UniSA | STEM

A Systematic Review of the Proton and Carbon FLASH Effect

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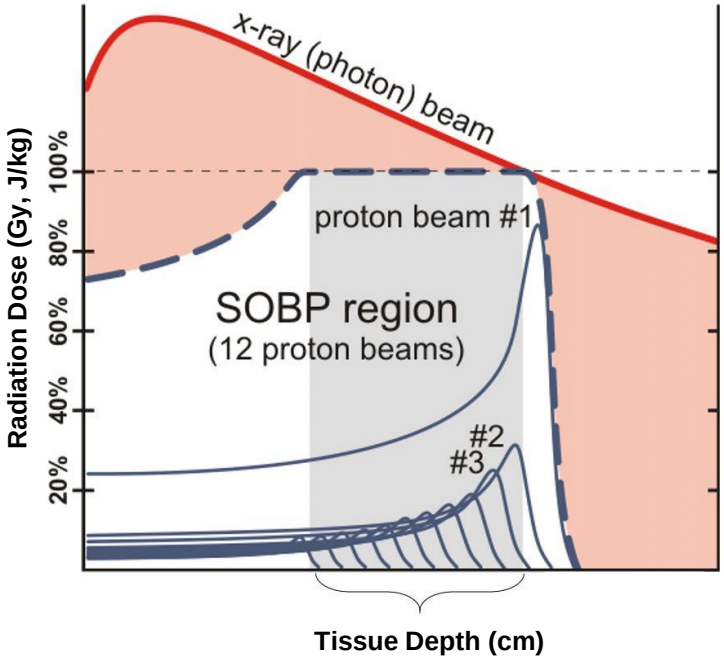


