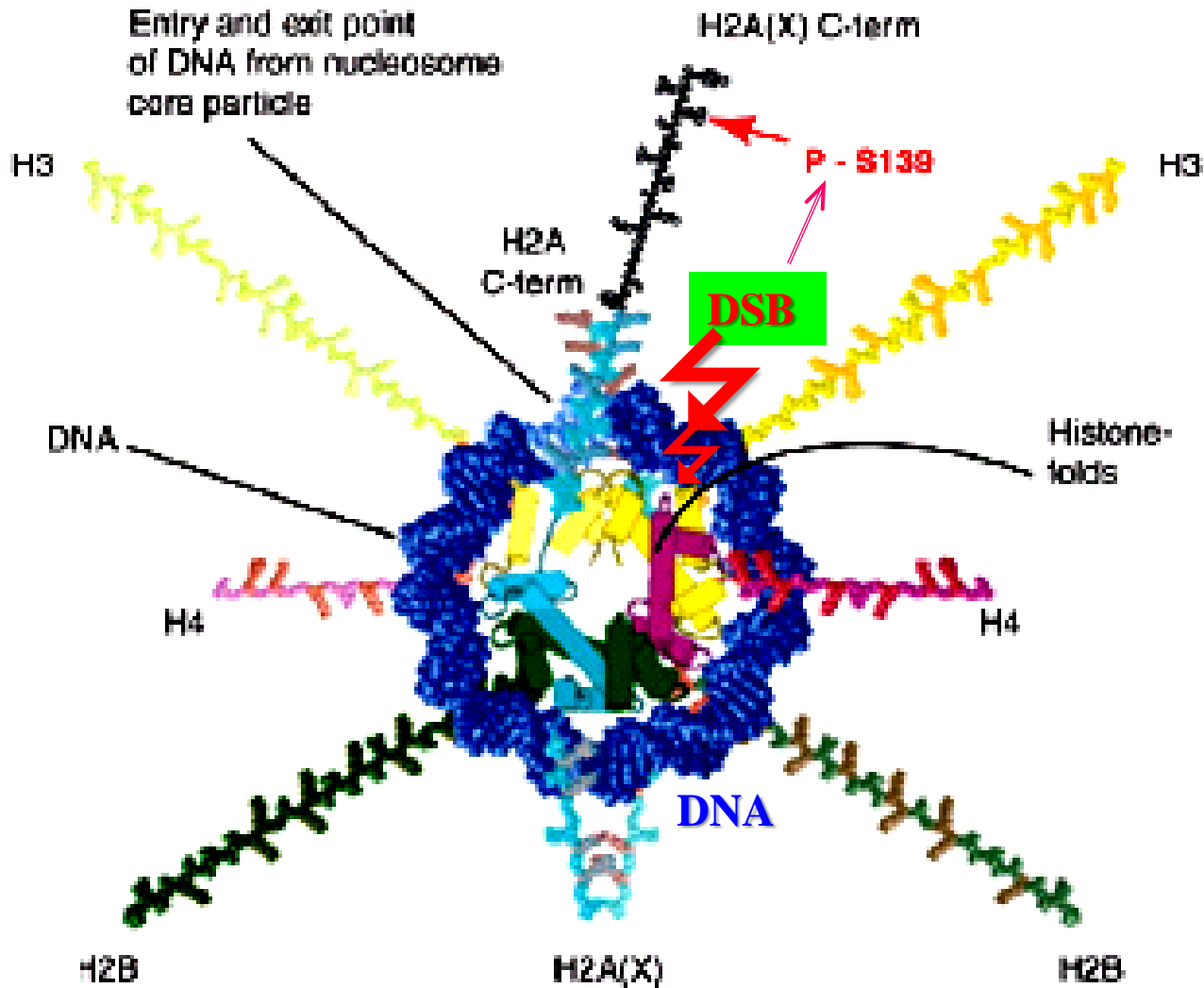
*repair in hours (days)*

=> chromosome aberrations

=> mutations, carcinogenesis or cell death



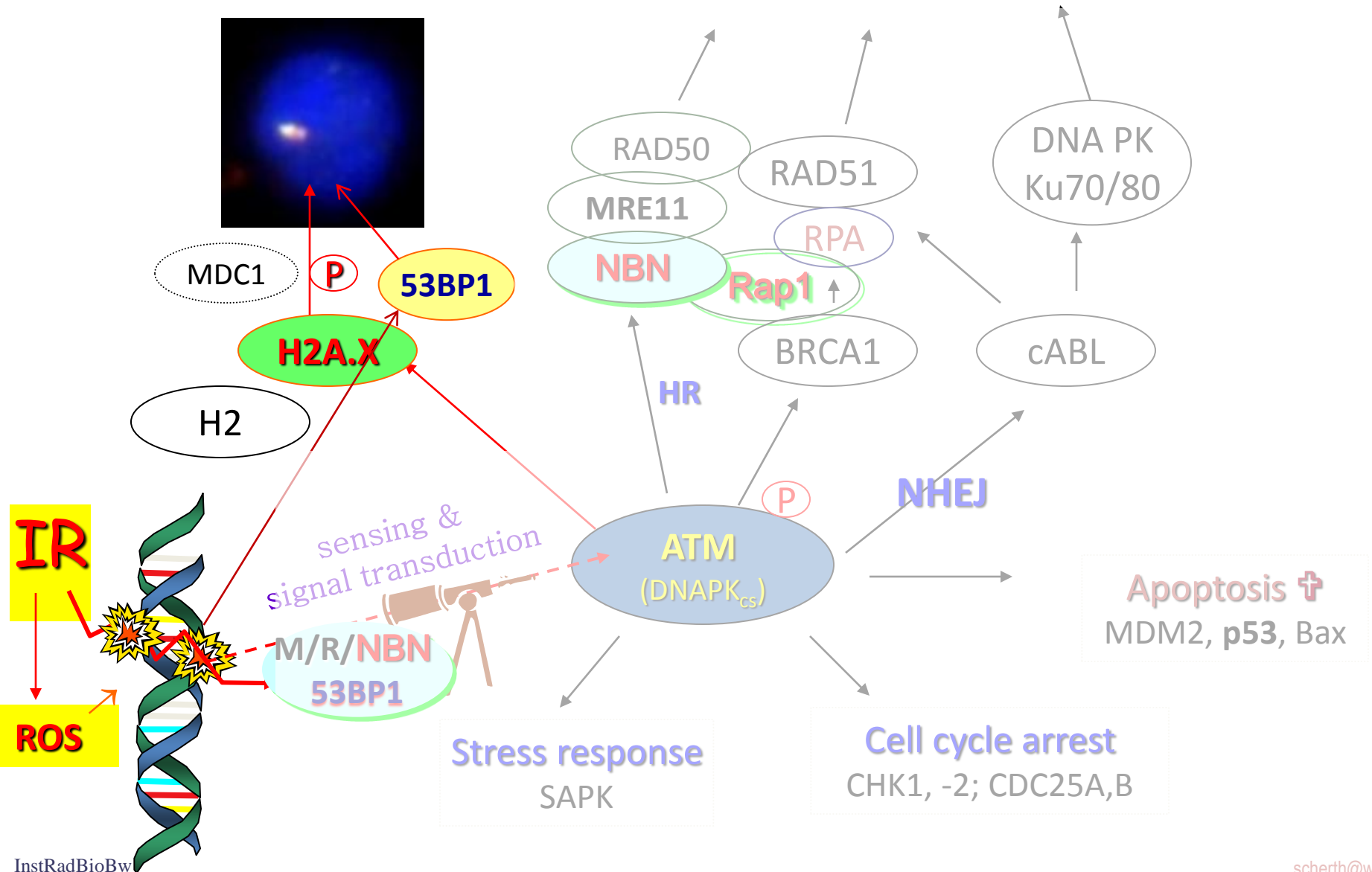
# DSB form in chromatin context: Histones & H2A.X



