the γ-H2AX assay – "untold stories"

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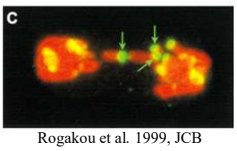
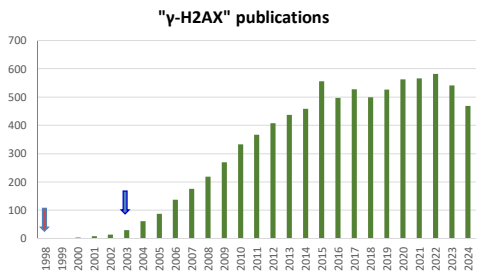
γ–*H2AX focus enumeration in the mic*

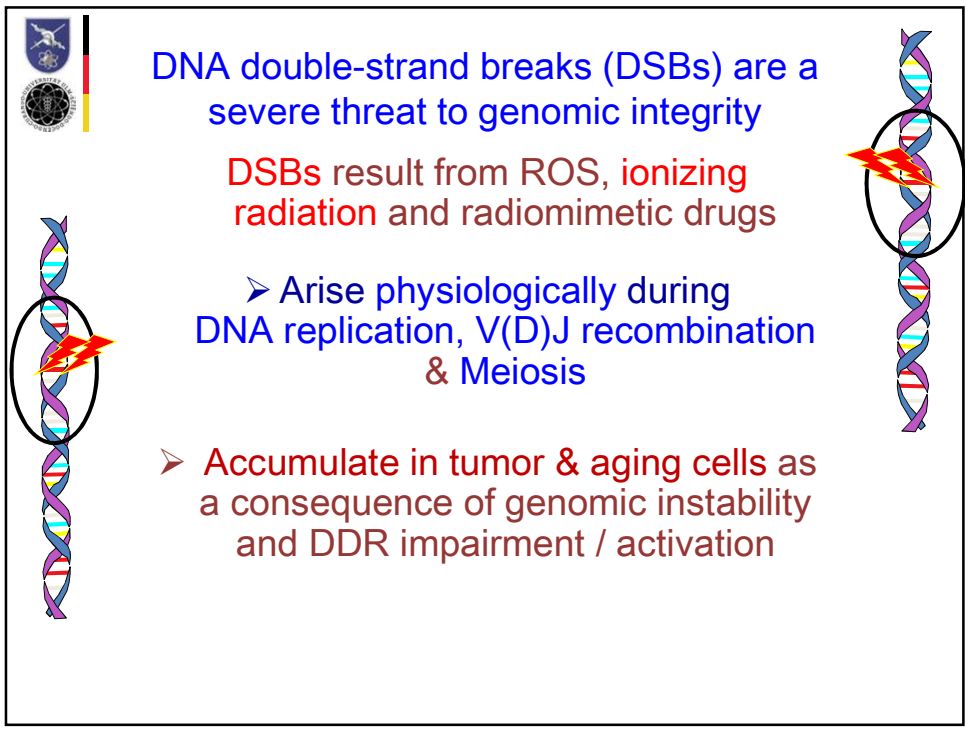
⇒ Method for quick detection of dsDNA breaks

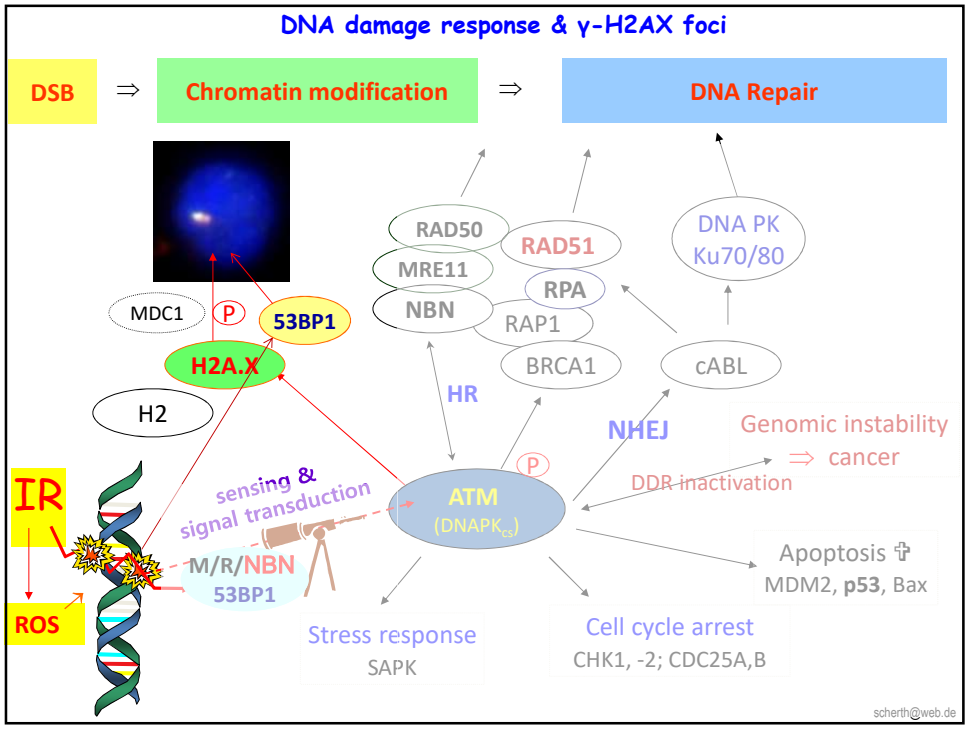


Rogakou et al. 1999, JCB

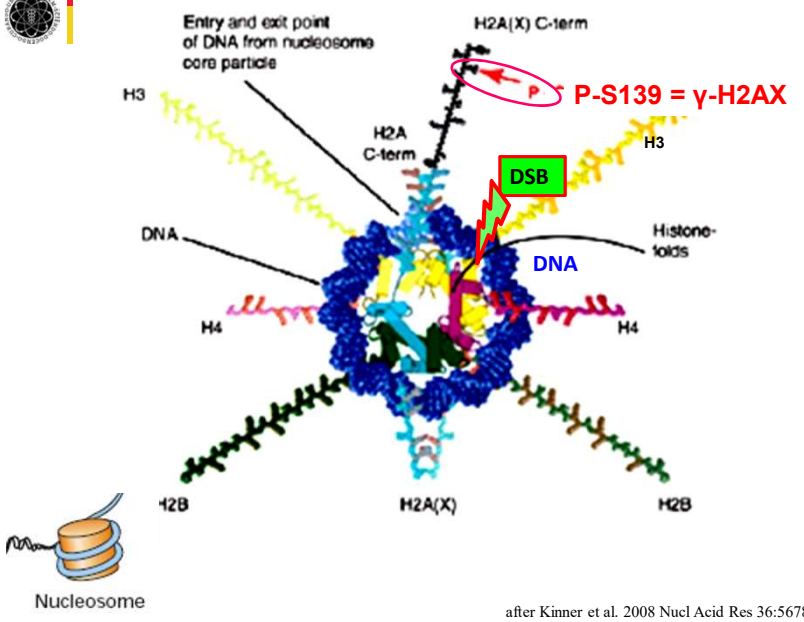


Rogakou et al. *JBC* 1998, 1999
Mahadevaiah et al. *Nat Genet*. 2001
Sedelnikova et al. 2002 RadRes
Rothkamm & Löbrich 2003 *PNAS*


DNA double-strand breaks (DSBs) are a severe threat to genomic integrity
DSBs result from ROS, ionizing radiation and radiomimetic drugs
➢ Arise physiologically during DNA replication, V(D)J recombination & Meiosis
➢ Accumulate in tumor & aging cells as a consequence of genomic instability and DDR impairment / activation


DNA damage response & γ-H2AX foci
DSB
⇒
Chromatin modification
⇒
DNA Repair
RAD50
MRE11
NBN
RAD51
RPA
RAP1
BRCA1
DNA PK
Ku70/80
cABL
MDC1
P
53BP1
H2A.X
H2
HR
NHEJ
Genomic instability
⇒ cancer
DDR inactivation
IR
ROS
sensing &
signal transduction
M/R/NBN
53BP1
ATM
(DNAPK$_{cs}$)
P
Apoptosis ✞
MDM2, p53, Bax
Stress response
SAPK
Cell cycle arrest
CHK1, -2; CDC25A,B
scherth@web.de