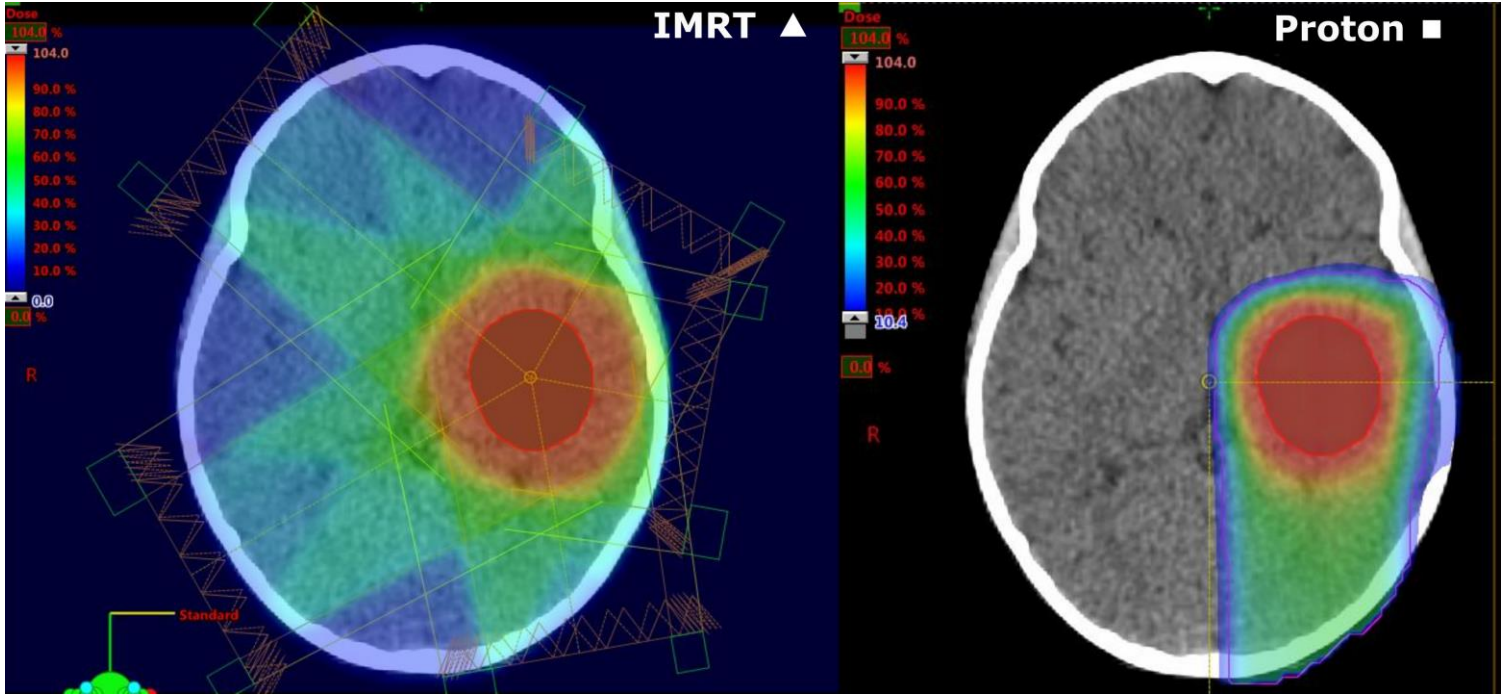
Radiation Therapy

- RT most common cancer treatment, ~50% patients in developed countries
- Limiting factor - healthy tissue damage
- Particle therapy benefits:
  - Protect more healthy tissues
  - Reduces damage to critical structures
  - Good for radioresistant tumours
  - Paediatrics - many years to manifest symptoms, emphasis on reducing normal tissue damage

Australia First!

- Australian Bragg Centre for Proton Therapy (SAHMRI 2)
- Expected completion: 2023
- First patient treatments: 2024
- Will contain ProTom's Radiance 330 akin to Massachusetts General Hospital – synchrotron
- Currently: 16.5 MeV GE PETtrace cyclotron
- Will be using for my experiments

X-rays versus Protons



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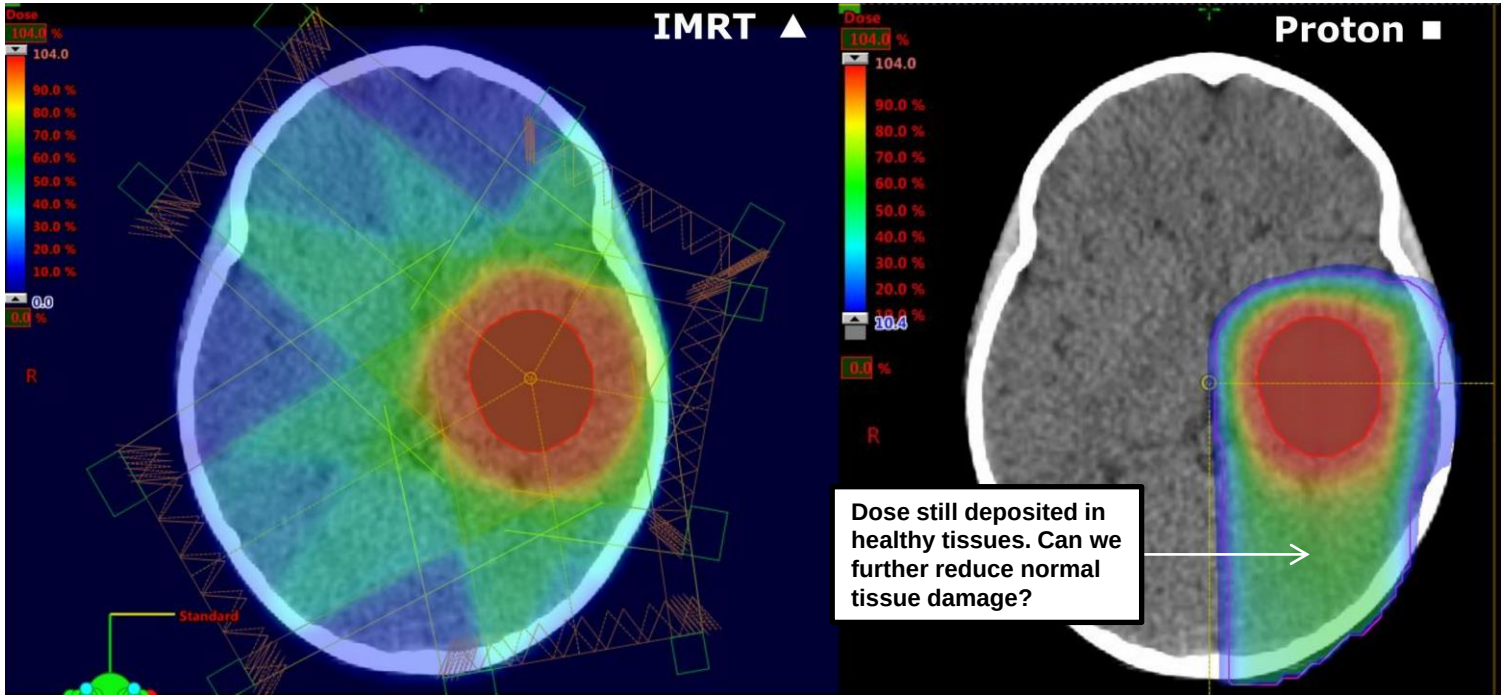