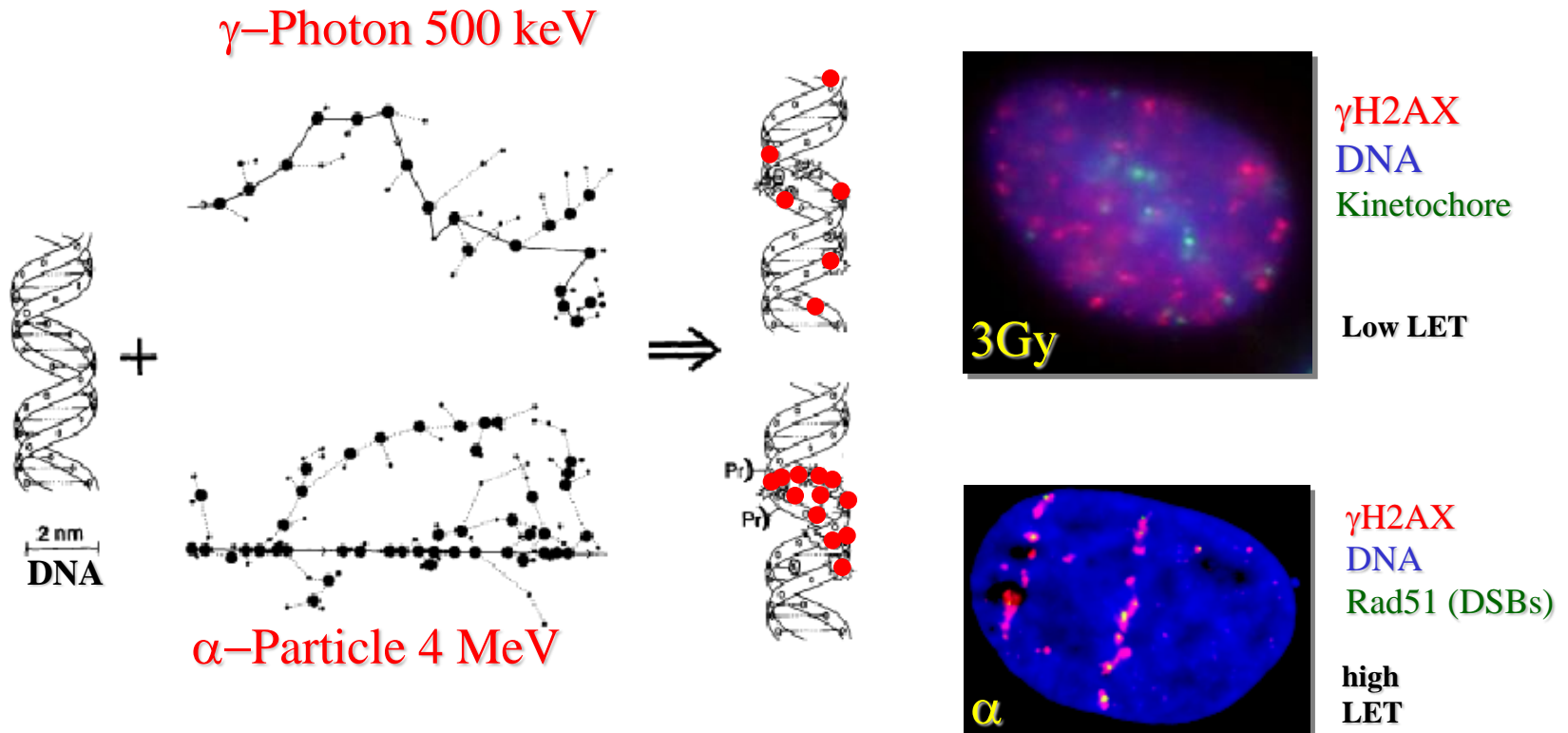
# Chromatin modification



# DNA Repair

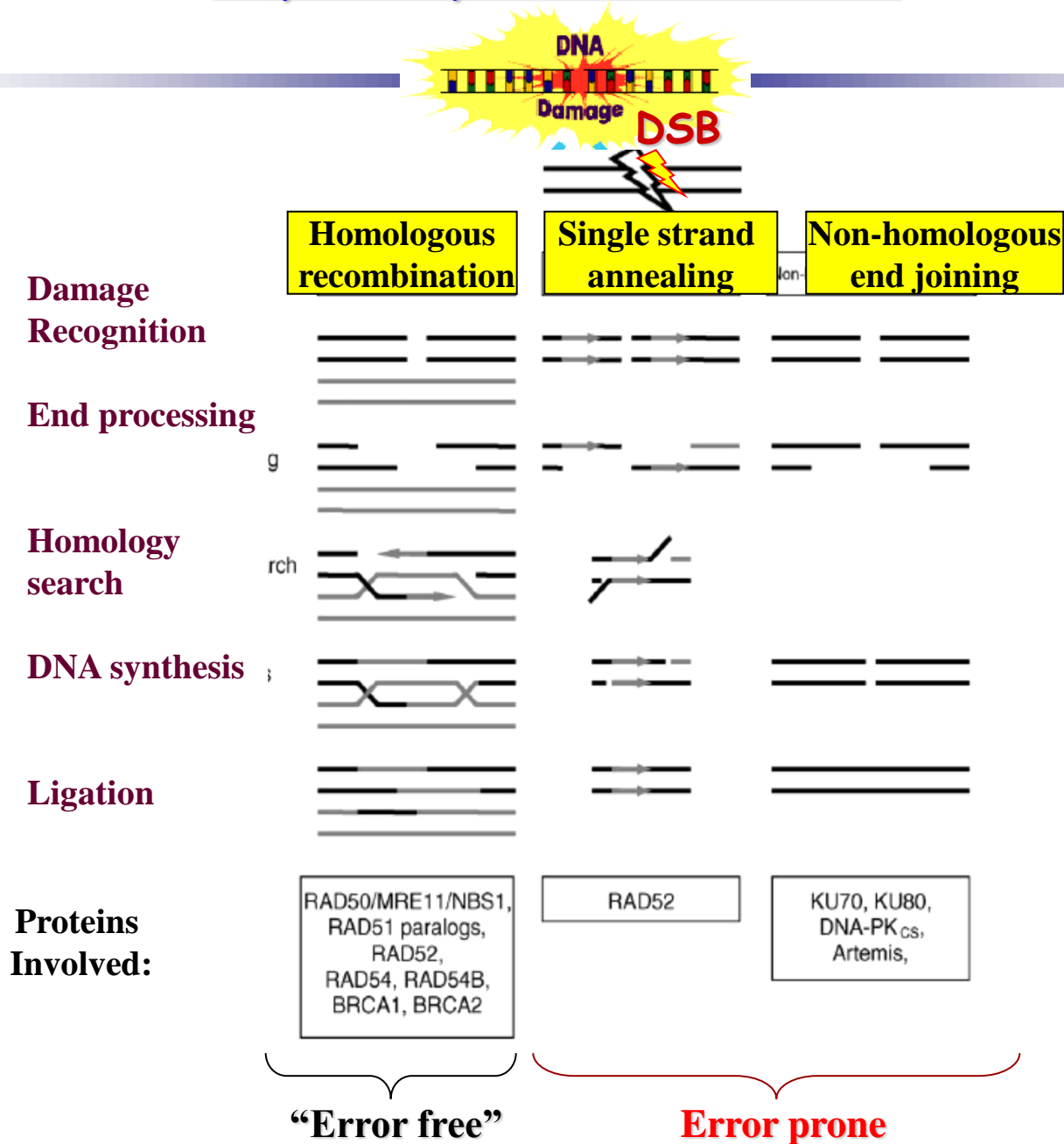


# IR induces chromatin & DNA damage



High LET: complex, potentially „lethal“ DNA/chromatin damage

# Repair Systems fix DSBs



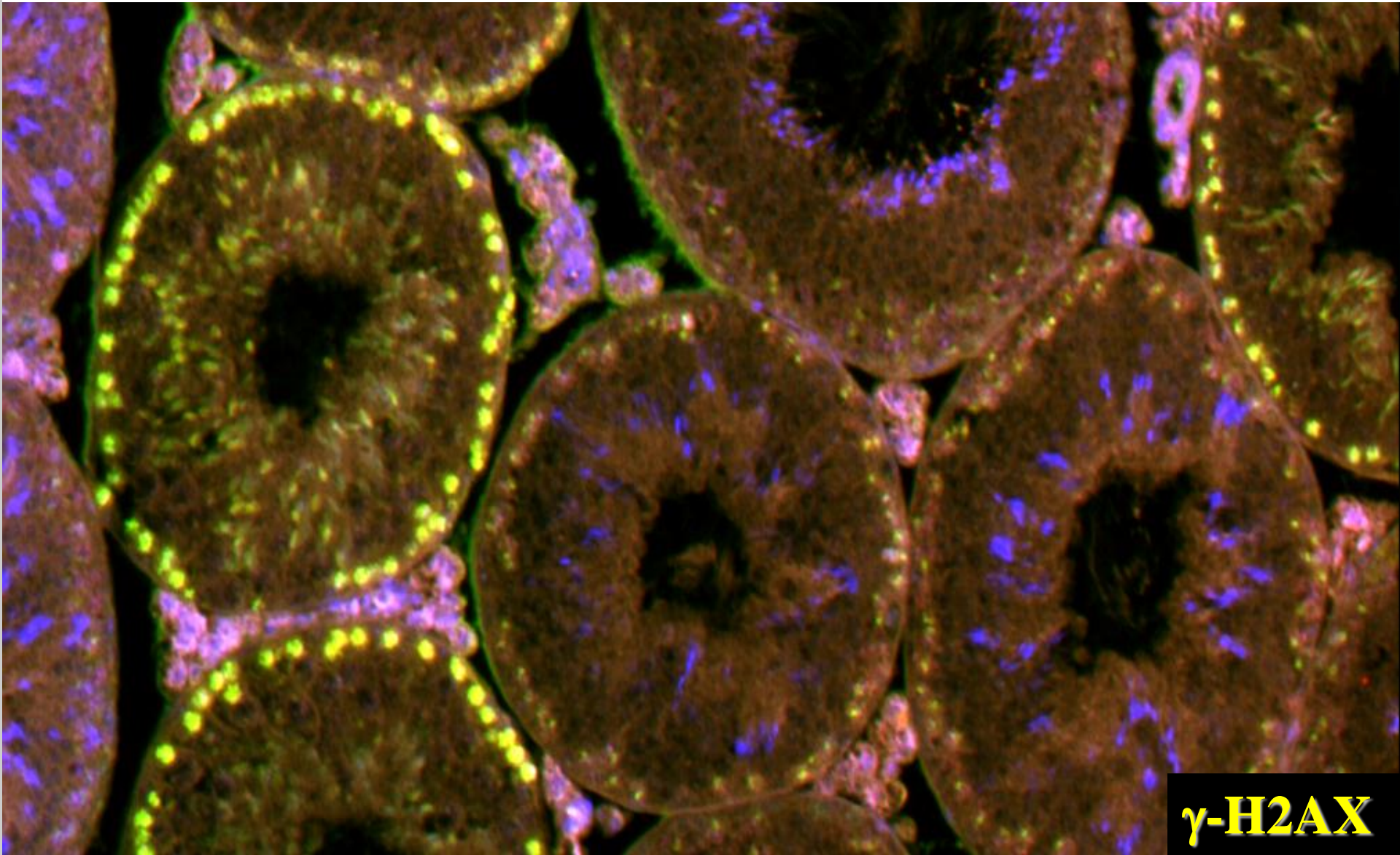
## ❖ $\gamma$ H2AX foci @ physiological DSBs

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- **MEIOSIS** (testis, fetal ovary)
- Lymphocyte maturation  
(VDJ recombination)



# DSBs & $\gamma$ H2AX in ♂ mamm. meiosis

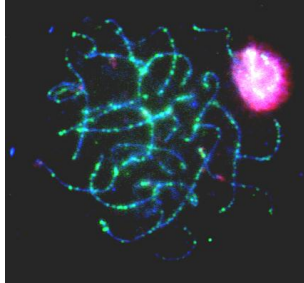
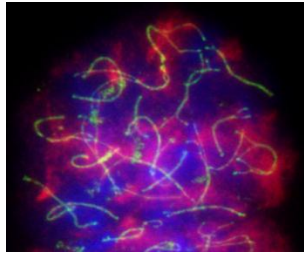
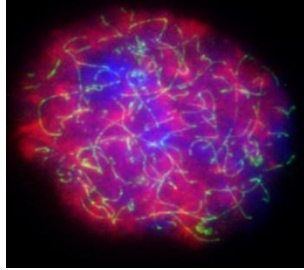


DSBs => repair => silencing => chromatin remodelling

**$\gamma$ -H2AX**  
**Nuclei**  
M.mu.

# Recombinational DSB Repair during Prophase I

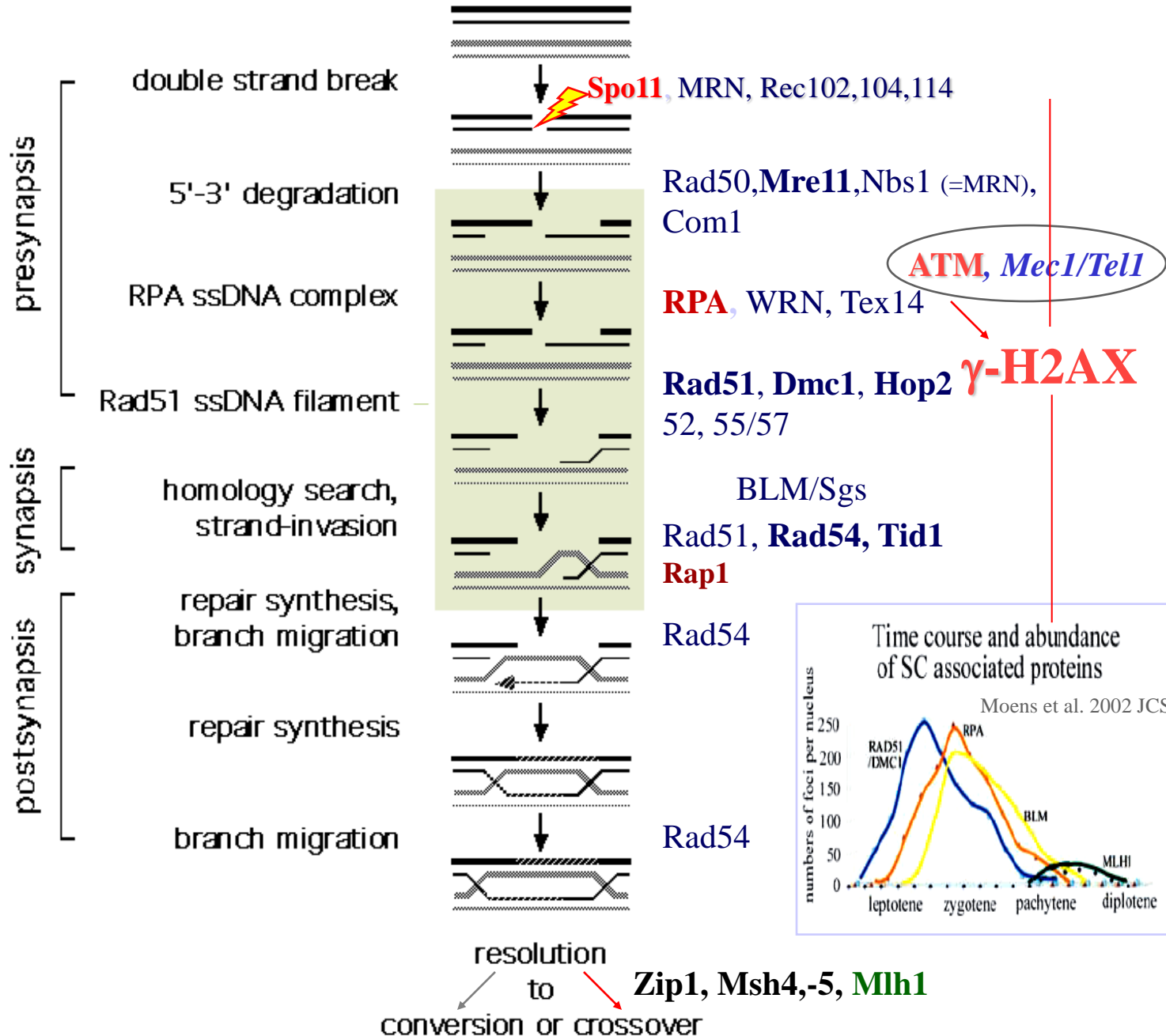
## $\gamma$ -H2AX Cytology



$\gamma$ -H2ax, SCP3

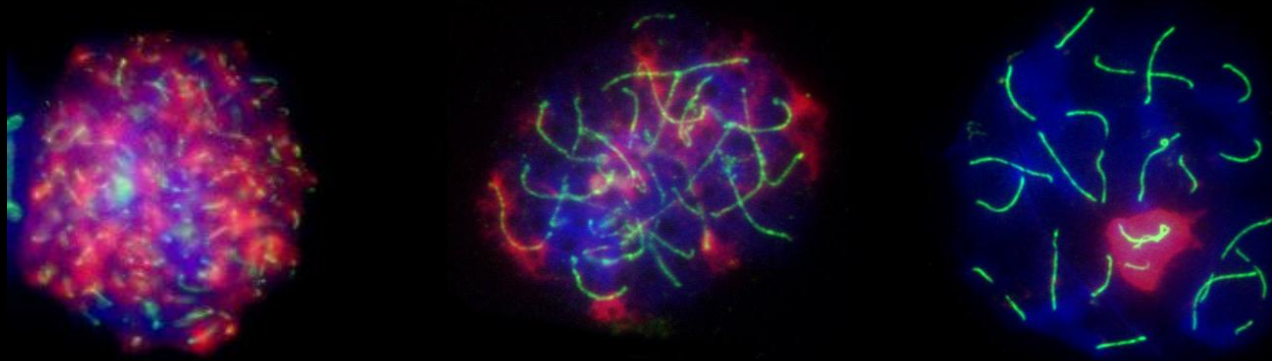
♂ mouse

Liebe et al. 2006 ECR

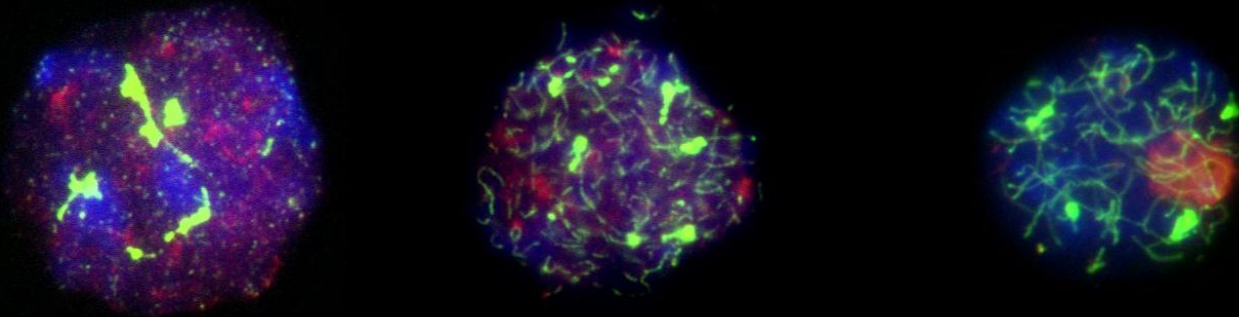


# IR-induced DSBs modulates chromatin phosphorylation & prophase I progression

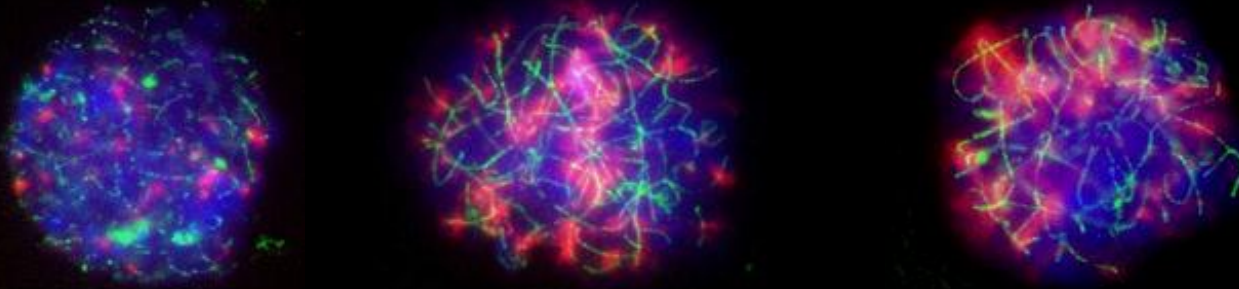
*Spo11*<sup>+/+</sup>  
 $\gamma$ H2AX



*Spo11*<sup>-/-</sup>  
(no DSBs)