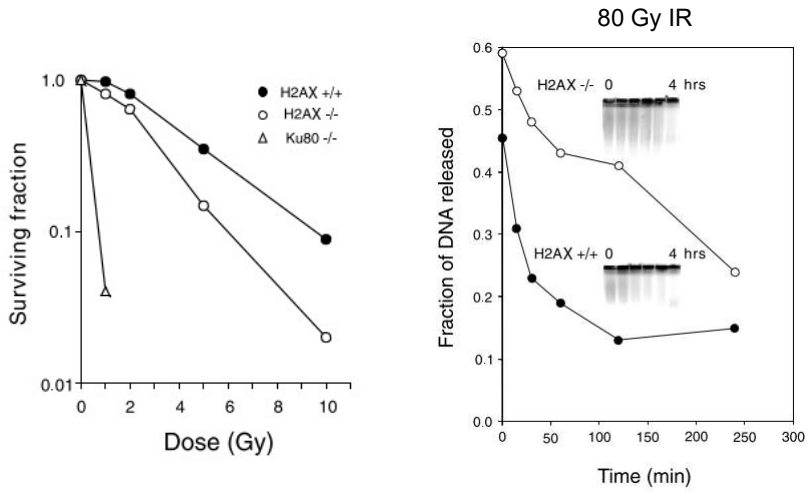
Histone H2A.X P-Ser-139: indicator of DSBs



after Kinner et al. 2008 Nucl Acid Res 36:5678

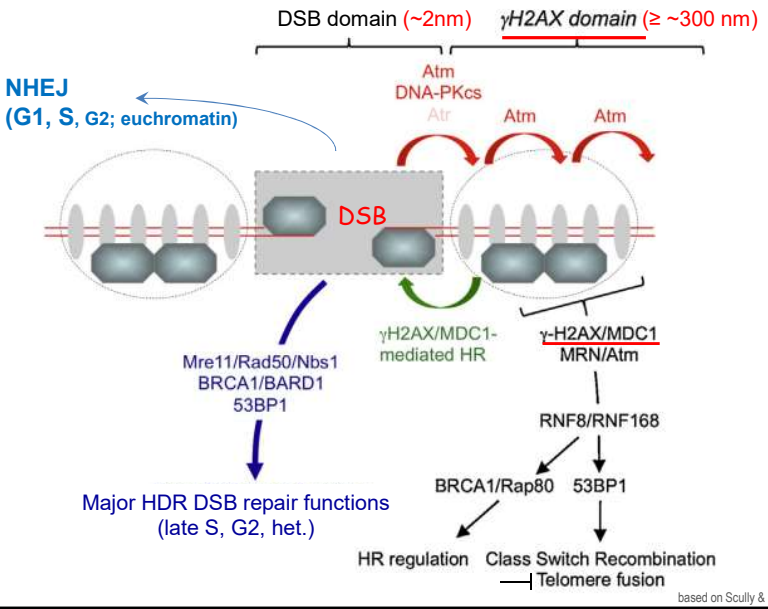
Histone H2AX is required for DSB repair

⇒ *H2ax*$^{-/-}$ knockout mice are radiosensitive



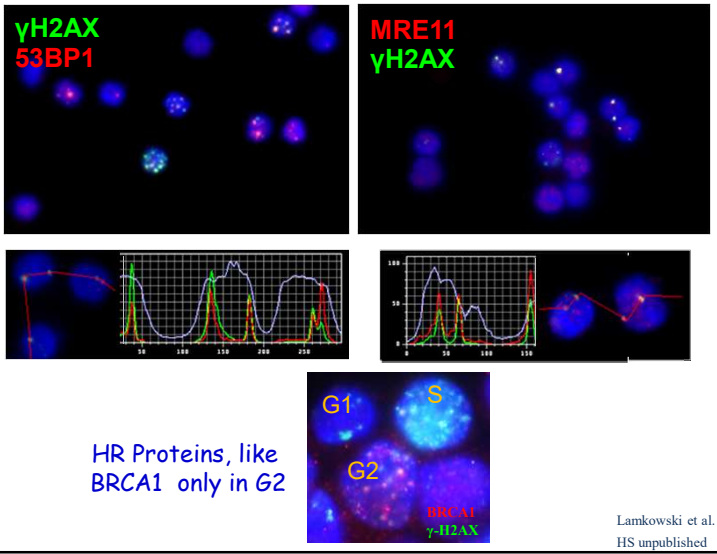
Celeste et al. 2002, Science

γH2AX forms a chromatin domain around a DSB



based on Scully & Xie MR 2013

DSB repair proteins form foci @ DSBs

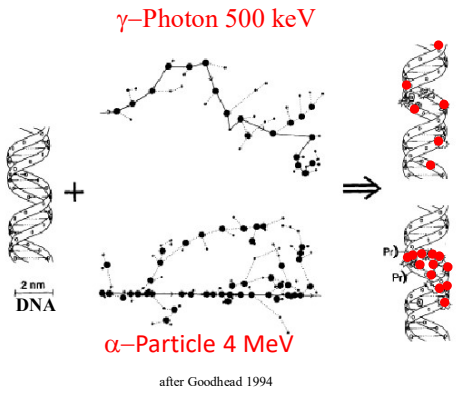


HR Proteins, like
BRCA1 only in G2

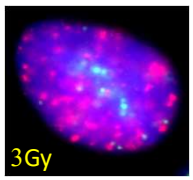
Lamkowski et al. 2014 PlosOne.
HS unpublished

IR: DNA damage patterns reflect LET

γ-H2AX forms DSB-containing foci or tracks

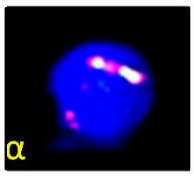


after Goodhead 1994



Low LET

γH2AX
DNA
Kinetochore



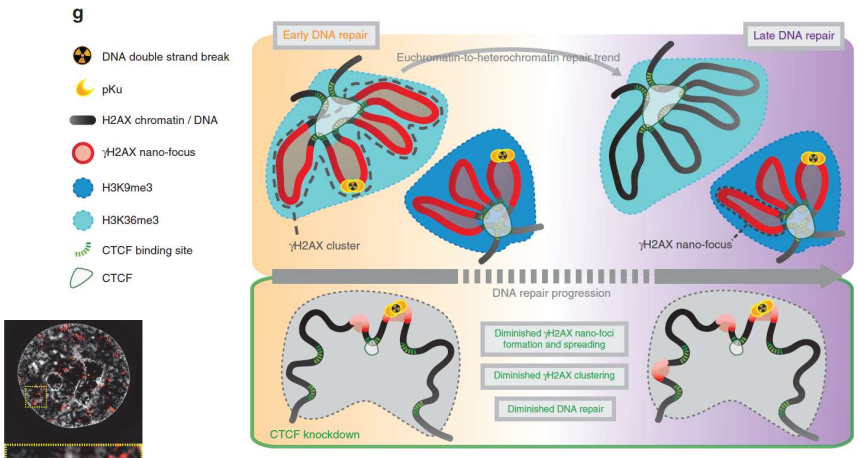
High LET
(**^{223}Ra**)

γH2AX
DNA
53BP1

after Goodhead 1994; Schumann, Scherthan 2019 SciRep

Ultra Resolution Microscopy & CHIP Seq:

γ-H2AX foci ⇒ clusters of CTCF-dept. nano-foci



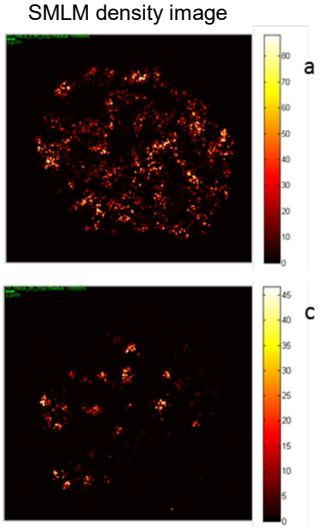
CTCF: CCCTC-binding factor

Natale et al. 2017 NATURE COMMUNICATIONS | 8:15760 | DOI: 10.1038/ncomms15760 | www.nature.com/naturecommu

γ-H2AX foci consist of γ-H2AX nano-clusters covering DSB sites

Single Molecule Localization (Fluorescence) Microscopy (nm range)

SMLM density image | Nanocluster map



2Gy
1h
(~90 clusters)

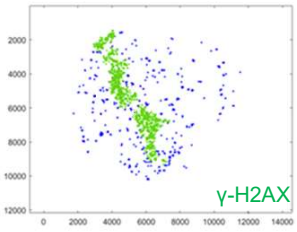
2Gy
3h (16 clusters)

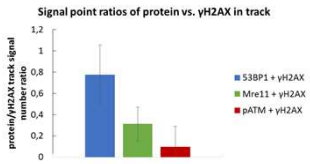
Point intensity => next neighbor frequency

Hausmann, Scherthan, et al. 2018 *Nanoscale* 10:4320

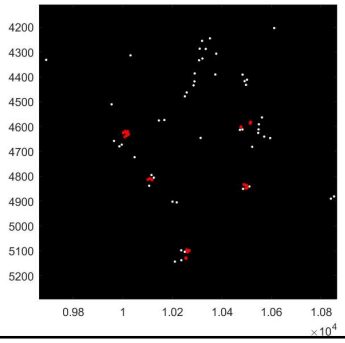
Alpha-track nano-structures by Ultra Microscopy

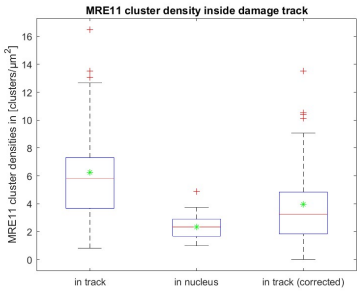
(SMLM, cooperation w Hausmann Lab, HD)





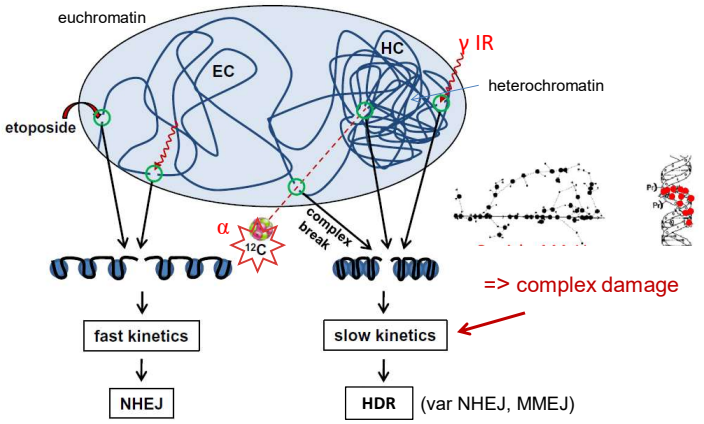
MRE11 ⇨ ~12 (± 9) nano-clusters (~DSBs)/α-track @10nm res.