What is FLASH?

Use of single pulses of radiation at ultra-high dose rates to treat solid tumours (>40 Gy/second)

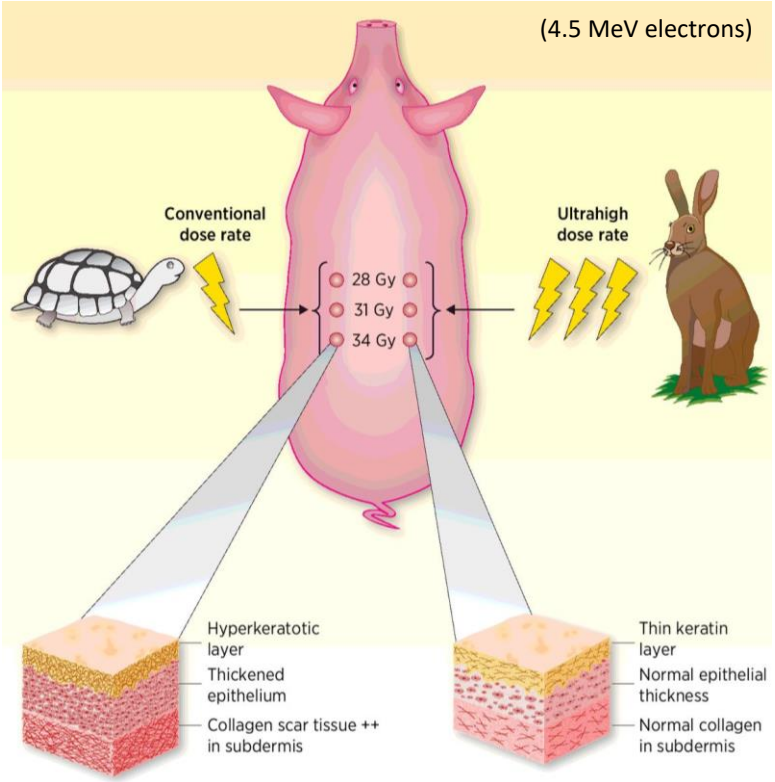
Contrasts conventional (CONV) therapy which uses low dose rates (Gray/minute) in multiple pulses or ‘fractions’

Equivalent tumour kill efficacy, but **improves the sparing of healthy, normal tissues**

Biological and physical mechanisms unclear…

Predicted mechanisms:

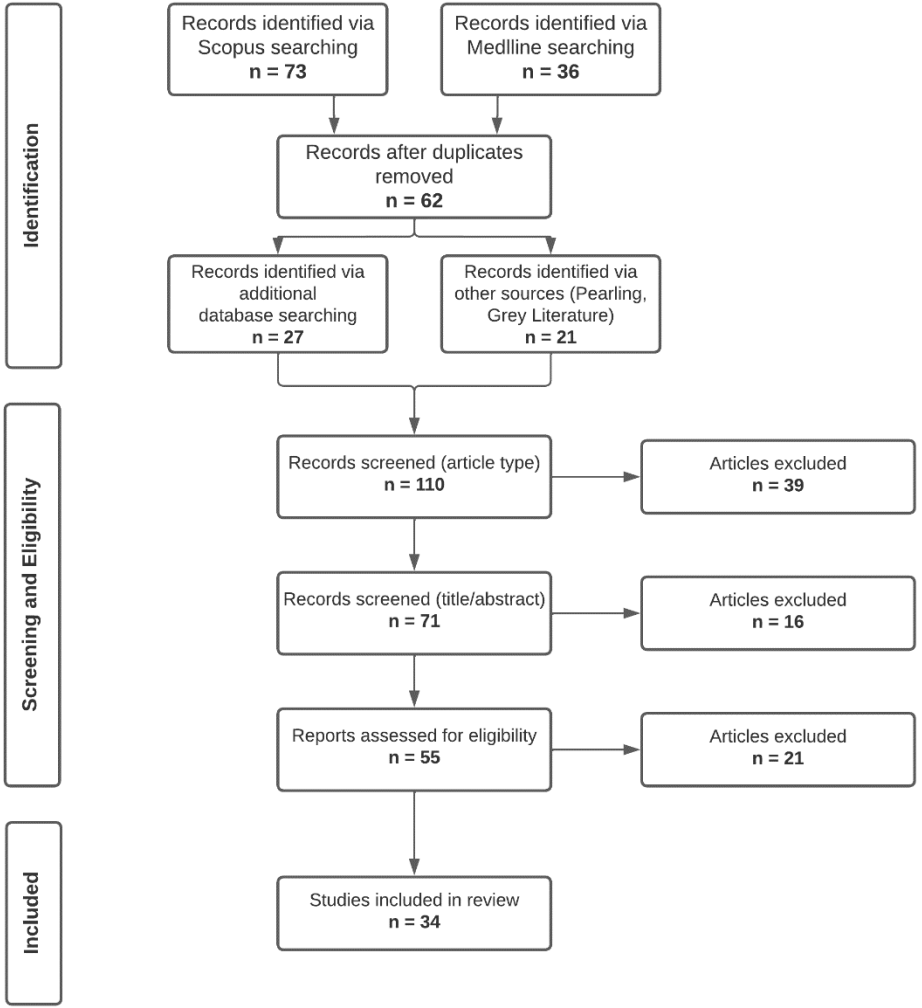
1. Oxygen depletion
2. DNA Damage Repair & Response
3. Immune Cell Modulation and Inflammation



Vozenin MC et al. 2019, Clin Cancer Res

Systematic Review

- What is it?
  - A reproducible method to collect all available research on a specific topic
- Review follows PRISMA Guidelines
- Preferred Reporting Items for Systematic Reviews and Meta Analyses
- Titled: The *Current Status of FLASH Particle Therapy: A Systematic Review*
- **Exclusion criteria:**
  1. Only proton and/or carbon FLASH papers
  2. Must cite use of > 40 Gy/s
  3. Radiobiological effect measured