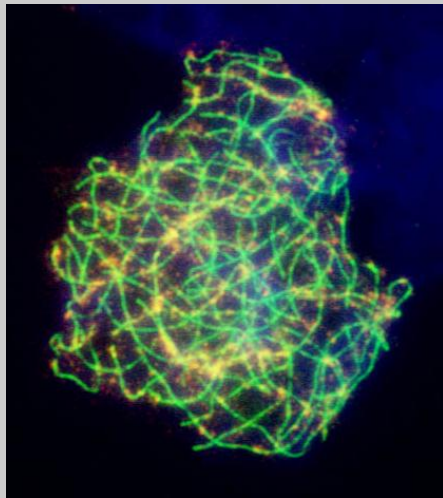
*Spo11*<sup>-/-</sup> IR  
3Gy X-rays



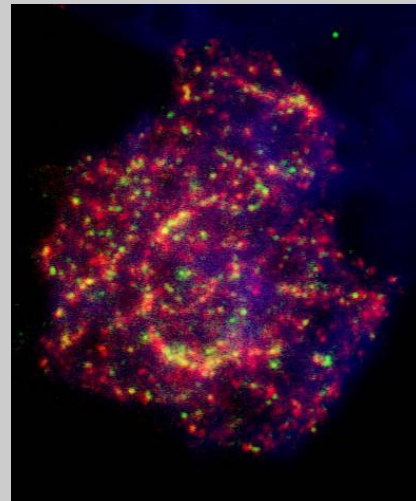
$\gamma$ H2AX  
SCP3

# Human meiosis: DNA repair and $\gamma$ H2AX

♀ pachytene

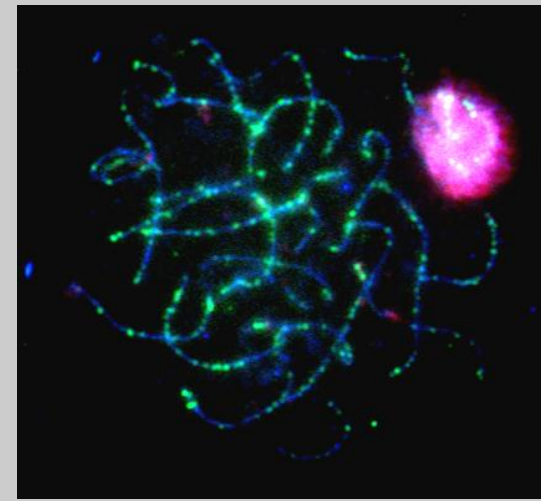


♀ SCP1,  $\gamma$ -H2AX, DNA



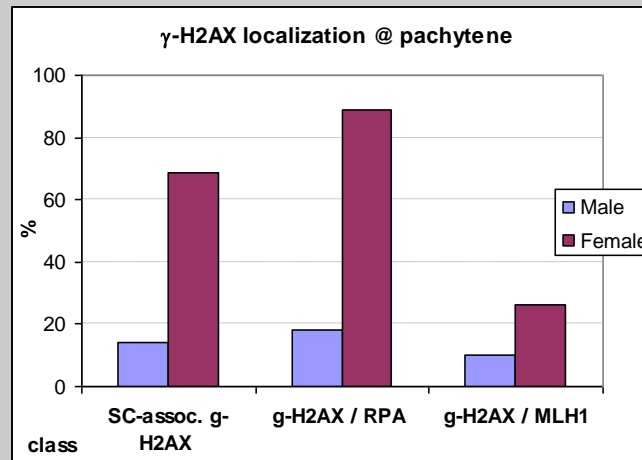
♀  $\gamma$ -H2AX, RPA

♂ pachytene



XY body

♂  $\gamma$ -H2AX, RPA, SCP3

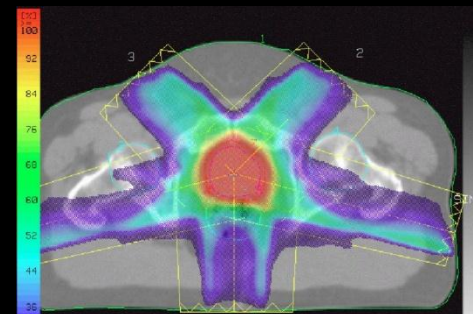
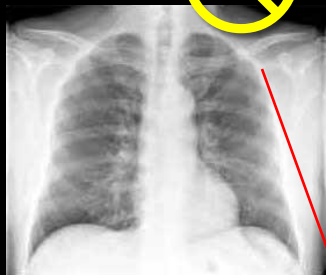
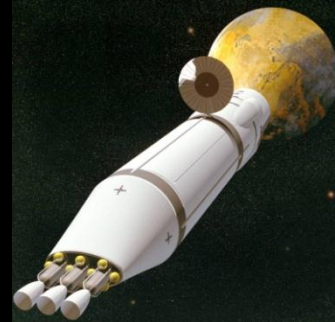




# ***Ionizing radiation exposure: $\gamma$ H2AX focus assay***

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





10  $\mu$ Gy

100  $\mu$ Gy

1 mGy

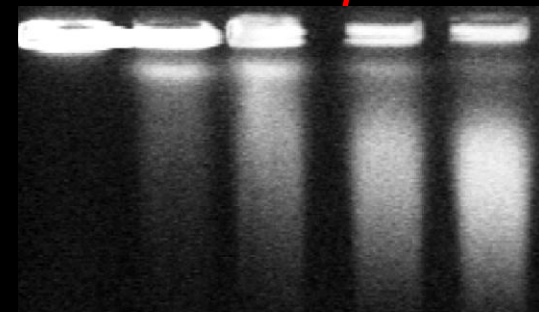
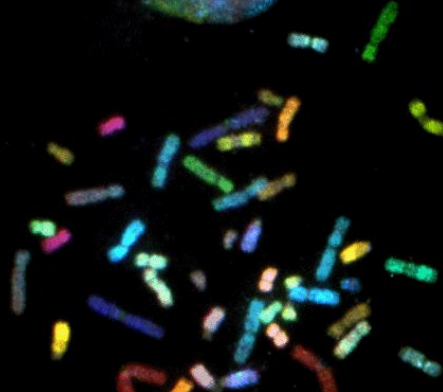
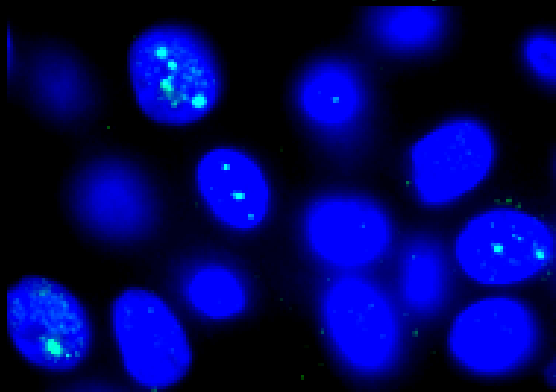
10 mGy

100 mGy

1 Gy

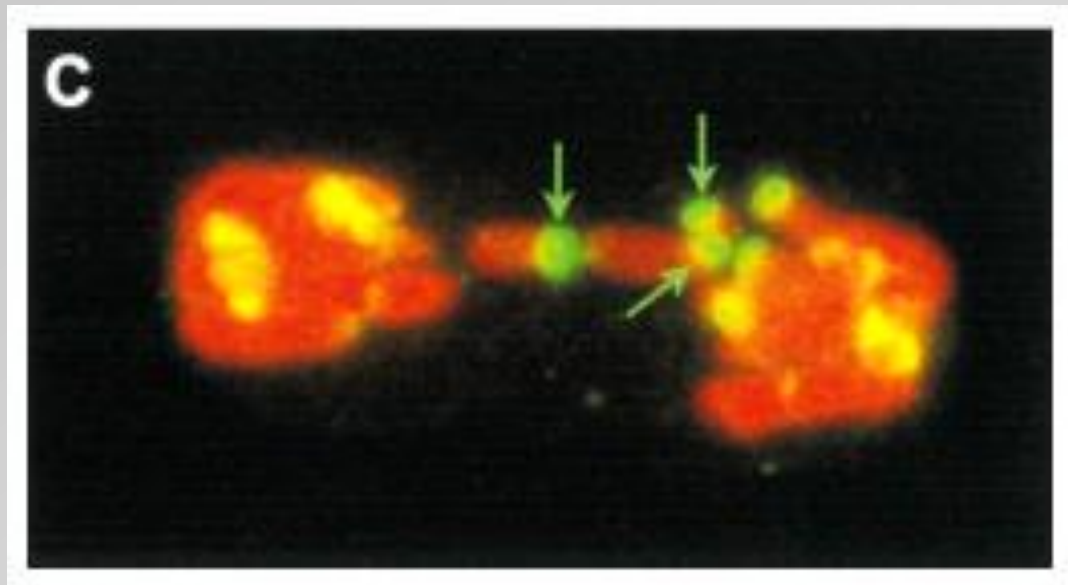
10 Gy

100 Gy



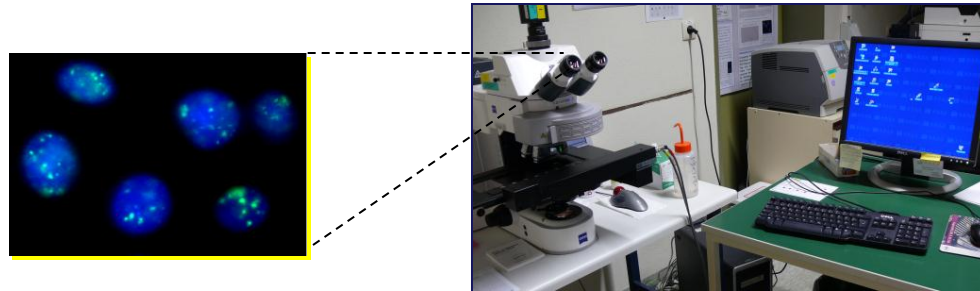
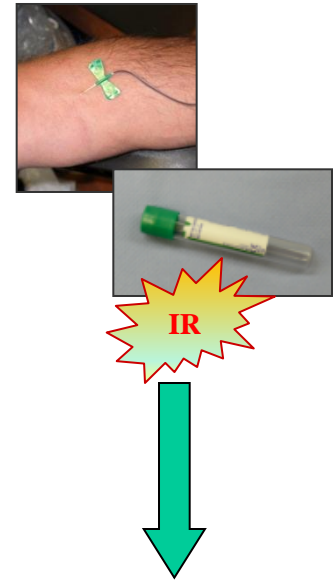


# gH2AX detects megabase pair domains around dsDNA breaks



# $\gamma$ -H2AX: ionizing radiation-induced foci

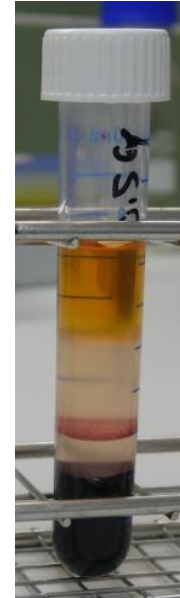
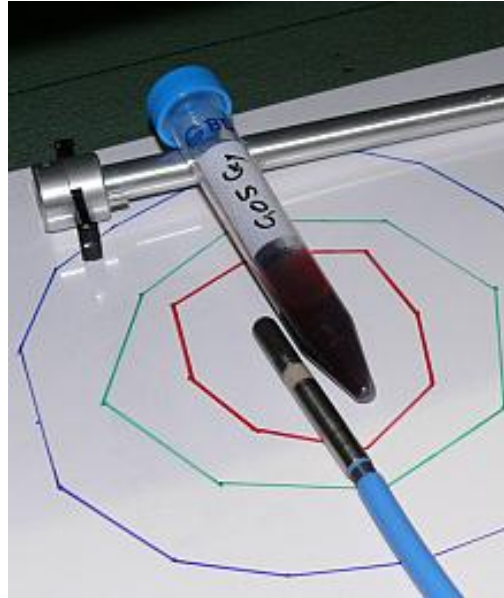
1. Blood sampling
2. Leukocyte isolation
3. Fixation (stop repair/de-phos.)
4. Immunofluorescence staining
5. Analysis