Scherthan, Lee et al., 2019 *Cancers*

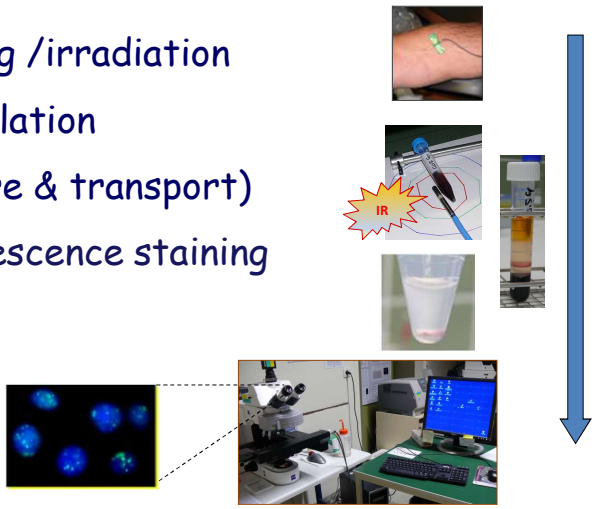
Chromatin context: important!
Slow HDR repair for complex, high LET-induced DSBs & DSBs in heterochromatin



After Löbrich et al.

γ-H2AX Assay in Biodosimetry - Method

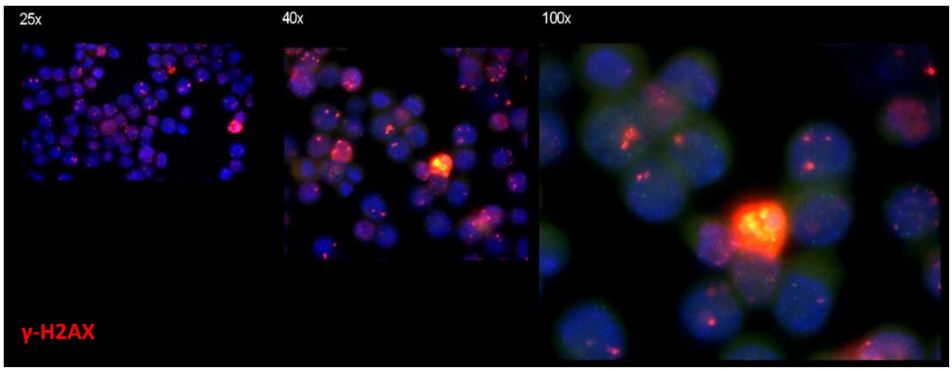
1. Blood sampling /irradiation
2. Leukocyte isolation
3. Fixation (store & transport)
4. Immunofluorescence staining
5. Foci Analysis



Scherthan et al. 2007 *Wehrm.Mschr.*
Lassmann et al. 2010 *J Nuc Med*

γ-H2AX Focus assay: **analysis**

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



Initially: 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** (e.g., Multibiodose, & others**)**

γH2AX dose response: linear
@ doses < 2Gy (30min pIR, fibroblasts)

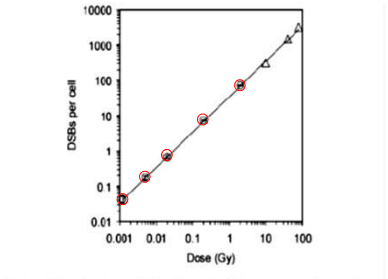
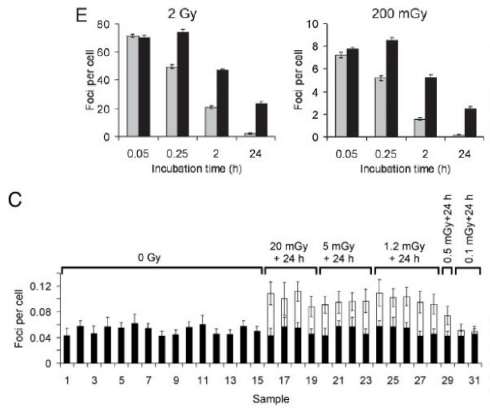
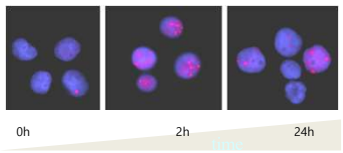


Fig. 2. DSB induction in MRC-5 cells. γ-H2AX foci were counted 3 min after irradiation, and the mean values of foci per cell are shown (circles). Triangles represent DSB induction data obtained from PFGE analysis. The line is a linear fit to the data points with a slope of 35 DSBs per cell per Gy.

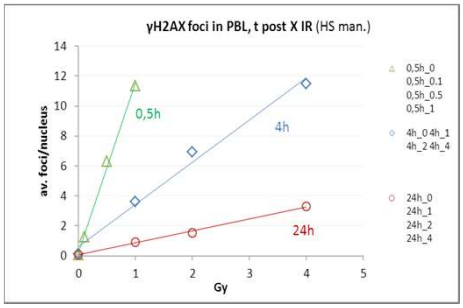


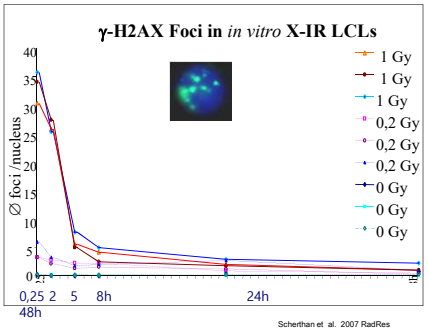
- Rothkamm and Löbrich 2003 PNAS: ~40 γH2AX foci /Gy in fibroblasts (5' pIR)
- Löbrich et al. 2005 PNAS: ~20 foci/Gy in lymphocytes

DSB-Repair Foci in leukocytes: sensitive, but rapidly declining marker of external exposure



Repair foci
DNA
(nuclei)

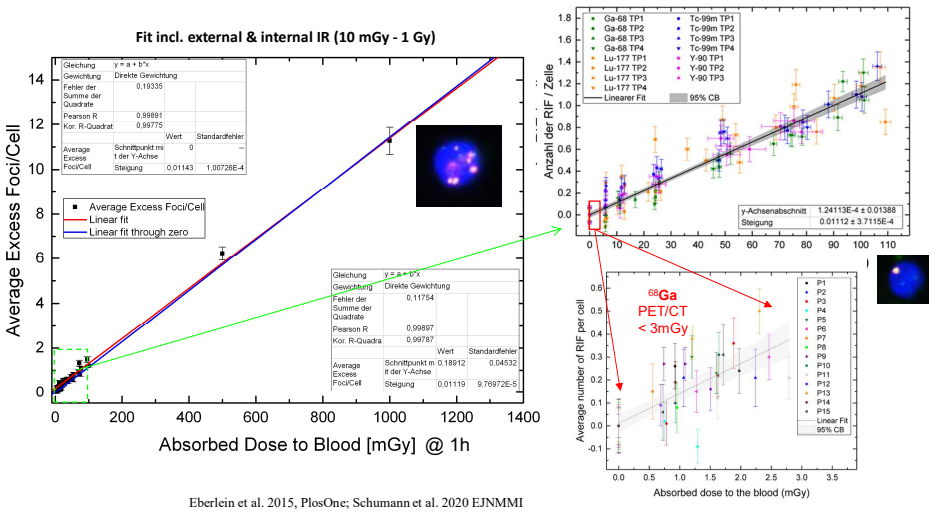




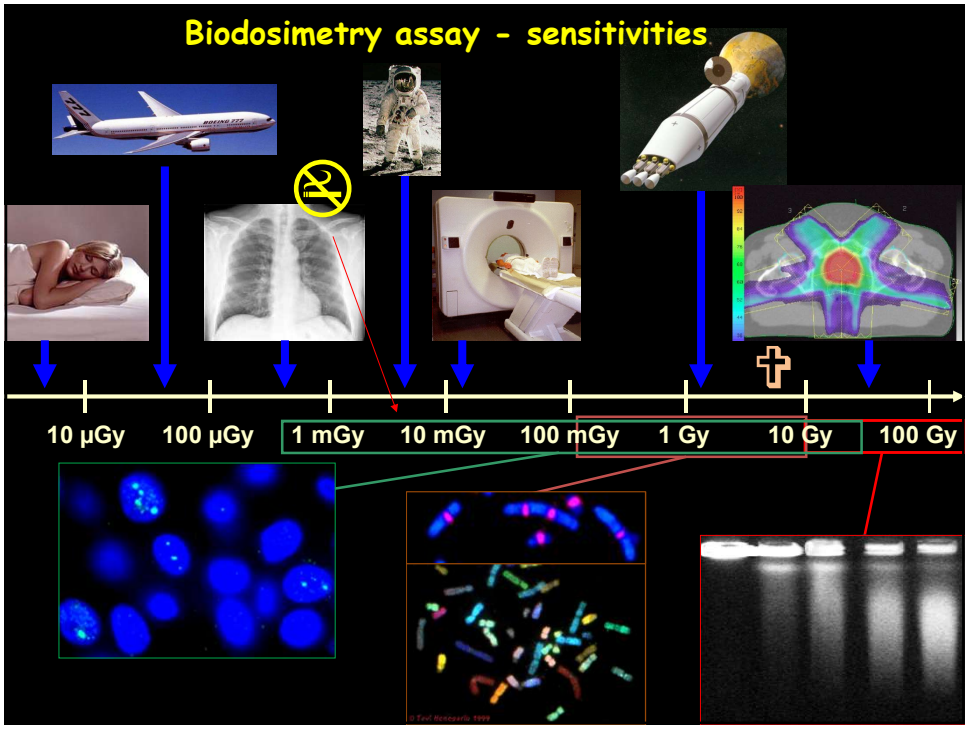
Scherthan et al. 2007 RadRes

dose response after very low dose internal IR with β & γ emitters ^{177}Lu, ^{131}I, ^{90}Y, ^{68}Ga, $^{90}Tc_m$