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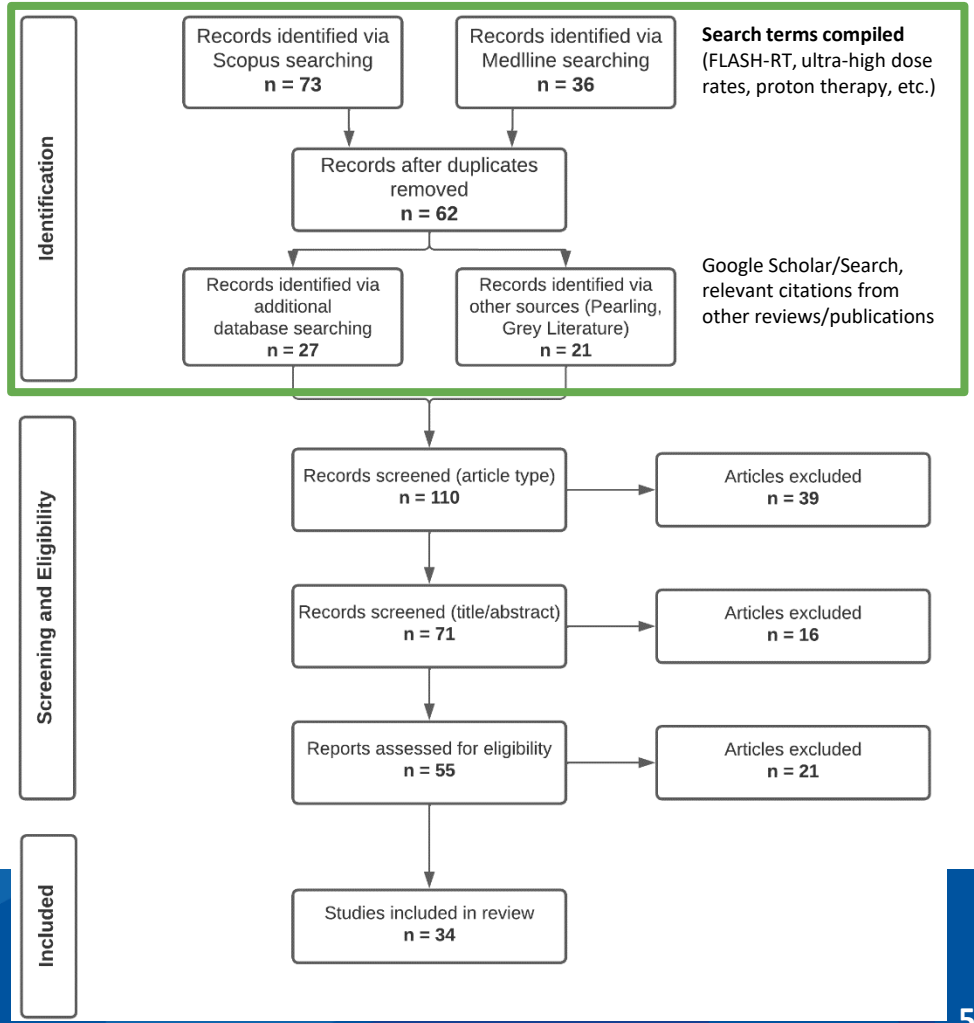