# Assay -1- in vitro blood irradiation & blood cell isolation



**Beware of erythrocytes !**





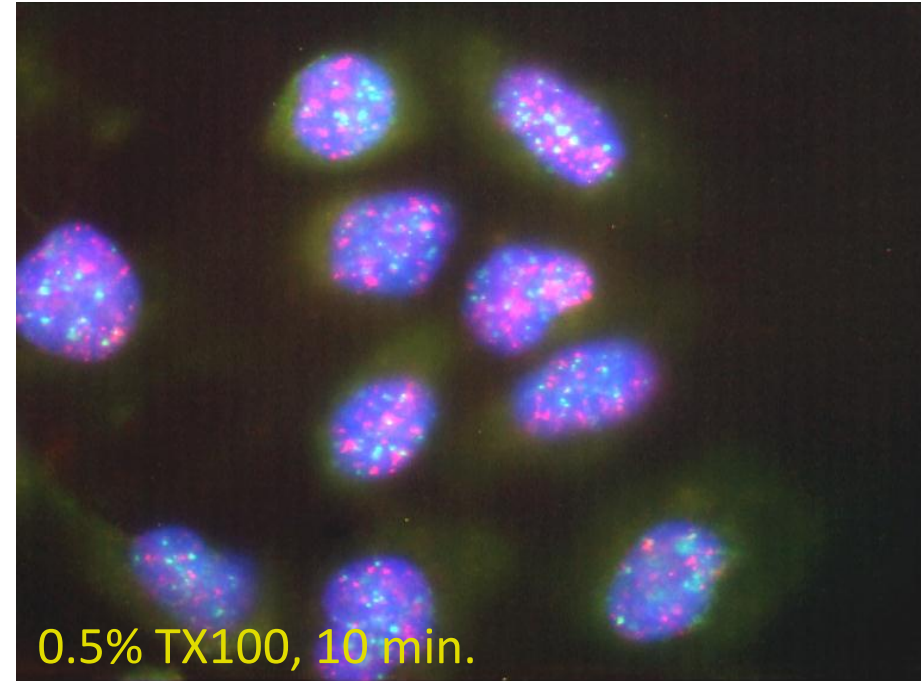
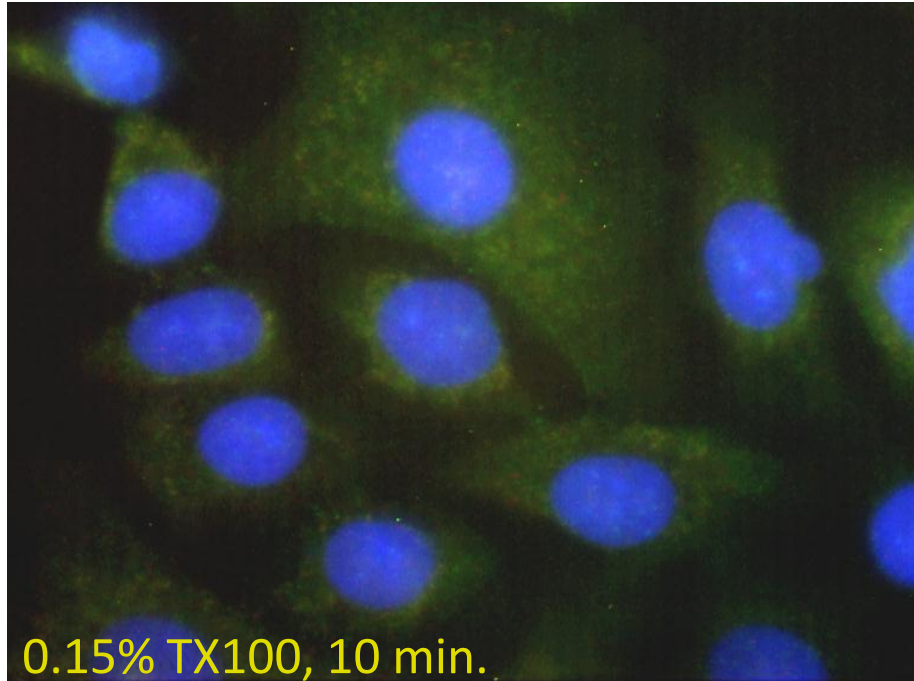
# Focus Assay - Staining

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 3.7% formaldehyde
- 3) Extract with TritonX100
- 4) add primary antibody  $\Rightarrow$  detection



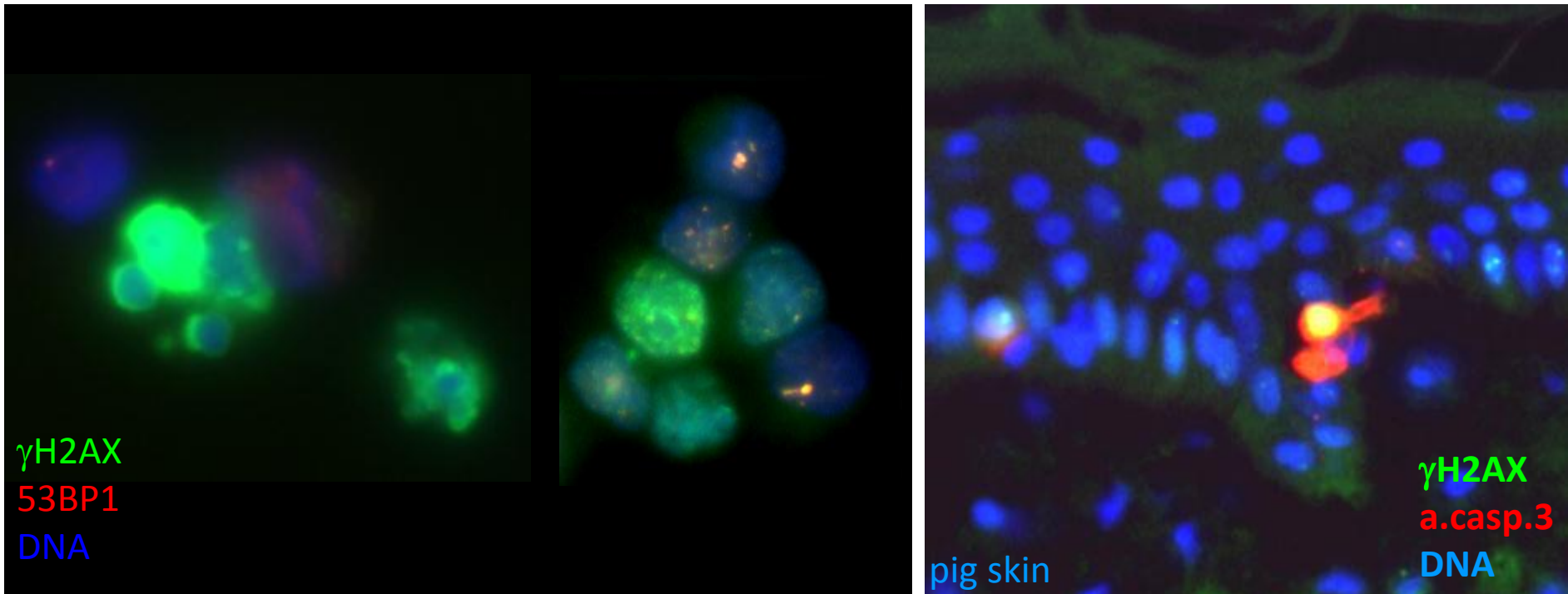
# Methodological pitfalls: Fixation/extraction determines the success of $\gamma$ -H2AX detection



kinetochores  
 $\gamma$ -H2AX



# Apoptotic cells display strong $\gamma$ H2AX fluorescence



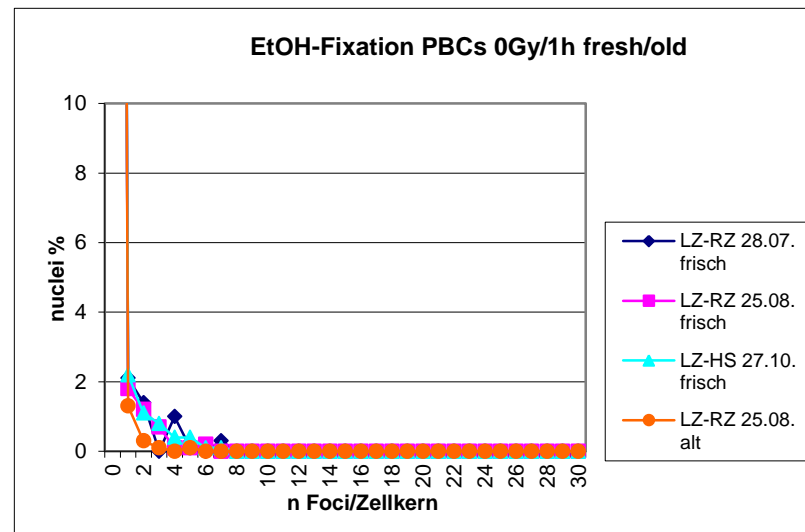
Another source of background: **granulocyte autofluorescence**



# Sample storage ? Yes !

Ethanol fixed cells can be stored and shipped

! only compare with similarly treated (time & temp) controls





# $\gamma$ -H2AX Focus assay: IF



n nuclei = 68 (x4,25)

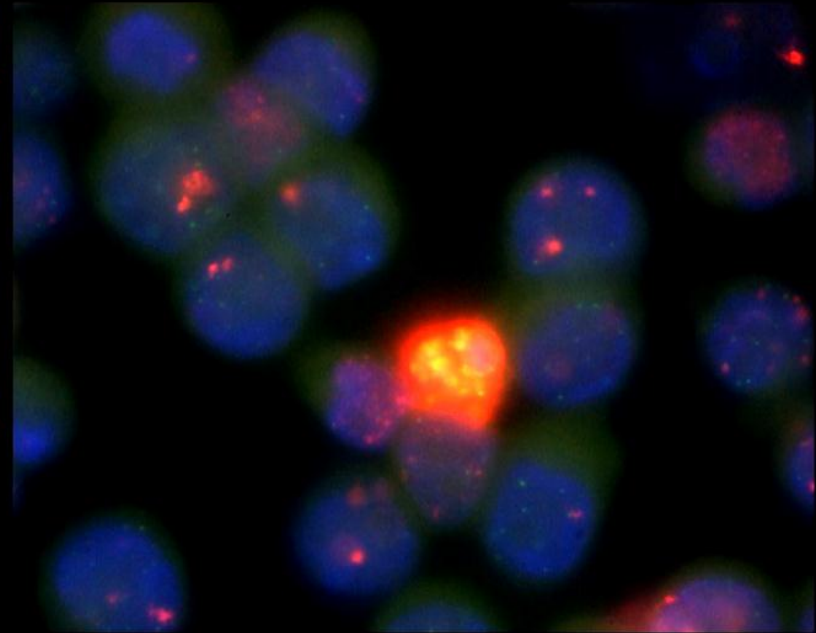
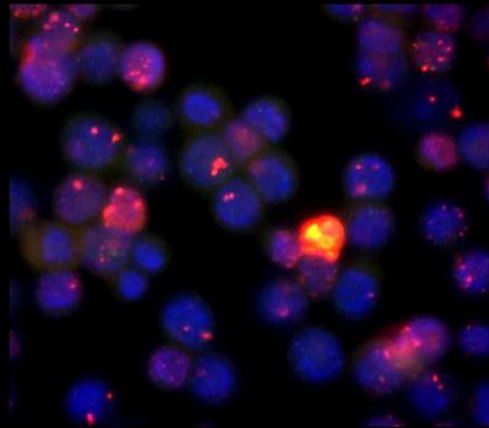
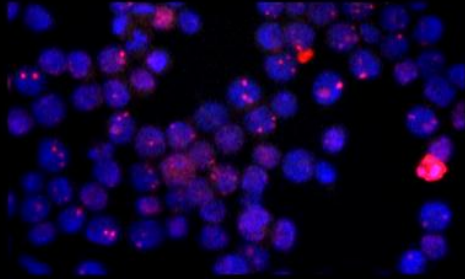
42 (x2,6)

16

25x

40x

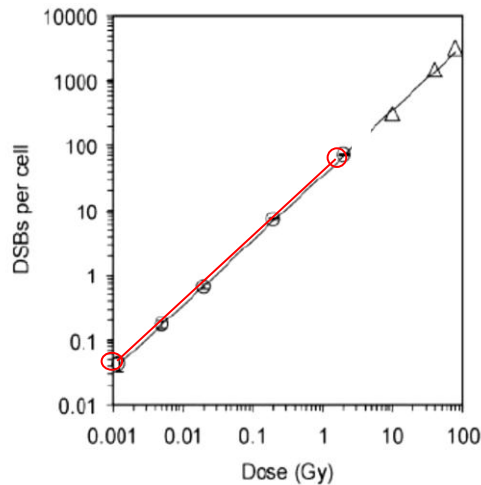
100x



**Enumerate (manually) foci numbers in 40 positive cells,  
or in up to 800 negative cells**

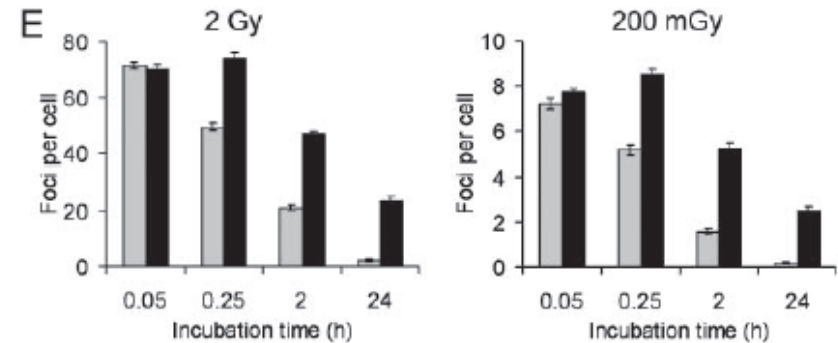


# Av. $\gamma$ H2AX Foci # linearly correlate with dose < 2Gy

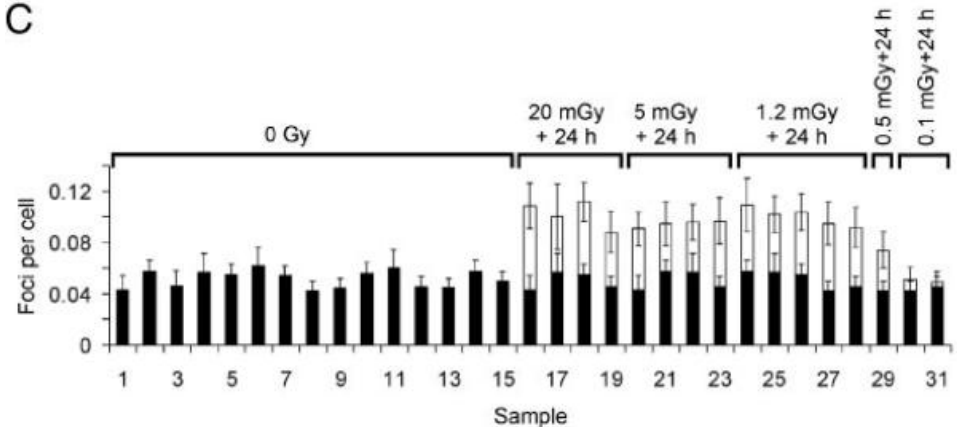


**Fig. 2.** DSB induction in MRC-5 cells.  $\gamma$ -H2AX foci were counted 3 min after irradiation, and the mean values of foci per cell are shown (circles). Triangles represent DSB induction data obtained from PFGE analysis. The line is a linear fit to the data points with a slope of 35 DSBs per cell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