(cooperation w NucMed WÜ)



Eberlein et al. 2015, PlosOne; Schumann et al. 2020 EJNMMI



γ-H2AX lab inter-comparisons: variability

NATO exercise lab comparison of γ-H2AX focus assay
(relative to individual calibration curves)

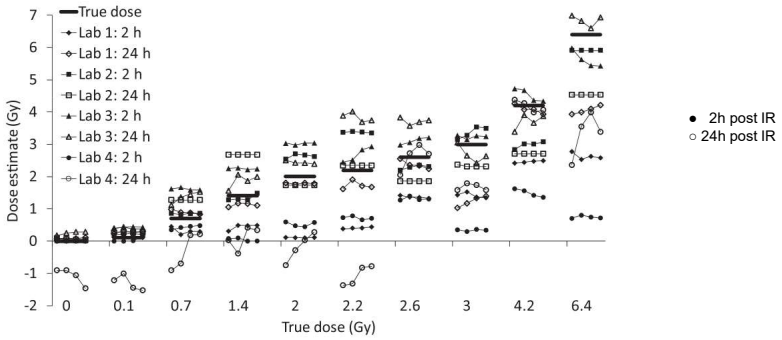
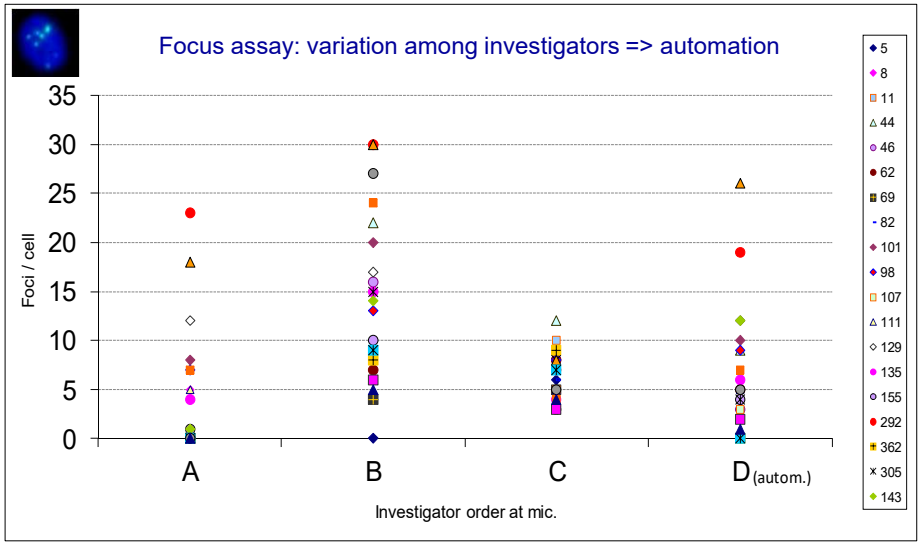


FIG. 2. Reported dose estimates are shown relative to the true absorbed doses per sample. Each chain of symbols shows dose estimates after scoring 20, 30, 40 and 50 cells (from left to right).

Rothkamm et al. 2013 Rad.Res.

Manual focus analysis down the mic: operator variability



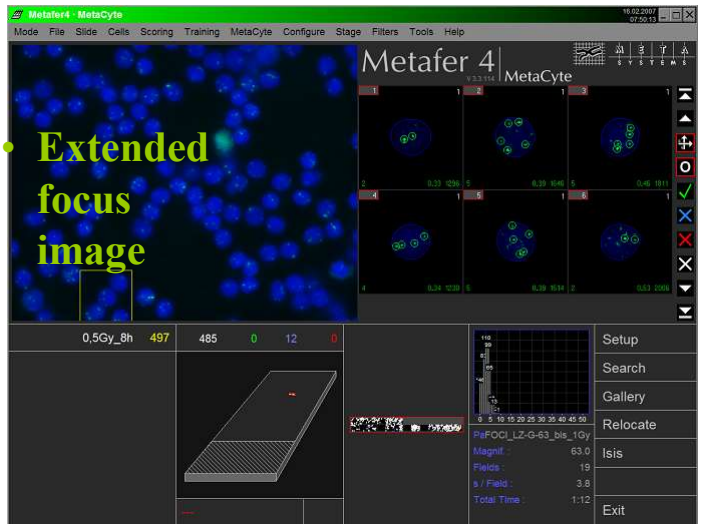
InstRadBioBw, unpublished

Does semi-automated image capture and processing help?

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

Ca. 6 published software solutions for foci analysis, ~4 diff. systems (2020)

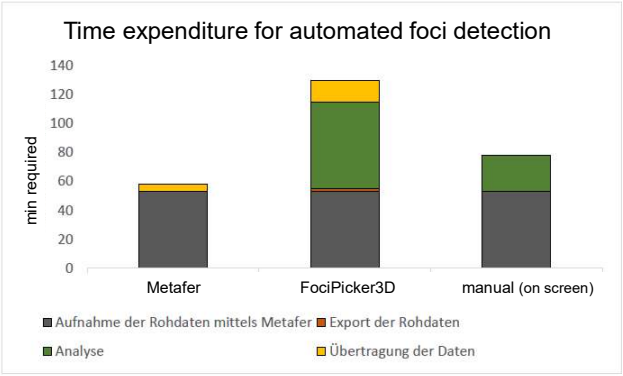
Computer aided focus analysis



- Single cell analysis
- Data output

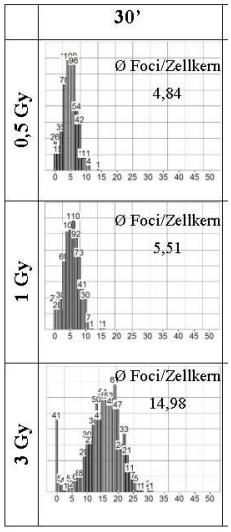
InstRadBioBw

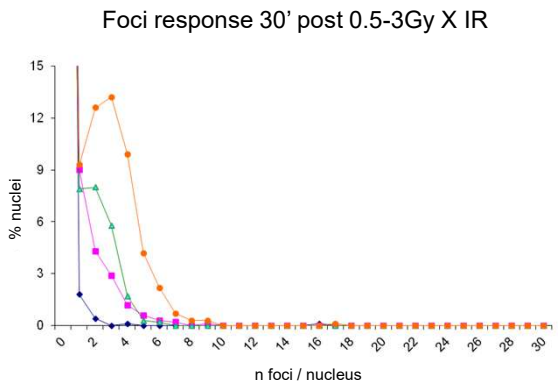
Time requirements of automated foci-counting workflows



- Automation: good for high doses

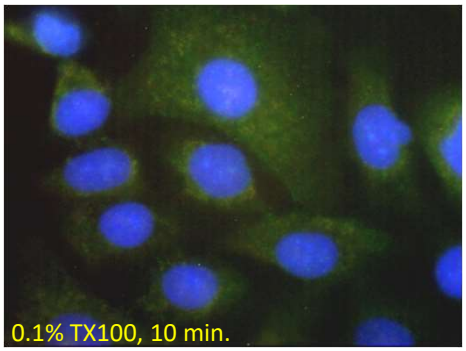
Automated scanning & image analysis: not so variable?