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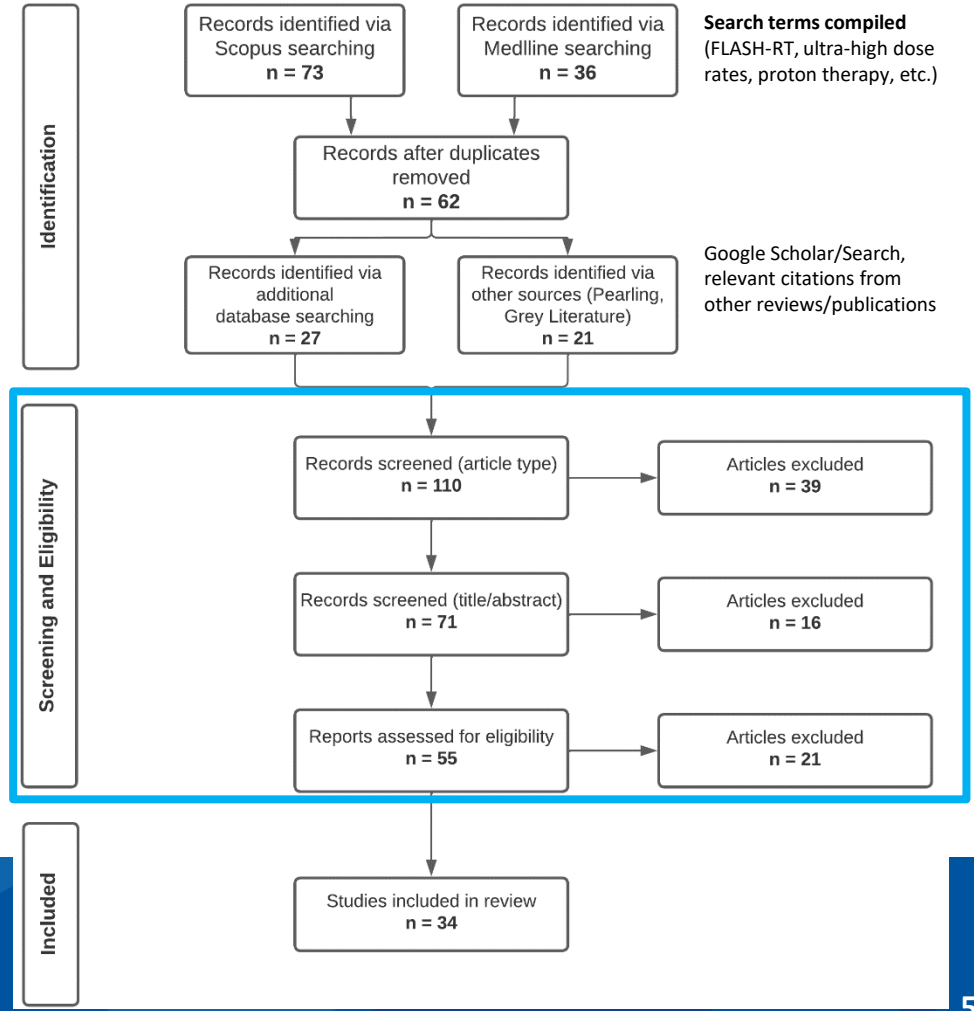