Rothkamm and Löbrich 2003

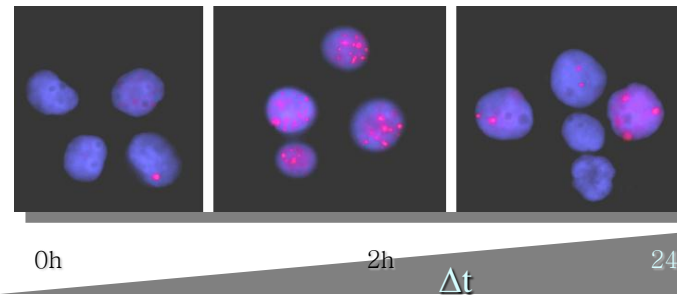


C

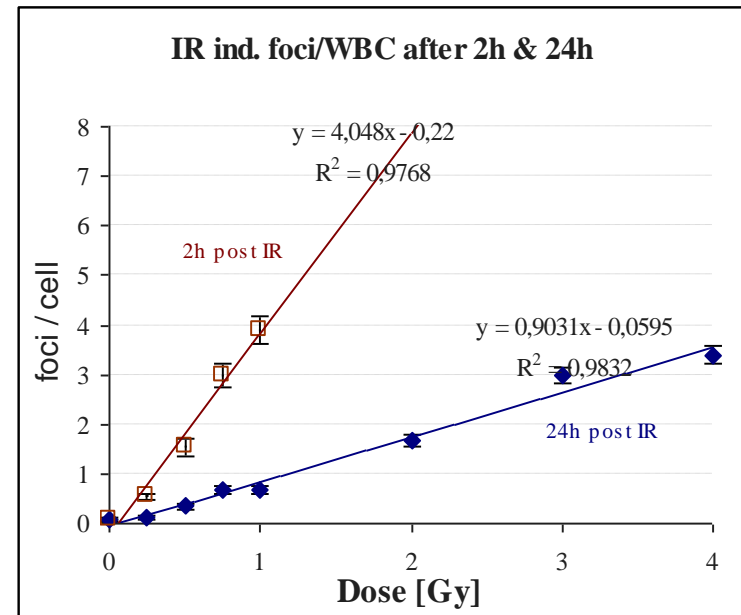
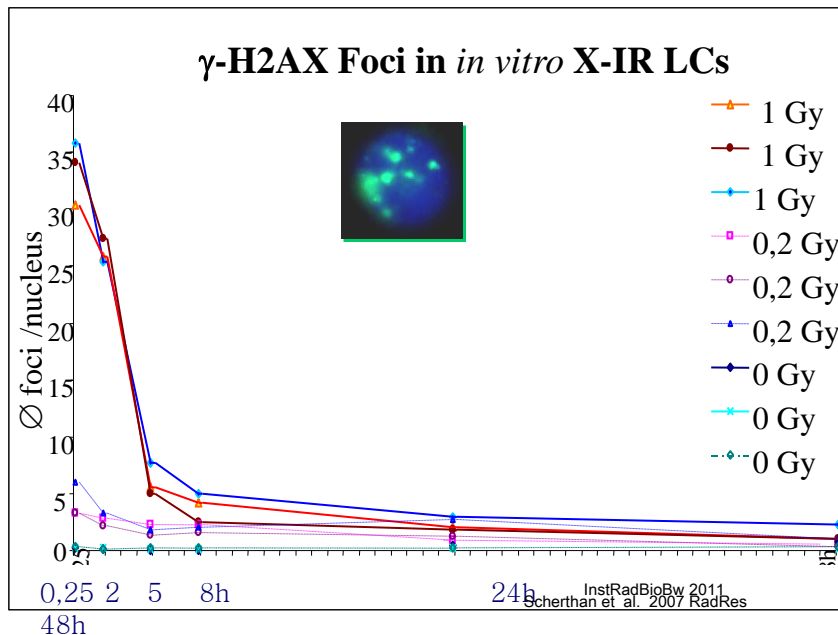


- Rothkamm and Löbrich 2003 PNAS:  
~40 foci at 5' @1Gy & ~7foci @ 0.2Gy in fibroblasts
- Löbrich et al. 2005 PNAS: ~20 foci/Gy in lymphocytes

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



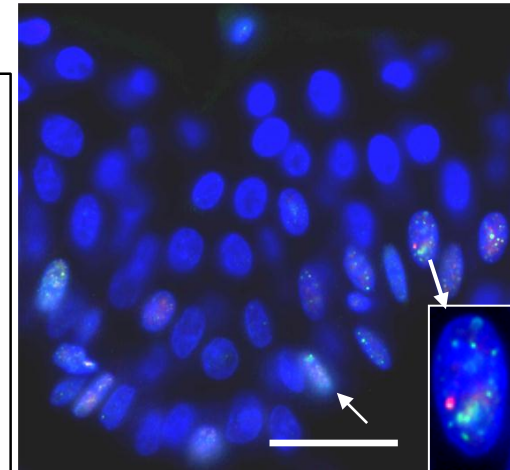
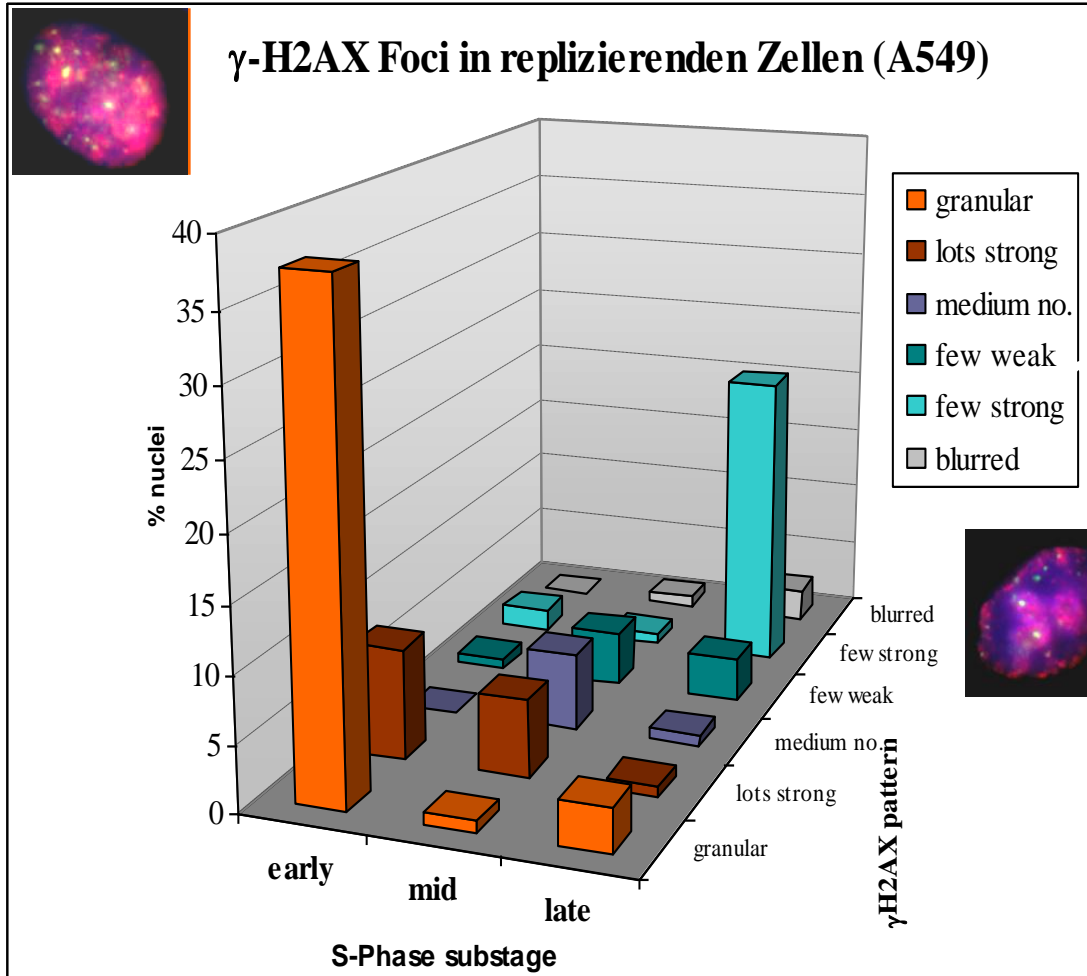
Repair foci  
DNA  
(nuclei)



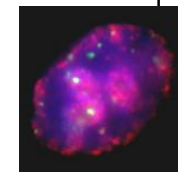




# Replicating cells contain different classes of foci



Minipig skin, PLoS one, in press



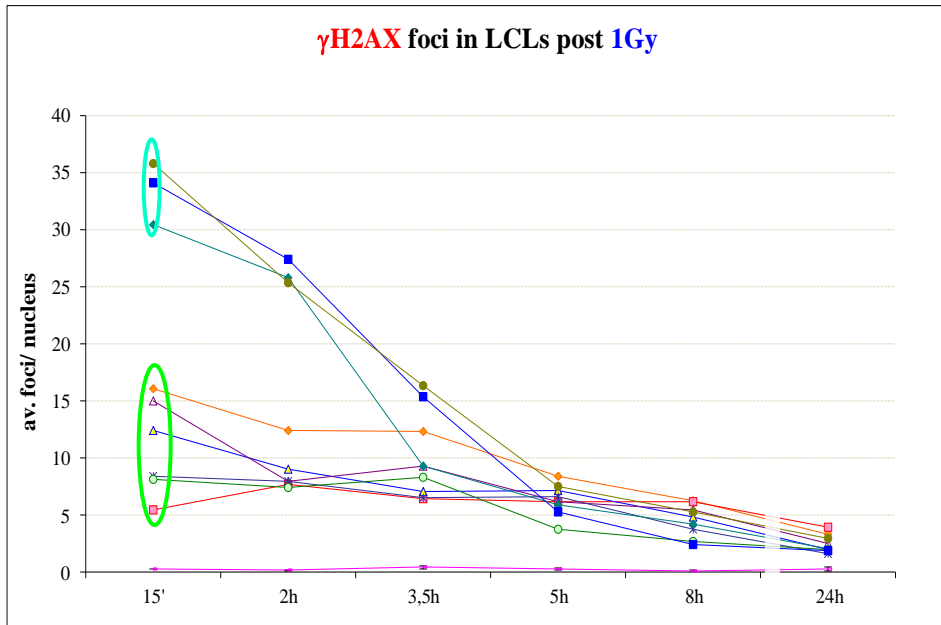
BrdU  
 $\gamma$ H2AX  
 DNA



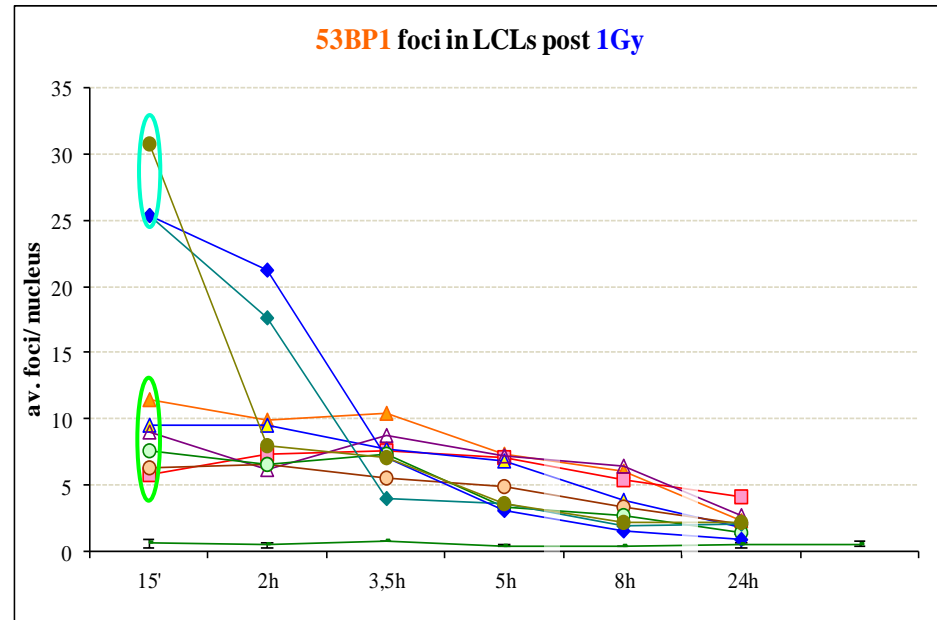
# Genetic background influences RIF formation – effects of NBS1 / ATM deficiencies