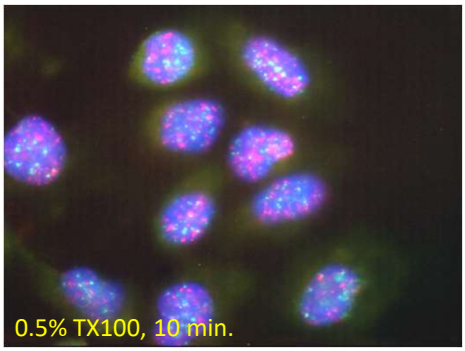




InstRadBioBw

Methodological pitfalls/limits
- fixation/extraction determines the success of γ-H2AX detection

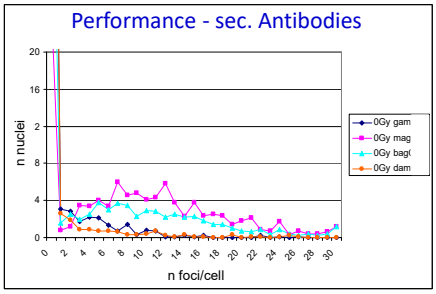


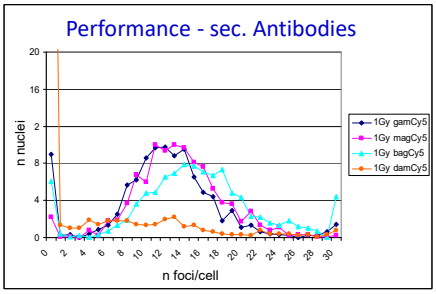


kinetochores
γ-H2AX
3 Gy X rays

Reagent-induced variation

different secondary Abs => variation





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

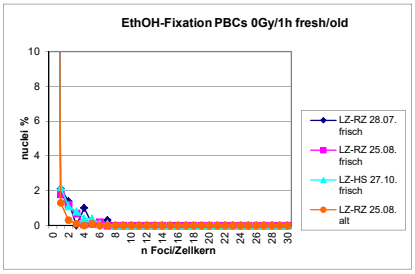
Sample storage ? – yes (but) !

+ Ethanol fixed cells can be stored and shipped

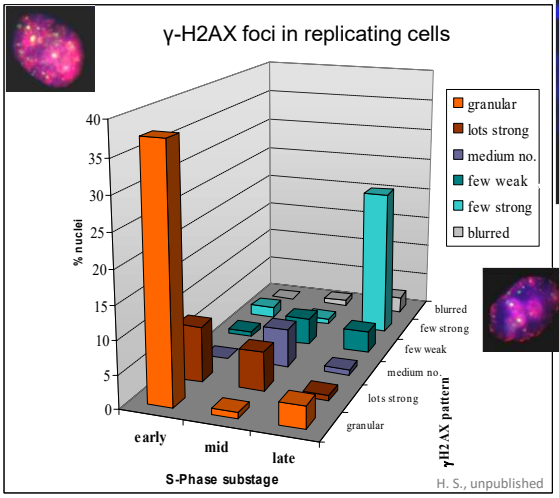
- ! Only compare with similarly treated (time & temp) controls

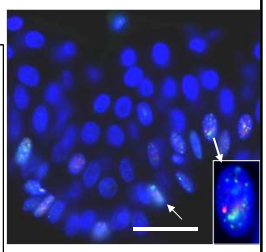
- We use 70% Ethanol @ -20°C

(Scherthan et al. 2007 RadRes; Lassman et al. 2010 J.Nucl.Med.; Schumann et al. 2018 Sci Rep, …)

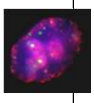


factors influencing the assay –
replicating cells display var. classes of foci



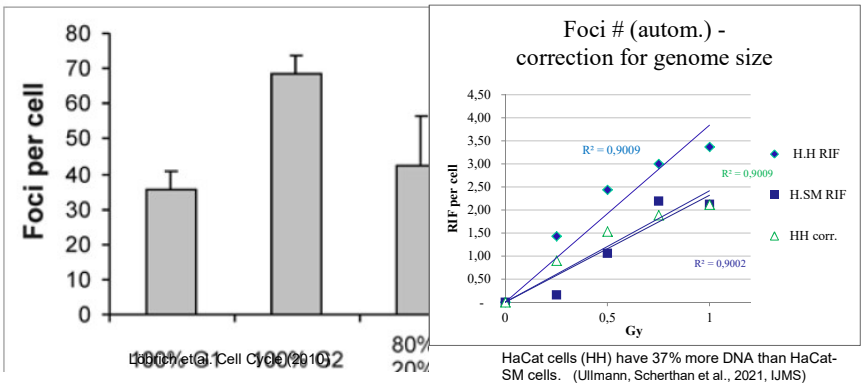


In vivo minipig skin, Ahmed et al . 2012, PLoS One 7:e39521



BrdU
γH2AX
DNA

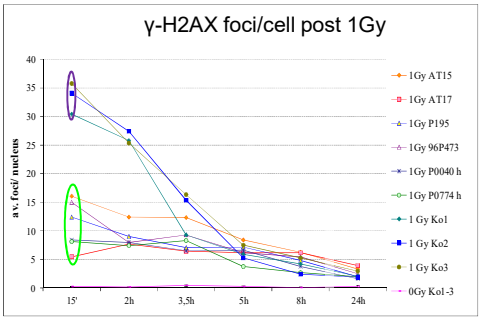
Rad. induced foci numbers –
Cell cycle and genome size matter

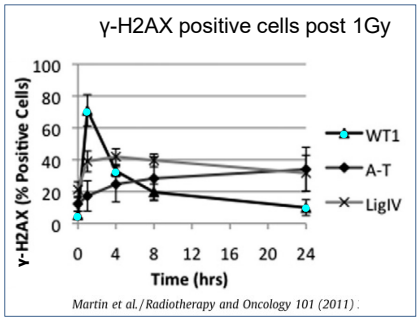


Lobrich et al. Cell Cycle (2010)

HaCat cells (HH) have 37% more DNA than HaCat-SM cells. (Ullmann, Scherthan et al., 2021, IJMS)

Genetic background influences foci formation – effects of NBS1, ATM deficiencies



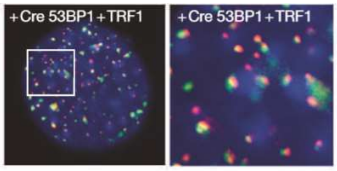


Martin et al./Radiotherapy and Oncology 101 (2011) :

Cell lines that lack ATM or NBS1 fail to induce the full level (≥30) of γH2AX or 53BP1 foci 15' after 1Gy IR. Control Atm-, NBS1-deficient LCLs

H. Scherthan, unpublished

γH2AX Foci are induced by dysfunctional (short) telomeres - TIFs



Celli & de Lange 2005 NCB

TIF: Takai *et al.*, Curr Biol. 2003

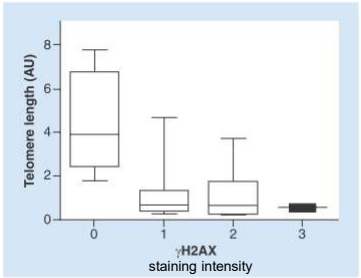
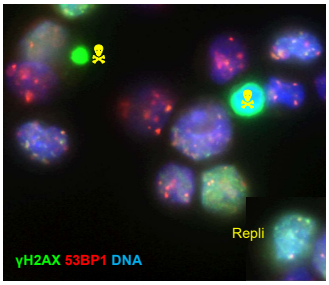


Figure 3. Box and Whisker plot showing median, 25th and 75th percentile, and min–max values: telomere length. Telomere length (as established by qPCR in AU, y-axis) in *p53* mutated breast cancer cell lines is negatively correlated with endogenous γ-H2AX staining intensity (0, n = 5; 1, n = 15; 2, n = 7; 3, n = 4; x-axis) as established by immunohistochemical staining in a breast cancer cell microarray of 54 breast cancer

(Nagelkerke et al. 2015 Biom. Med)

Tumor cells express γ-H2AX

marker for genomic instability and replication stress,
pan-nuclear γ-H2AX tags apoptosis



EBV infected cell line.
Scherthan, unpubl.

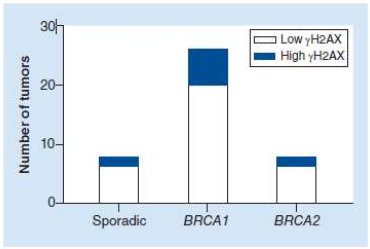


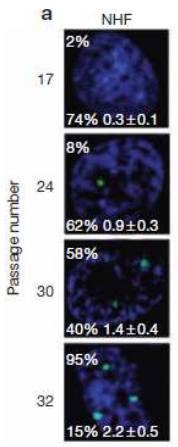
Figure 5. No significant difference in the proportion of tumors expressing γ-H2AX in sporadic or familial (*BRCA1* or *BRCA2*) breast cancer cases.

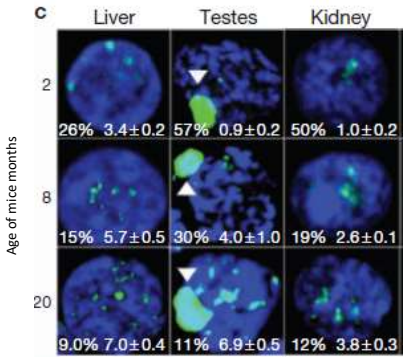
Nagelkerke et al. *Biomark. Med.* 2015, 9.