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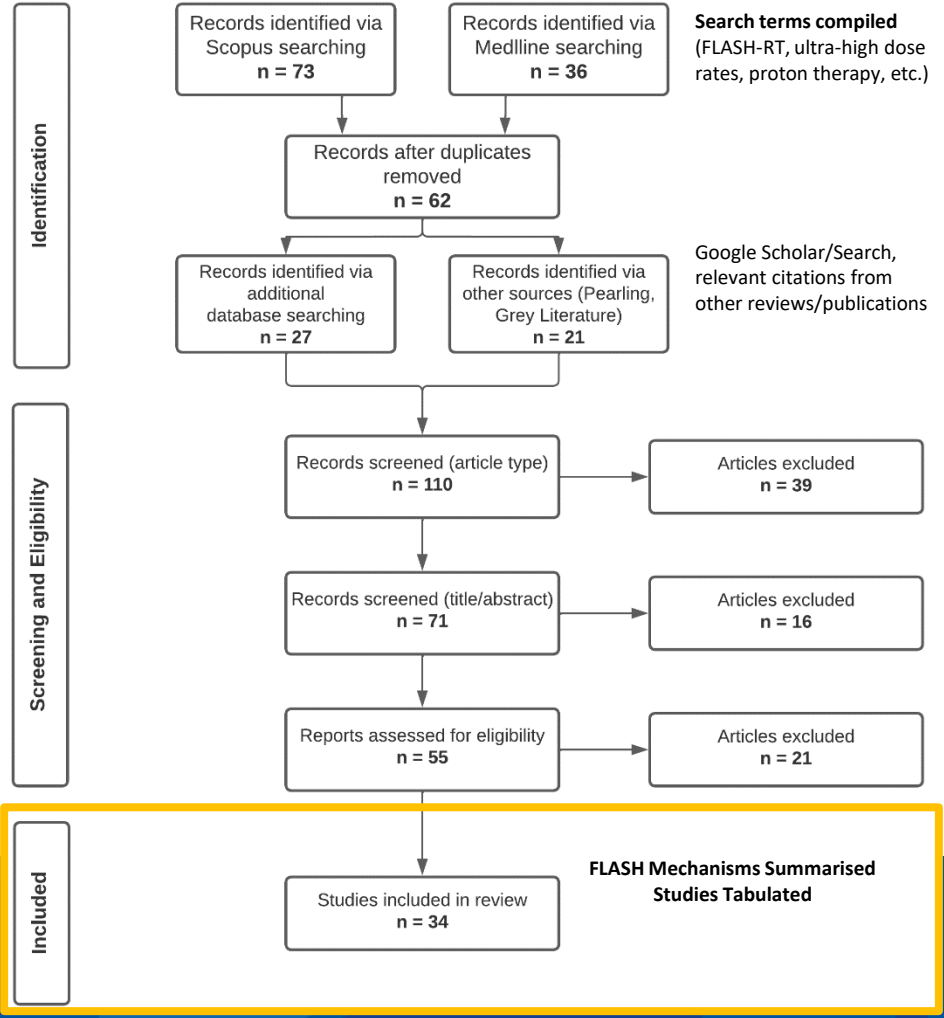
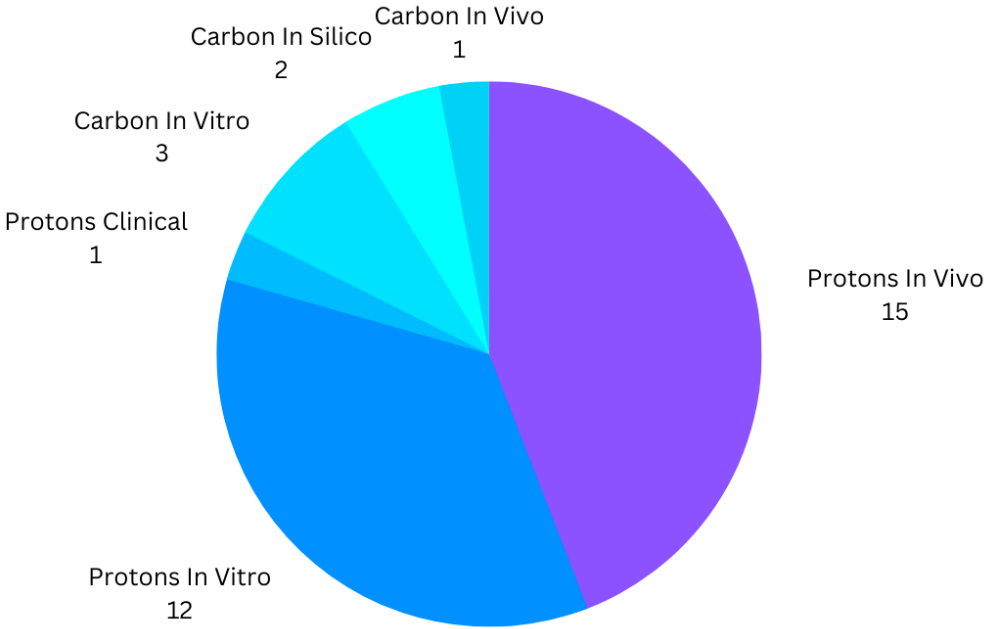