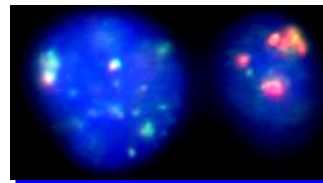
$\gamma$ H2AX foci in LCLs post 1Gy



53BP1 foci in LCLs post 1Gy



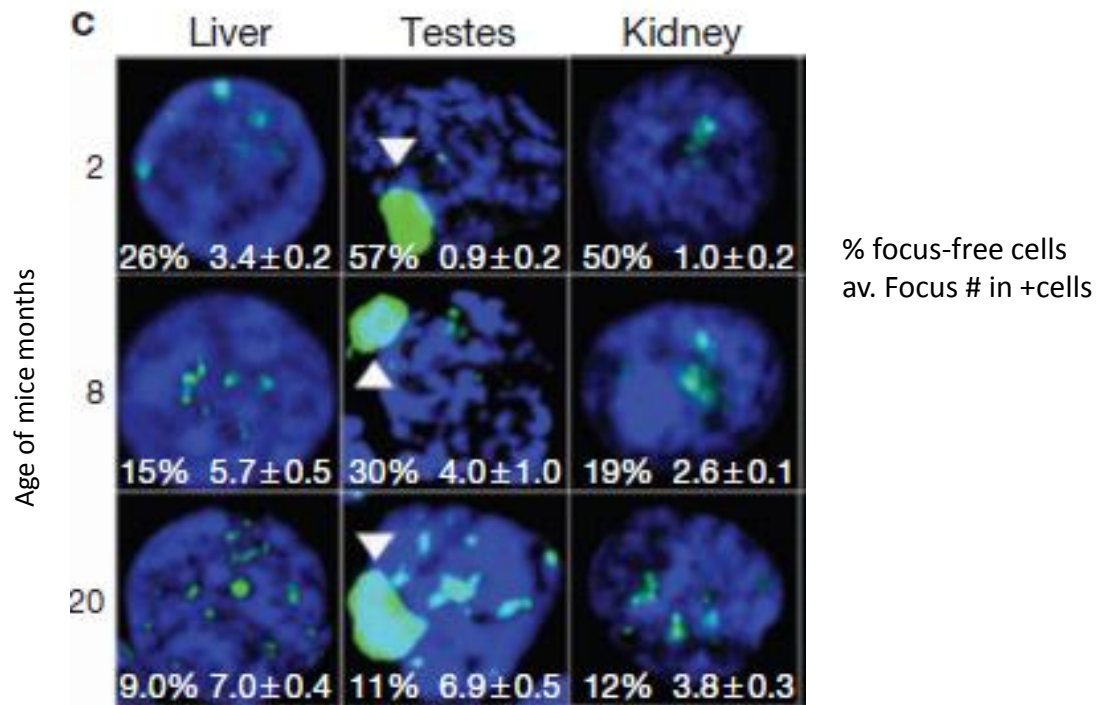
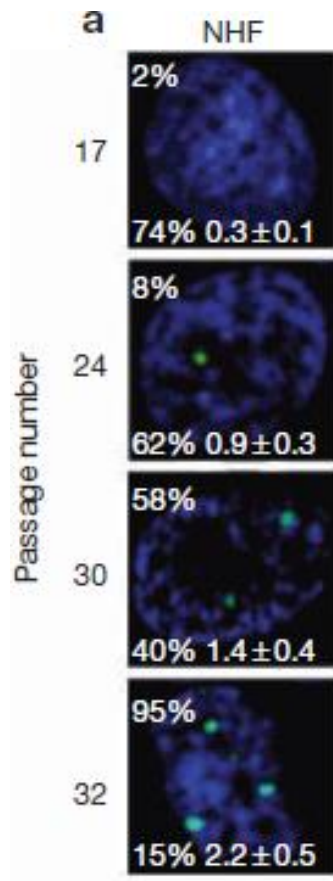
⊖ All cell lines that lack ATM or at least one copy of NBS1 fail to induce the full level ( $\geq 30$ ) of  $\gamma$ H2AX or 53BP1 foci 15' after 1Gy IR. ⊖ Control



# Senescent cells contain increasing # of persistent $\gamma$ H2AX foci

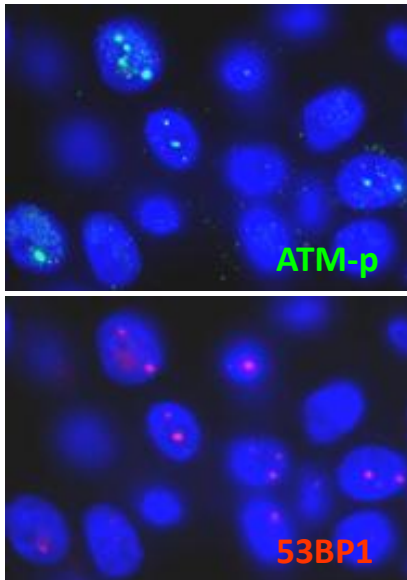
## Pitfalls:

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

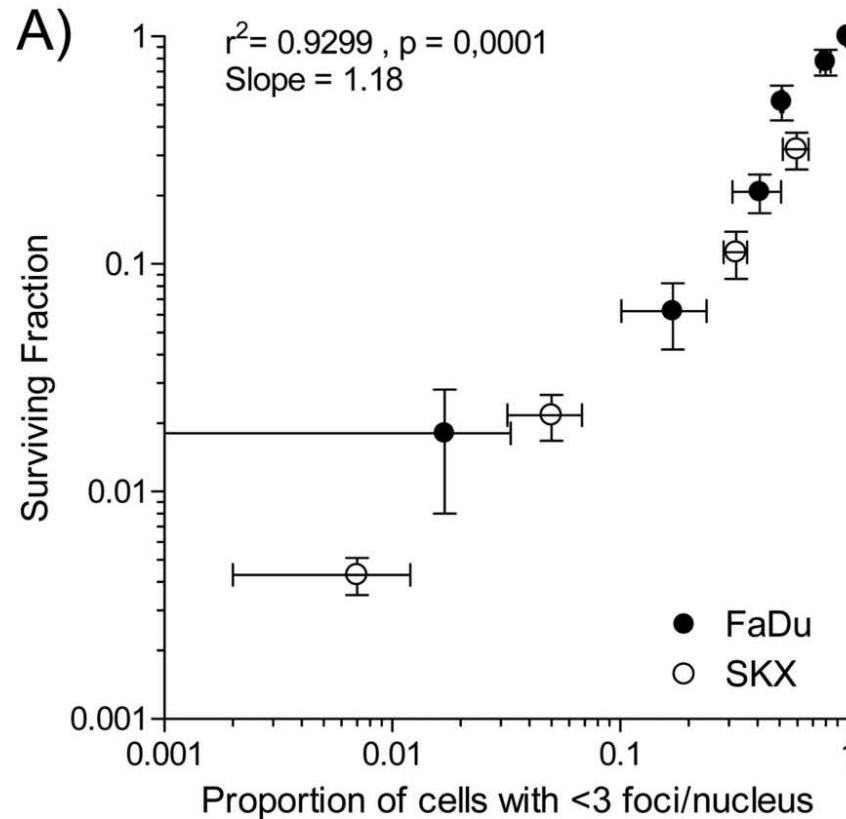




# The fraction of cells with “residual” foci 24h after IR correlates with clonogenic survival



Pig skin 48 days after 50 Gy  
Inst. Radbio. Munich

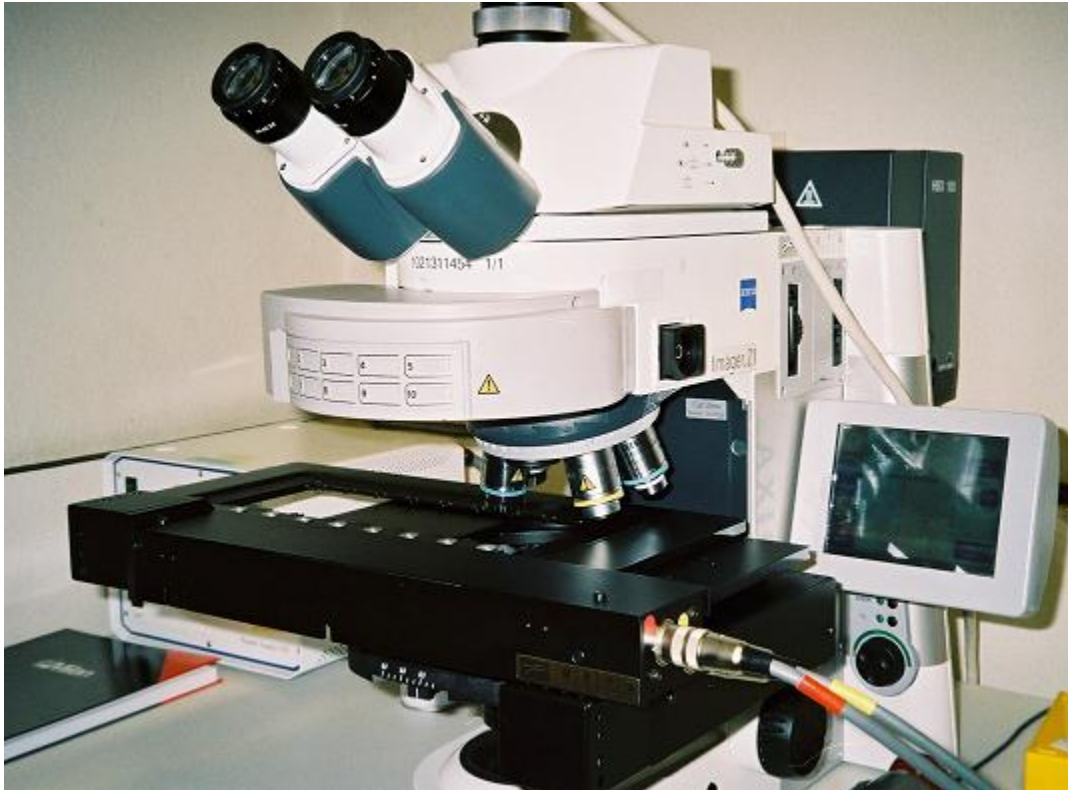


Menegakis et al. 2009 IJRB

**# of cells w >3 foci ↗ ⇒ cell death ↗**

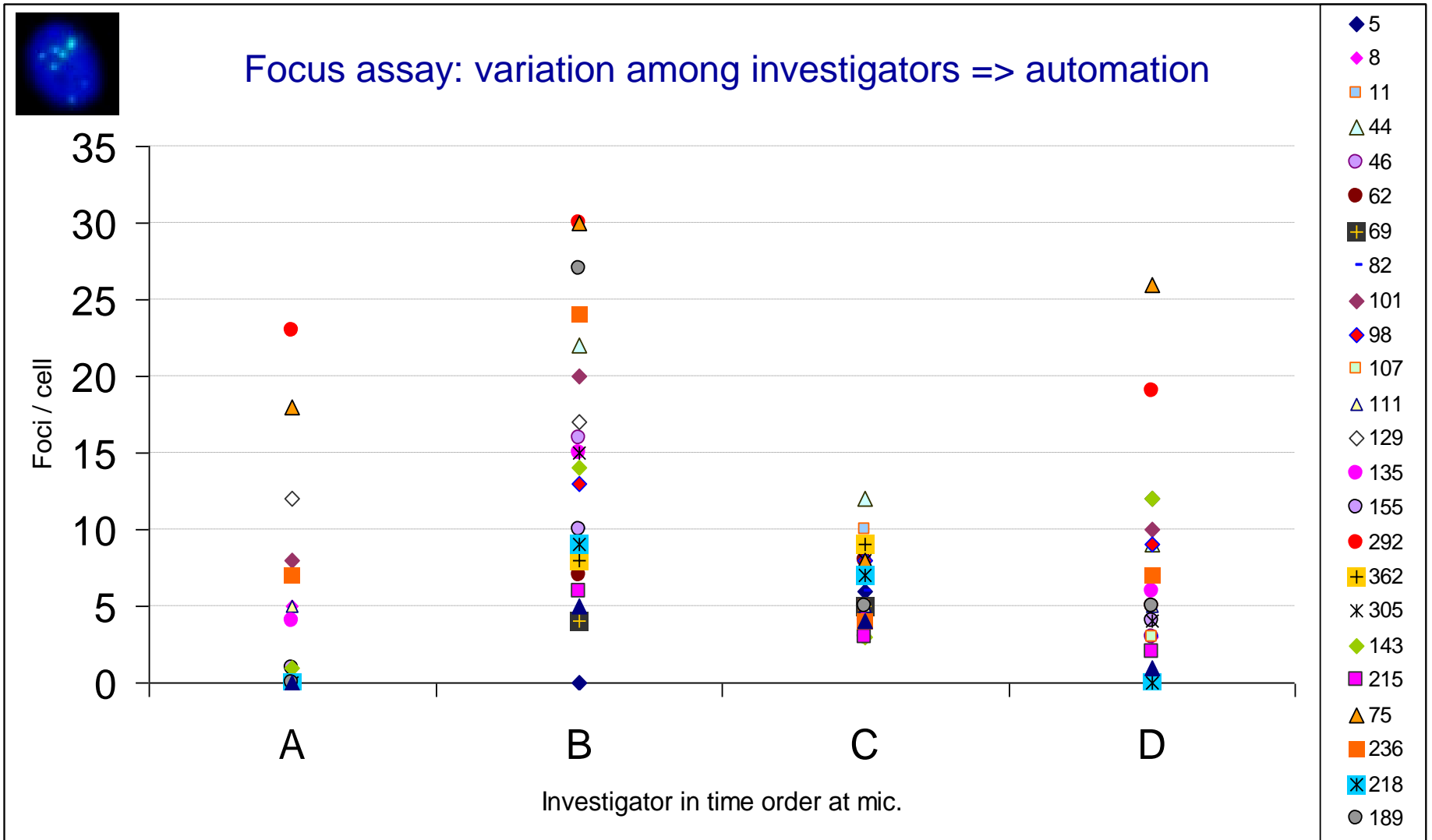


# *Make a wish: automated image capture and analysis !*



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

# Variability in manual analysis



# Computer aided focus analysis

- **Extended focus image**

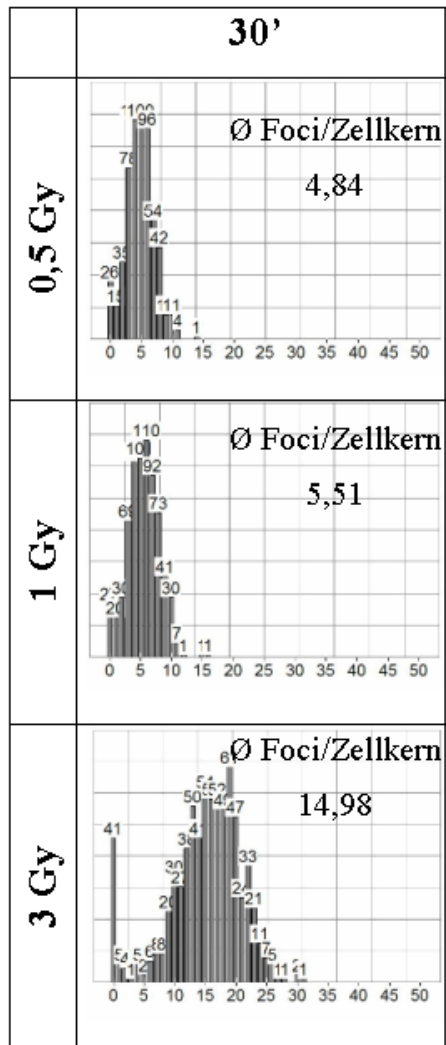
The screenshot displays the Metafer 4 software interface. The main window is titled "Metafer 4 | MetaCye" and includes a menu bar with options: Mode, File, Slide, Cells, Scoring, Training, MetaCye, Configure, Stage, Filters, Tools, Help. The interface is divided into several sections:

- Left Panel:** A large "Extended focus image" showing a field of blue-stained cells. A small white box highlights a specific region.
- Center Panel:** A 2x3 grid of zoomed-in cell images, numbered 1 through 6. Each image shows individual cells with green and red markers. Below each image are numerical values, such as "0.33 1298" for image 2.
- Right Panel:** A vertical toolbar with navigation and control icons, including arrows, a magnifying glass, a circle, a checkmark, an 'X', and a double 'X'.
- Bottom Panel:** A control panel with a histogram on the left showing a distribution of values. To the right of the histogram are several buttons: Setup, Search, Gallery, Relocate, Isis, and Exit. Below the histogram, the following data is displayed:
  - PaFOCI\_LZ-G-63\_bis\_1Gy
  - Magnif. : 63.0
  - Fields : 19
  - s / Field : 3.8
  - Total Time : 1:12
- Bottom Left:** A status bar showing "0,5Gy\_8h" and "497". Below it is a 3D schematic of a slide with a grid of cells.

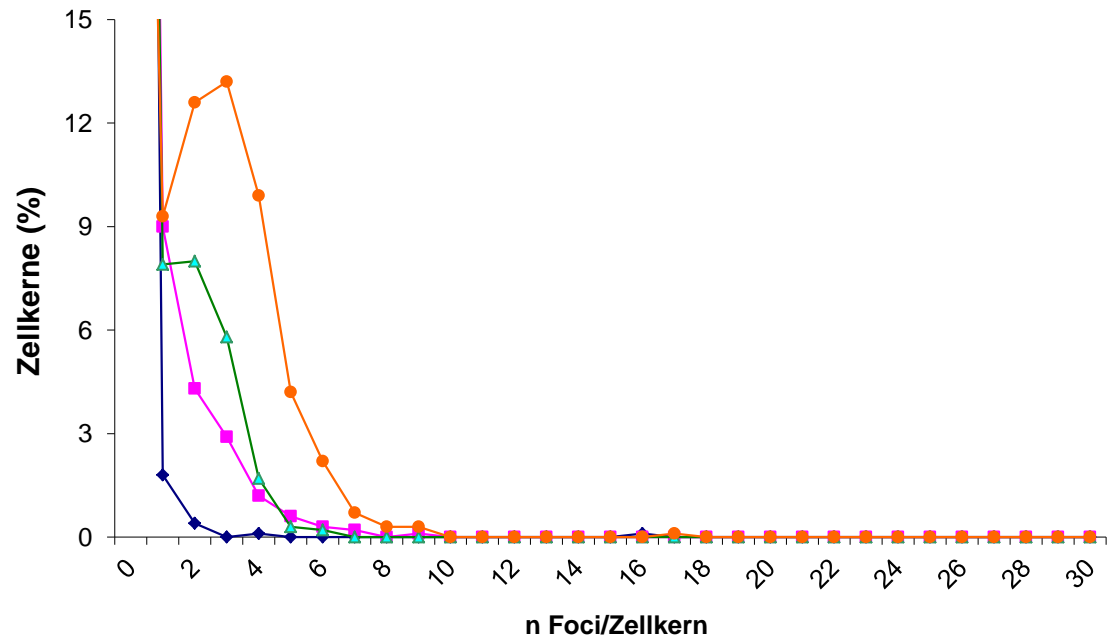
- **Single cell analysis**
- **Data output**

# Automated scanning & image analysis – not so variable?

Dosisbereich 0,5 bis 3 Gy:



Dose response LL X irrad.



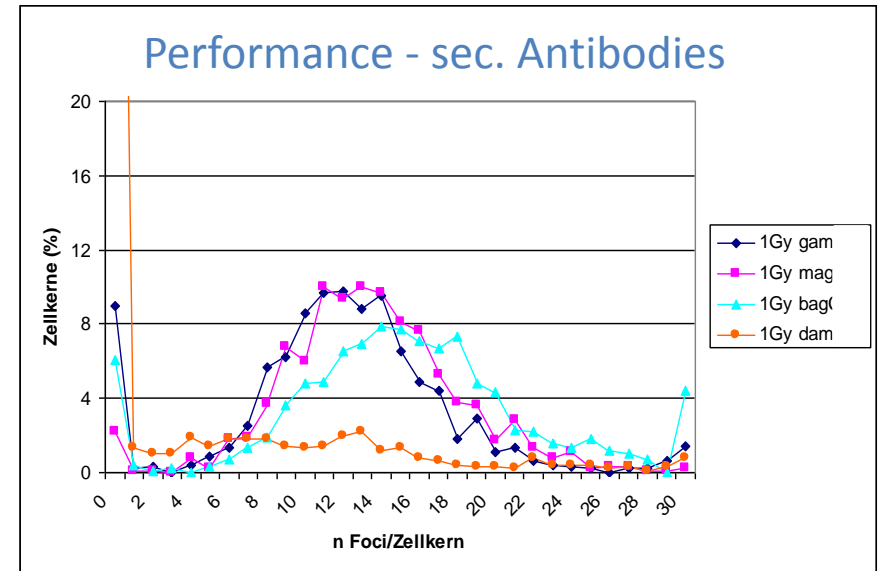
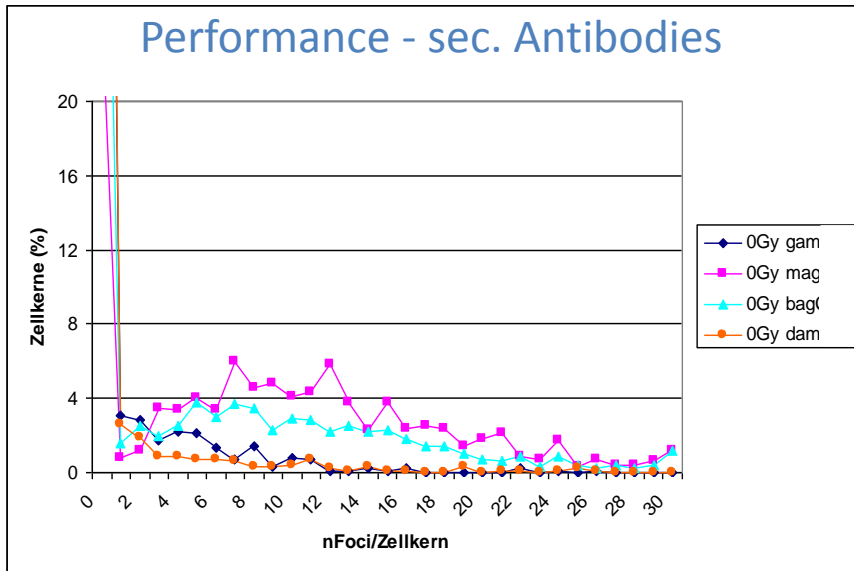




# Reagent-induced variation



different secondary Abs → variation





# Sample storage

*Problem:* when collecting samples over time:  
variation in staining

*Solution:* fix cells and collect samples,  
stain when appropriate

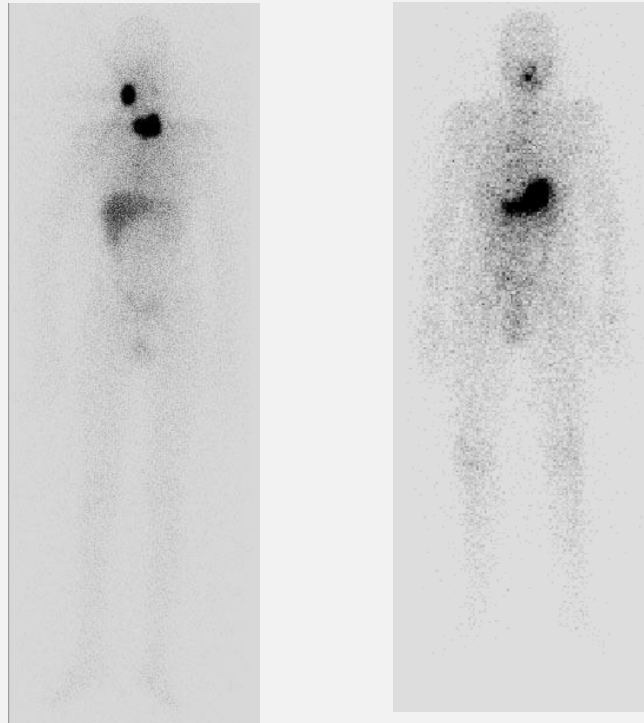
- We use 70% ethanol @ -20°C

(Lassman et al. 2010 Nucl.Med.)



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

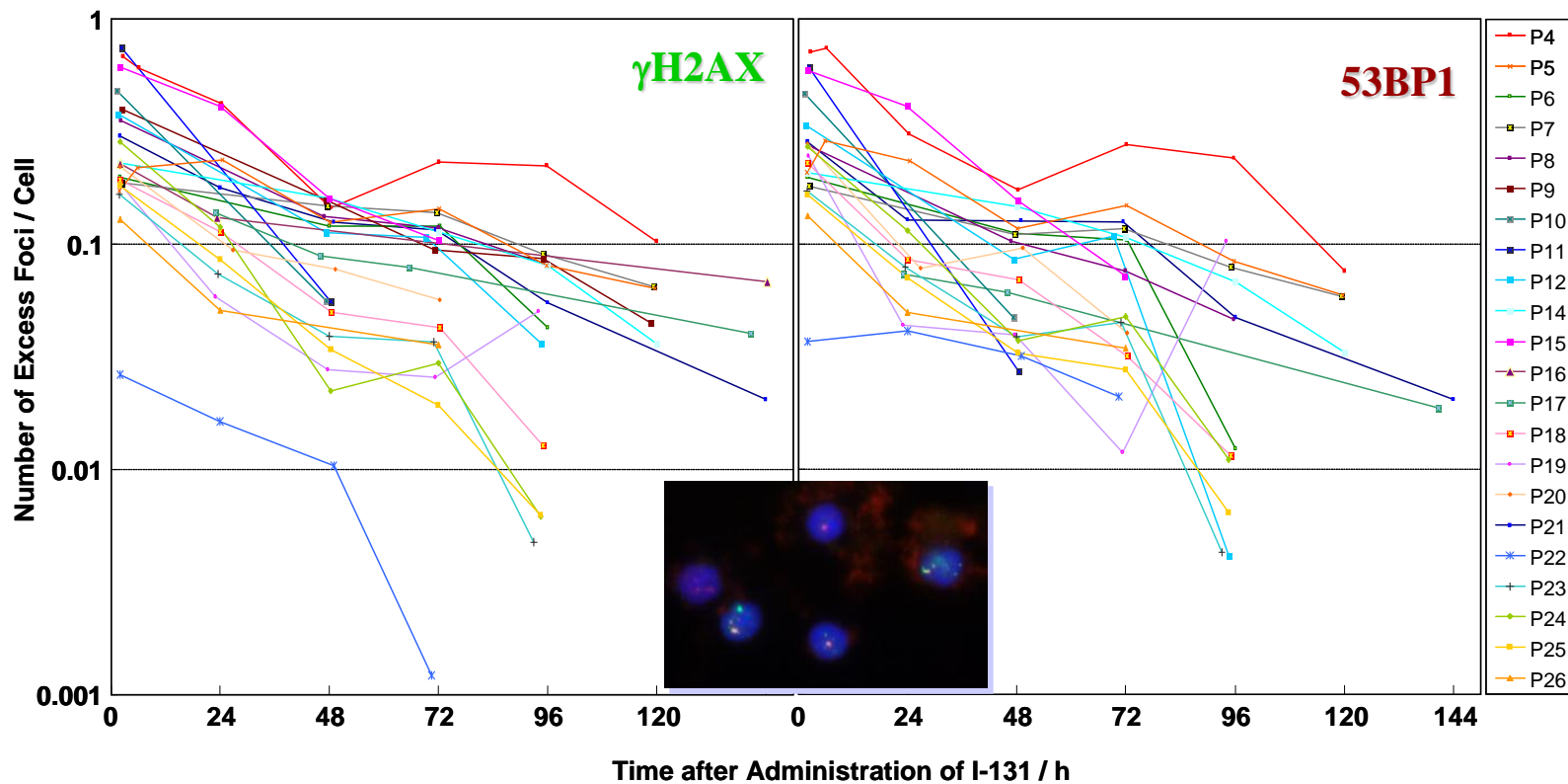
- M. Lassmann, Clinic of Nuclear Medicine, Univ. of Würzburg, GER