Aging increases persistent γ-H2AX foci

Pittfalls:

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

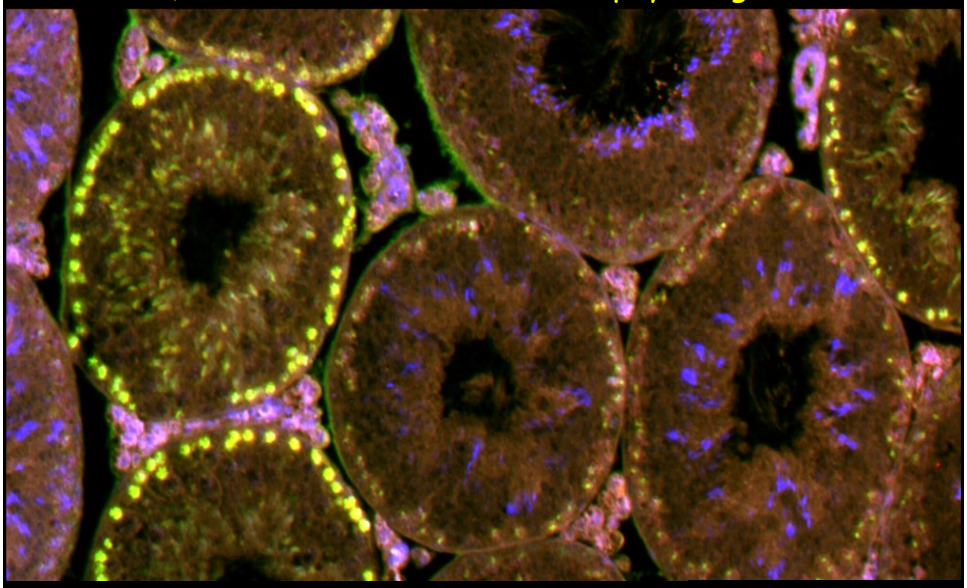




% focus-free cells
av. focus # in +cells

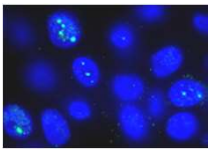
Sedelnikova et al. NCB 2004

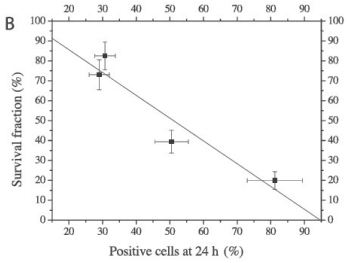
γH2AX in the mouse testis => physiological

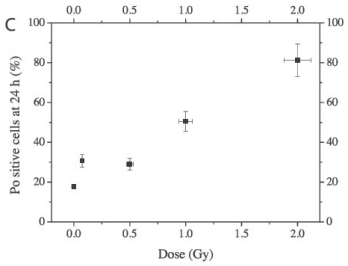


γ-H2AX
sperm heads (nuclei)
image: H.S.

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



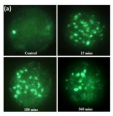


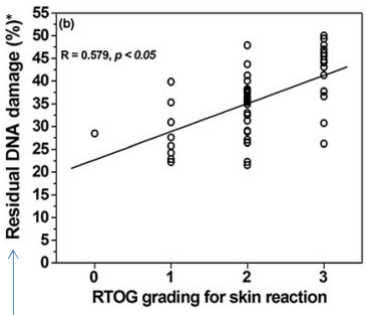


A measure for radiosensitivity?

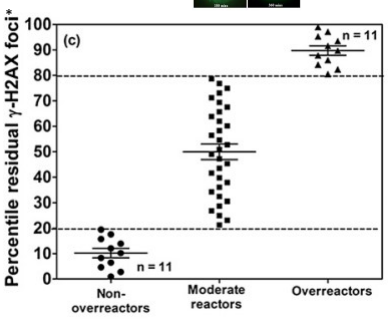
Wéra et al. Rad Res. 2013

Persistent γ-H2AX foci can correlate with radiosensitivity in head/neck cancer patients – but…





*Excess damage in neutral Comet Assay after 3h



*Excess Foci after 6h ranked according to fpc values

Goutham et al. Rad Oncol 2012

Radiosensitive patients are rarely encountered (~1%) and there is a high risk of false positives

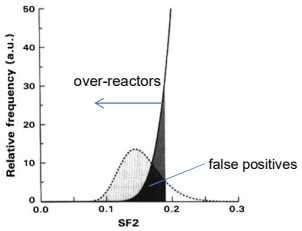
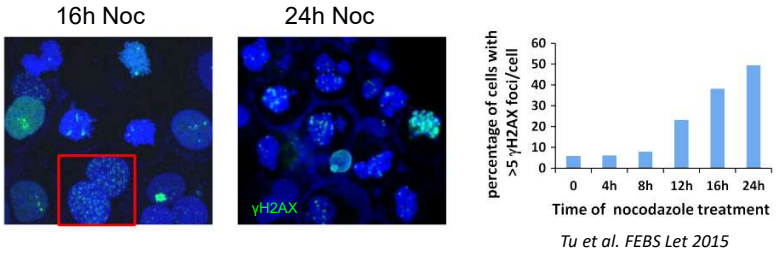


Fig 1. Log-normal distributions of the surviving fraction after 2 Gy (SF_2) in two hypothetical cohorts of 'over-responders' (stippled) and normal individuals (full line). For the clarity of illustration, only the left-hand tail of the latter distribution is shown in the graph.

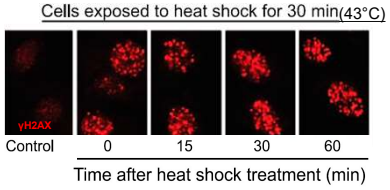
Bentzen 1997 RadOnc

… Since a complex interplay of various molecular processes contribute to (late) radiation-induced side effects, it is questionable whether one cellular marker (here γH2AX) alone can predict for normal tissue radiosensitivity… (apoptosis, survival, DDR assays etc.) …

! γ-H2AX formation in absence of DSBs: => MT-disrupting drugs or heat !

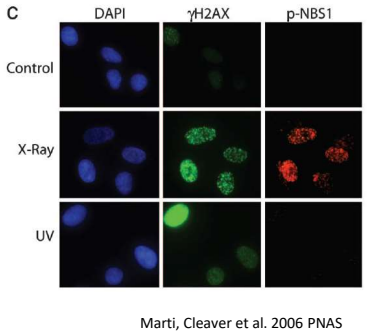


Tu et al. FEBS Let 2015

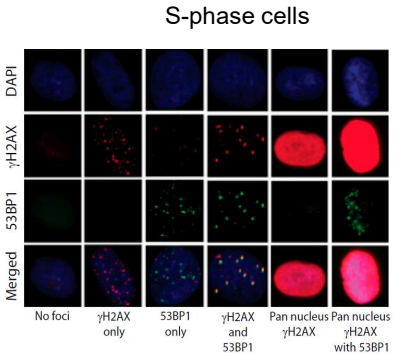


Hunt et al. Canc. Res. 2007

pan-nuclear of γH2AX in G1, S phase after **UV damage** depend on BER and not on DSBs



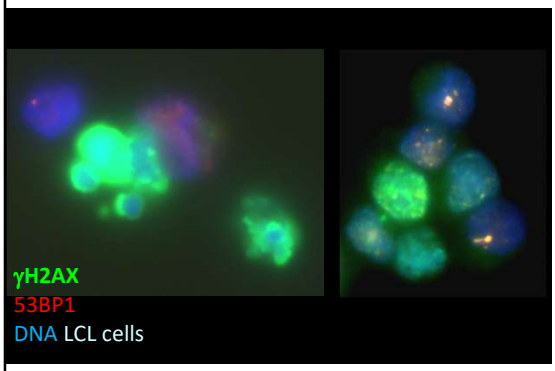
Marti, Cleaver et al. 2006 PNAS

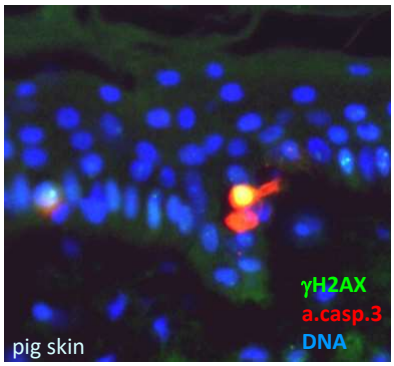


de Feraudy, Cleaver et al. 2010 PNAS

…UV irradiation induces nuclear pan-γ-H2AX staining in the absence of 53BP1 foci: DSBs represented only a small fraction of the total number of UV-induced γH2AX foci

Apoptotic cells display strong pan-nuclear γH2AX fluorescence



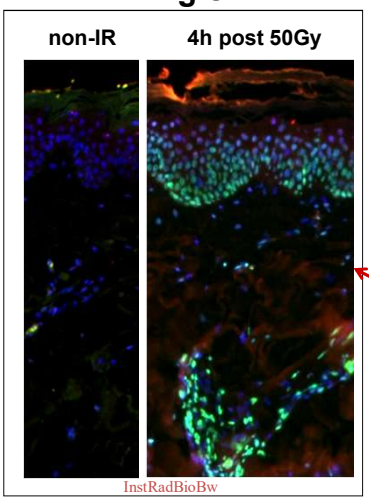


(Another source of background: **granulocyte autofluorescence** in whole blood)

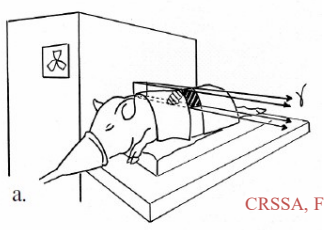
Ahmed et al . 2012 PLoS One 7:e39521
Scherthan et al. 2022 Cancers

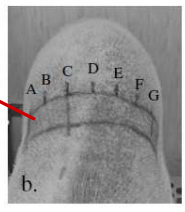
acute high dose γ-irradiation of pig skin: pan-γ-H2AX w/o apoptosis

Pig-skin



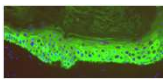
Pig model



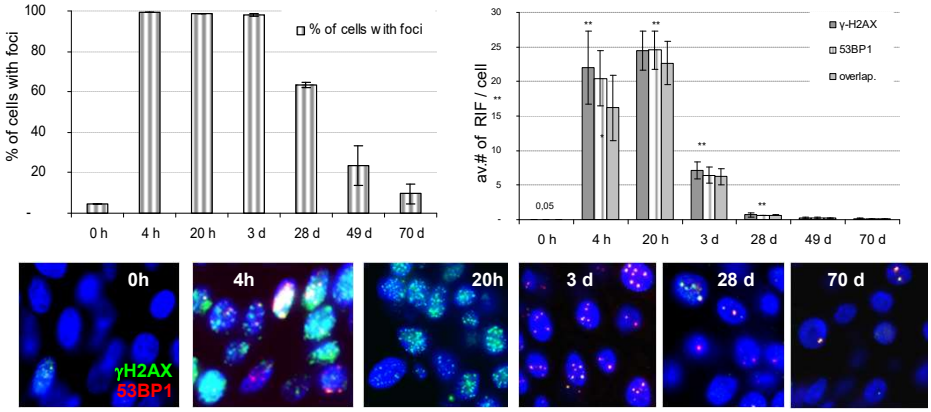


Agay, et al. Exp. Hemat. 2010

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

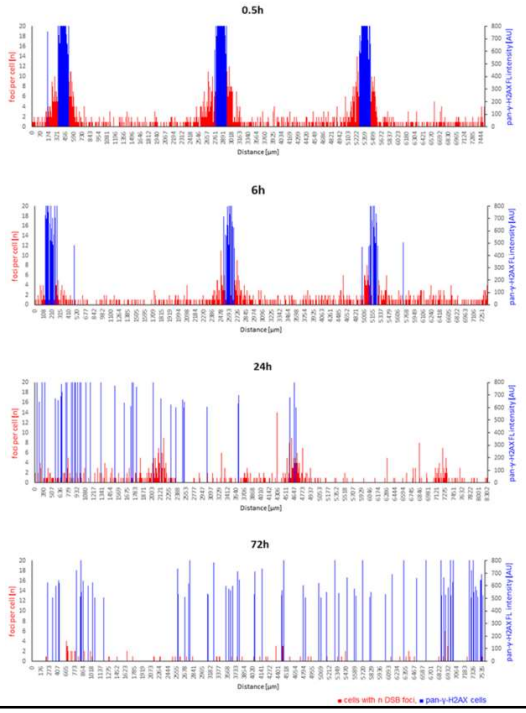


Epidermis
Keratinocytes

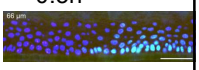


Ahmed et al. PloS One. 2012

Appearance of pan-γ-H2AX nuclei after focused p^+ minibeam IR of skin models



0.5h



pan-γ-H2AX & cells w DSB foci

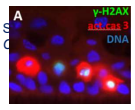
6h

Cell death

(pan-γ-H2AX & act. Casp. 3+)

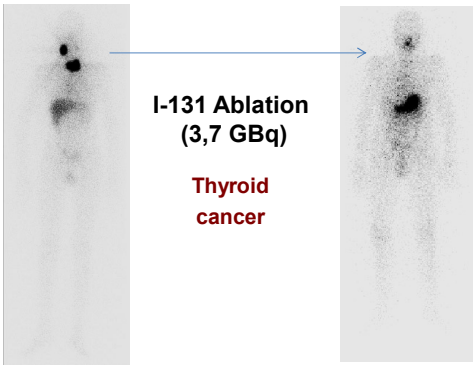
24h

72h



*In vivo, **low dose***: DSB focus assay in nuclear medicine patients

- Coop. with M. Lassmann lab, Clinic of Nuclear Medicine, Univ. of Würzburg, Ger.



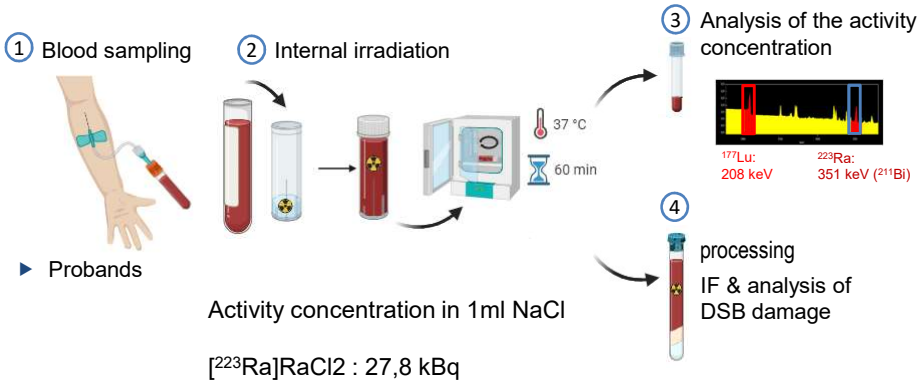
I-131 Ablation (3,7 GBq)

Thyroid cancer

ex vivo internal α; β irradiation

Uniklinikum Würzburg

Isabella Strobl



Activity concentration in 1ml NaCl

[^{223}Ra]RaCl2 : 27,8 kBq

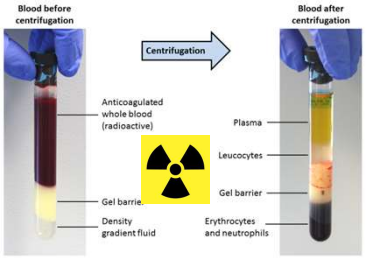
[^{177}Lu]LuCl3 : 5 MBq

=> abs. energy dose = 50 mGy @ 1h

Blood sampling & immunofluorescence analysis

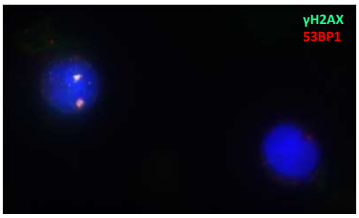
Nuclear Medcine Würzburg → shipping → InstRadBioBw Munich

- Blood sampling in CPT tubes
- Centrifugation → isolation of leukocytes, removal of radionuclides



- Fixation in Ethanol
- Storage/shipping

- IF staining for γ-H2AX + 53BP1
- Manual enumeration (HS) of co-localizing γ-H2AX and 53BP1 foci in 100 cells/sample

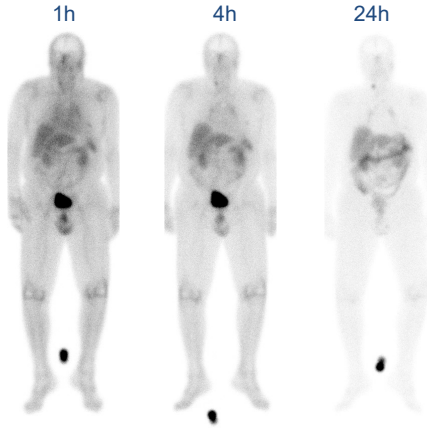


Radiation induced foci (RIF)
= foci IR samples – individual background foci

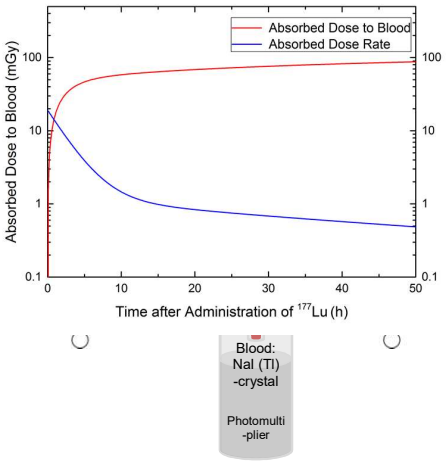
Physical dosimetry in ^{131}I-treated thyroid cancer patients

Medical Physics, Prof. M. Lassmann et al., NucMed Univ. Hospital Würzburg, DE

Gamma camera measurements



Dose rate & blood measurements



^{131}I (3.5 GBq) treated DTC patients – low dose & dose rate in blood

M. Lassmann, U. Eberlein, Nuclear Medicine, Univ. Würzburg, DE

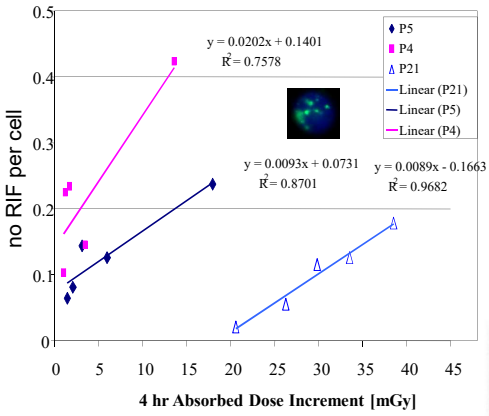
Mean specific absorbed dose to the blood: **0.1** ±0.067 **Gy/GBq**