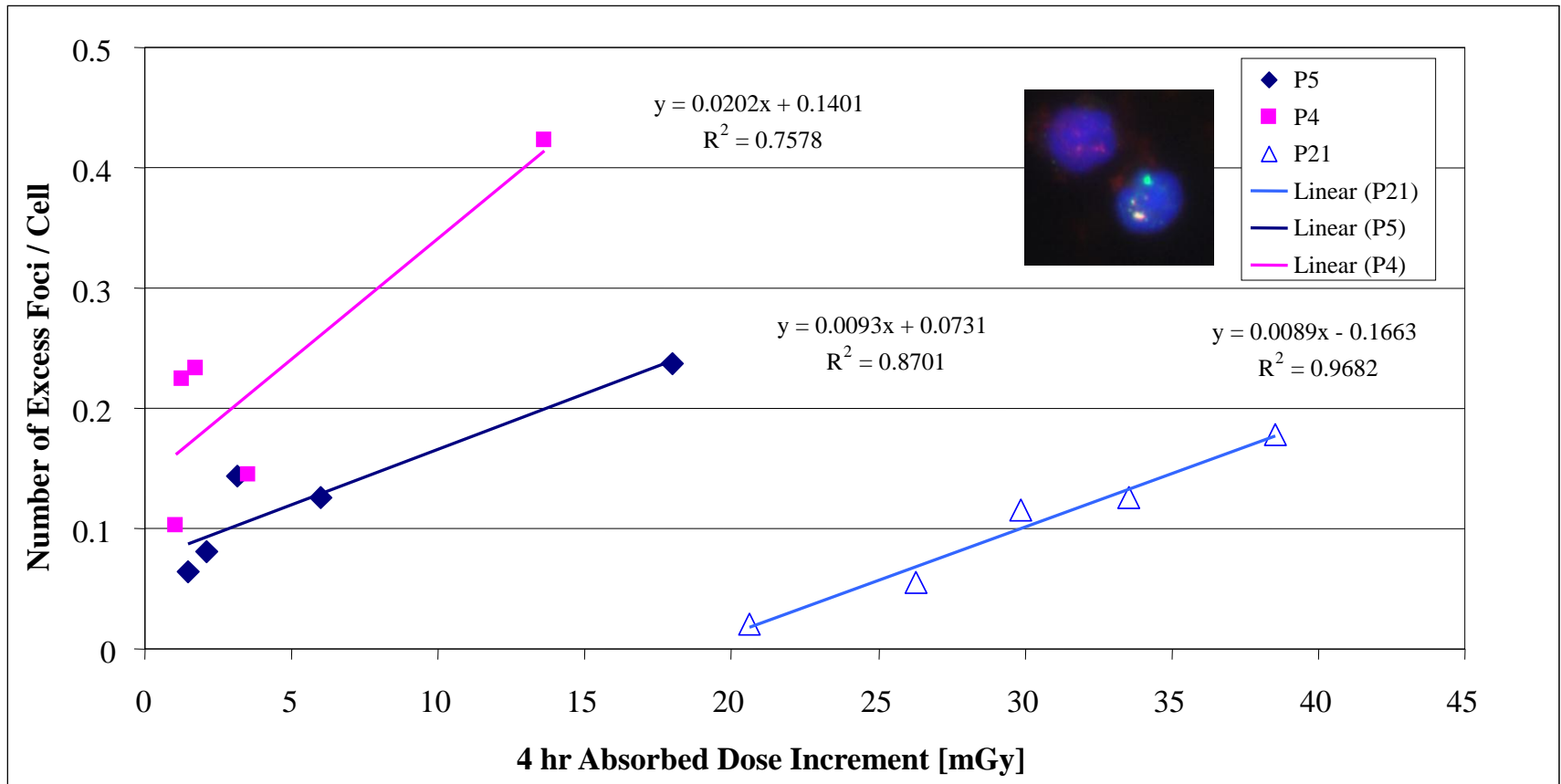
**DTC: Lymph node & thyroid rest treatment by ablation w 3,7GBq I-131**



# Focus analysis among DTC patients treated with $\sim 3.5$ GBq I-131: high inter-indiv. variability



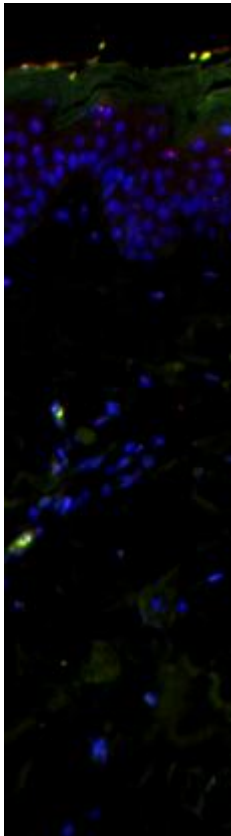
# Correlation foci / physical dosimetry



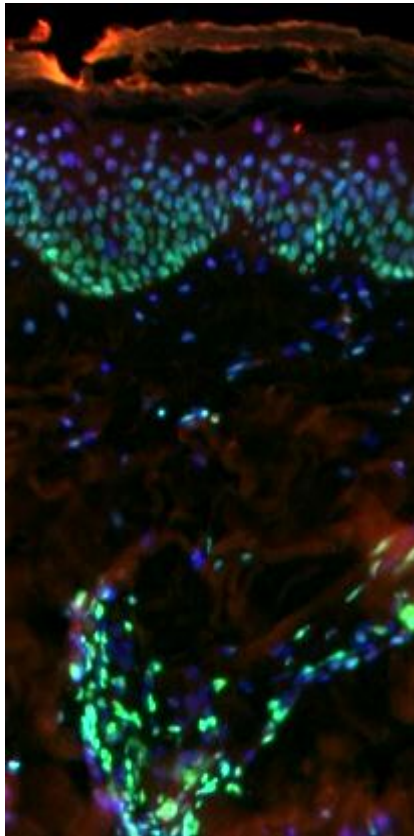
# Focus yield in skin & blood after 50Gy partial body $\gamma$ -irradiation

## Pig-skin

non-IR

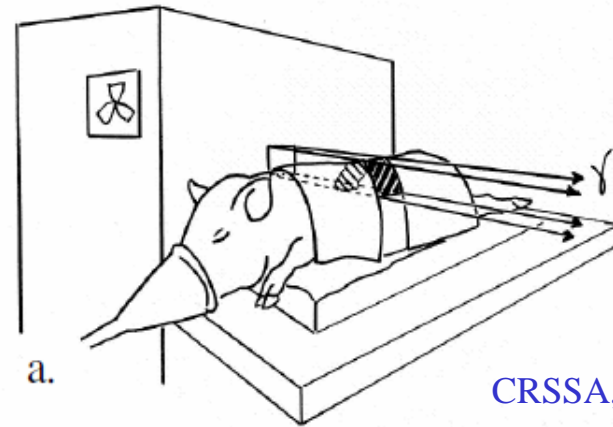


4h post 50Gy

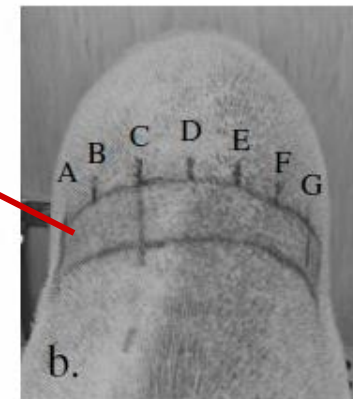


InstRadBioBw

## Pig model

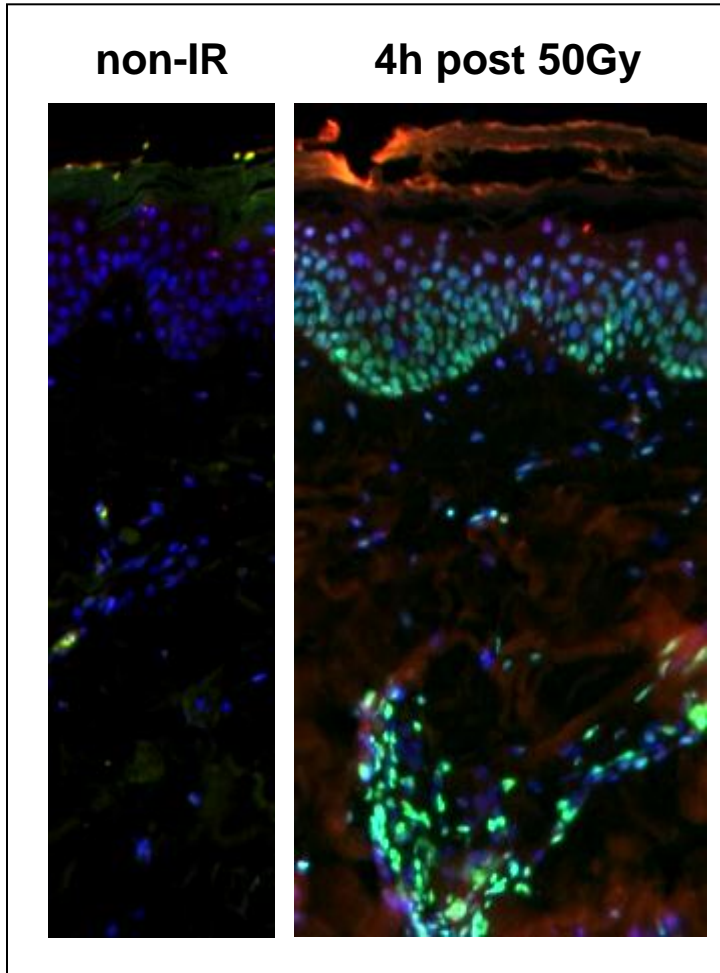


CRSSA, F

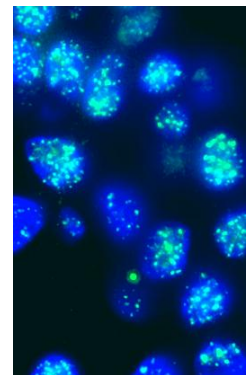
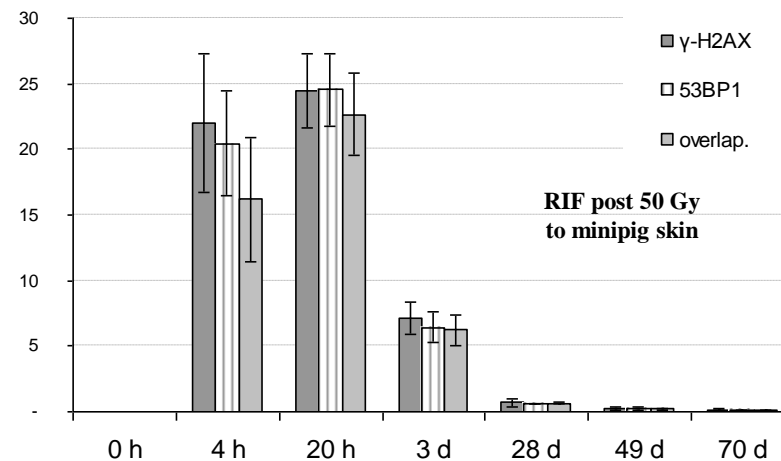
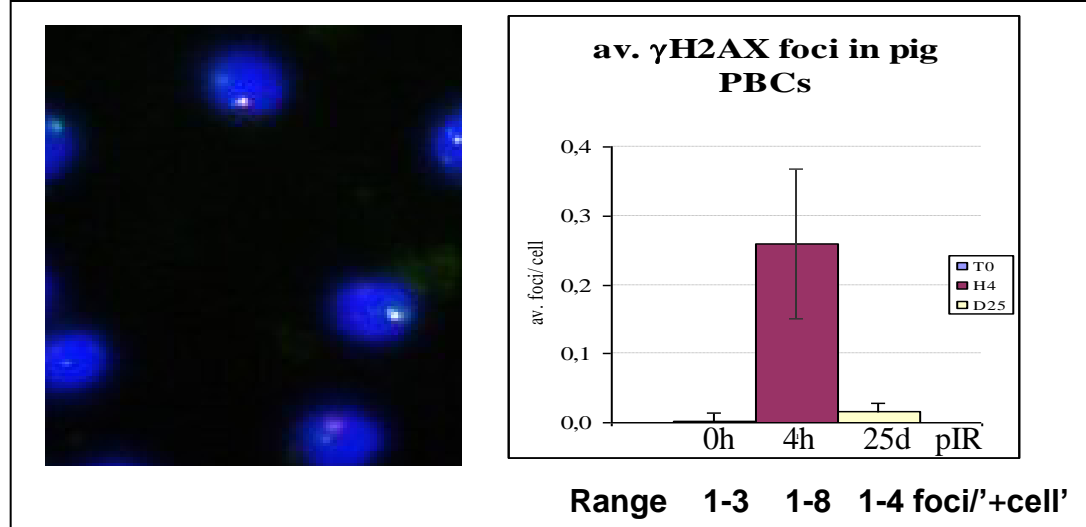


# No correlation of foci # in skin & blood after 50 Gy partial body $\gamma$ -irradiation

## Pig-skin



## PB Leukocytes

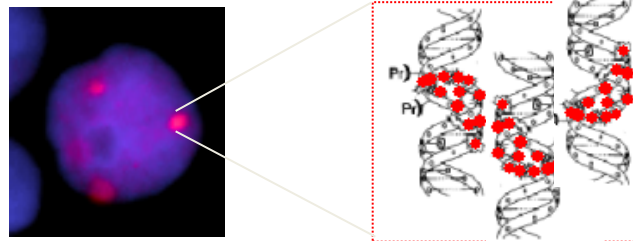


Ahmed et al. 2012, PloSOne, in press.

# Conclusions

## ➤ Repair ( $\gamma$ H2AX) Focus Test

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





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