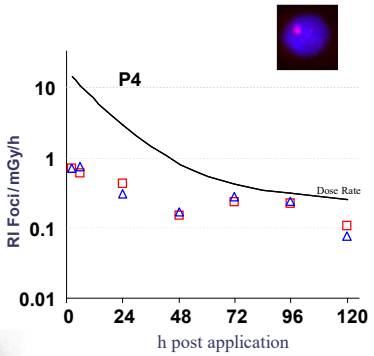
Mean **absorbed dose to the blood**: **0.39** ±0.40 **Gy**, range 0.2 – 2 Gy

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

Lassmann et al. J Nuc Med. 2010; Eberlein et al. JNM 2015

γ-H2AX DSB foci in thyroid cancer patients after ~3,7 GBq ^{131}I



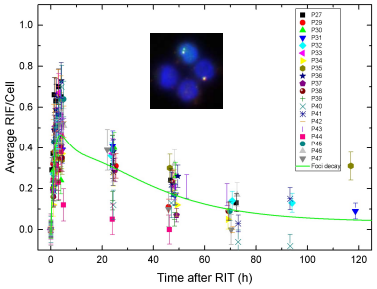


Ratio foci/absorbed dose rate increases with declining dose rate

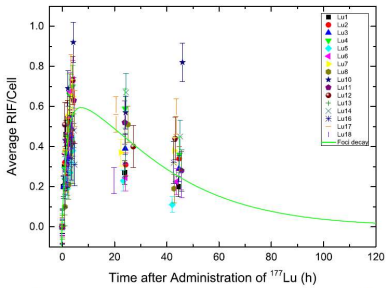
Persistent DSB foci after internal IR in vivo

^{131}I: DTC patients (n=20, 55.1+-17.1 y) **D** ~ 0.35 *Gy* to the blood @ 3.5 *GBq*

^{177}Lu: 16 NET patients (n=18, 61.2 ±10.5 y), D = 79 (±16) mGy @ 7 GBq



Fast repair component in ^{131}I patients induced @ D>20mGy in 1st h.



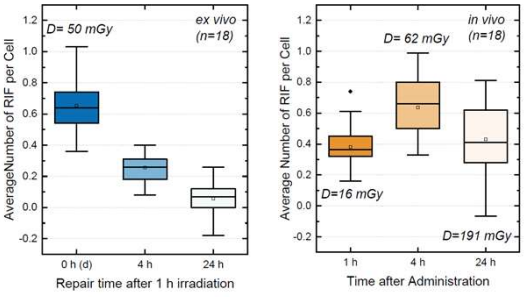
^{177}Lu DOTATATE/DOTATOC-treated NET patients

Eberlein, et al. 2015 EJNEMMI, 2016 J Nuc Med

in vivo/ex vivo internal ^{131}I irradiation *of same patient's blood: test tube situation does not reflect the DDR in vivo*

***Ex vivo* internal irradiation** (wash out of activity @1h): **max # of IR-induced DSB foci after IR, complete repair after 24h**

***In vivo* int. IR:** **Increase of RIF up to 4h after incorporation, remaining damage at 24h**

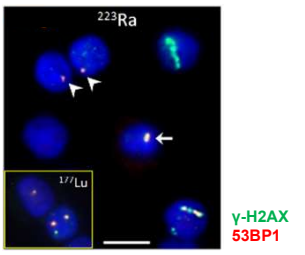


D = absorbed dose to the blood at Δt

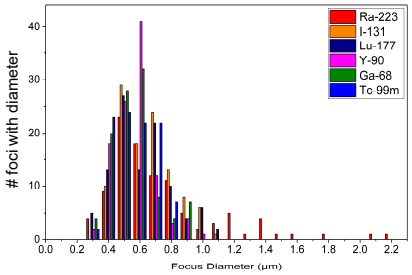
Schumann, S., Scherthan, H *et al. Eur J Nucl Med Mol Imaging* 49, 1447–1455 (2022)

high LET α-IR: DNA damage track nos in ^{223}Ra or ^{224}Ra i.v. exposed blood samples correlate w absorbed doses

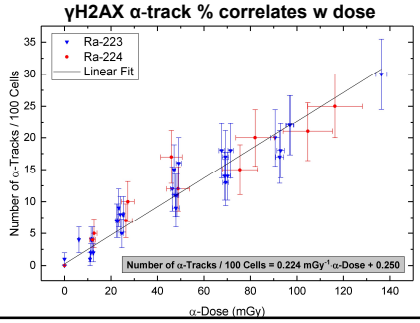
Coop. w S. Schumann, M. Lassmann, Med. Physics Nuc Med, Univ. WÜ

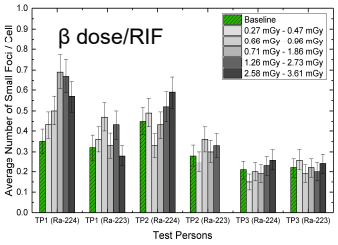


γ-H2AX
53BP1



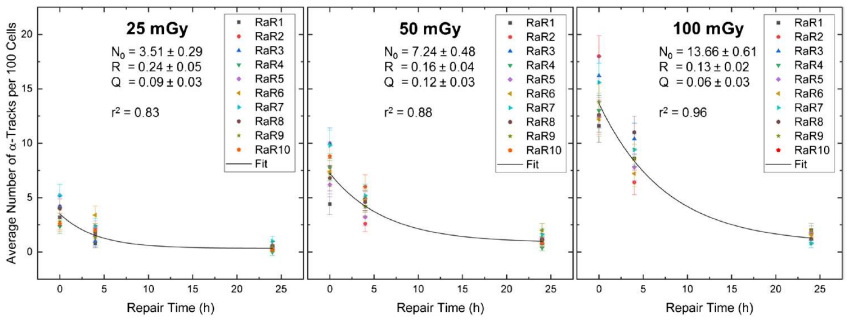
γH2AX α-track % correlates w dose





Schumann Scherthan et al. Sci.Rep. 2018 & EJNMMI 2018

Ex vivo α IR: α-tracks vanish with progress of DSB repair

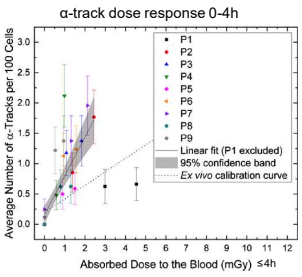


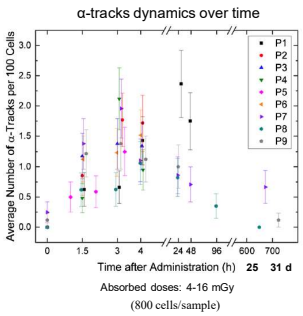
PBMCs only rarely (< 0.7%) showed pan-γ-H2AX patterns indicative of cell death

Göring et al. 2022 *EJNMMI* 49:3981-88

in vivo: α-tracks in PBMNCs ^{223}Ra-treated prostate cancer patients

Nuclear Medicine, Würzburg Univ. & InstRadBioBw München

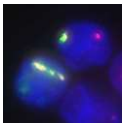


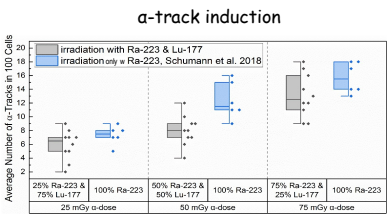


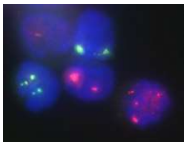
Absorbed doses: 4-16 mGy
(800 cells/sample)

Schumann S et al., EJNMMI 2021

RN Scenarios: mixed internal irradiation
α-tracks and DSB foci







- Energy dose 100 mGy 50% mix
  50 mGy α- + 50 mGy β dose

RN activity in isoton. NaCl:

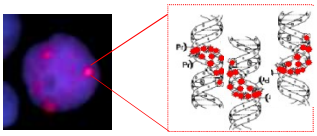
- [^{223}Ra]$RaCl_2$: 3,5 kBq · ml^{-1}
- [^{177}Lu]$LuCl_3$: 0.63 MBq · ml^{-1}

ratio ^{223}Ra / ^{177}Lu	Repair: **fraction of α-tracks @ 24h**
25% / 75%	0.10 ± 0.09
50% / 50%	0.10 ± 0.09
75% / 25%	0.05 ± 0.06

Strobel, I et. al. 2024, *Int. J. Mol. Sci. 25, 8629.*

Conclusions I

- **γH2AX DSB-indicating Foci**
  - **High sensitivity, inter-individual variability, rapid decline**
  - **Good indicator of WB exposure, dose categorization.**
    In skin: indicator for weeks; after intern. IR: days
  - **Focus geometry indicative of alpha emitters**
  - Residual damage (>24h) can correlate with radiation sensitivity. Indicative of incorpor. of high LET emitters
  - **Cancer cells have high γ-H2AX levels** (foci, area)
  - Dose reconstruction difficult, but triage possible

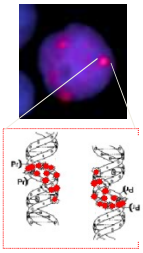


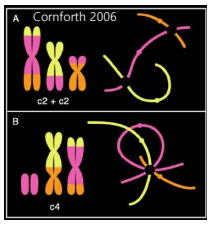
Conclusions II

DNA Repair occurs in foci ("factories")

- **Foci consist of nano chromatin domains**
- **Not always a linear dose relationship**
- **DSB No. / focus increases with dose**
  ⇒ lots of DSBs along particle tracks

⇒ source of mutation and complex exchanges @ high doses





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Thank you

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