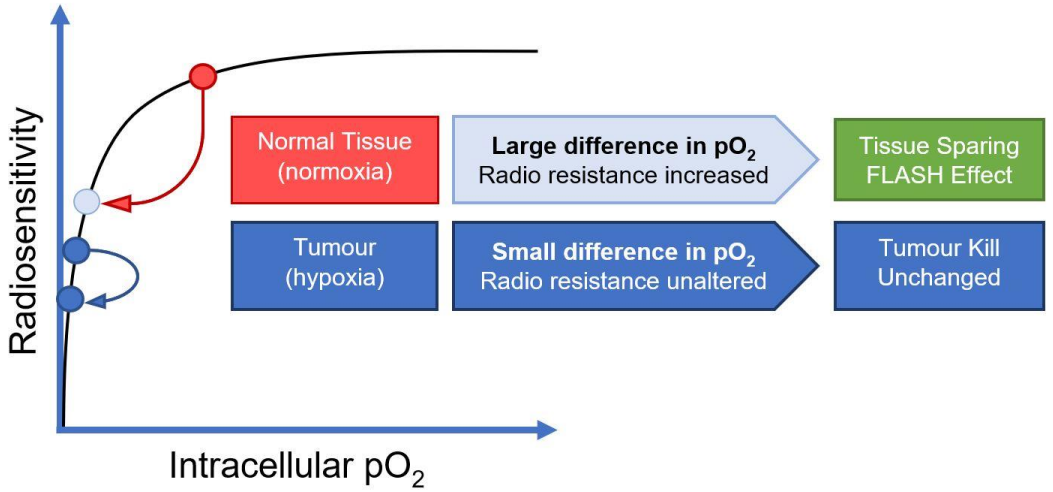


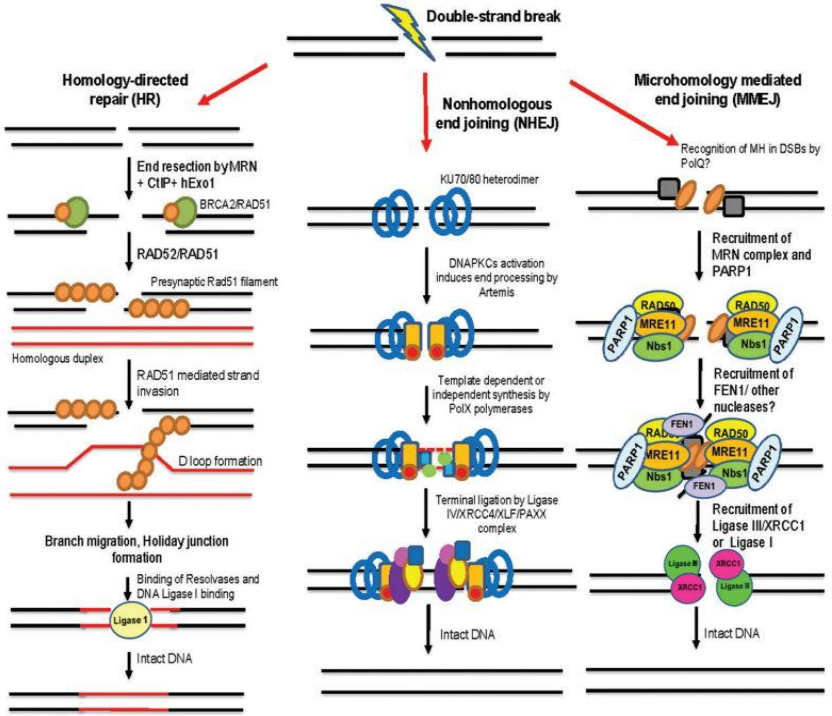
Key Findings

1. In vivo proton therapy studies are most prevalent with a total count of 15
2. Six studies utilise the Bragg peak for irradiations, rest use transmission
3. Stringent verification of dosimetry is rarely provided, not yet standardised
4. Biological assays are prone to limitations which need greater acknowledgement
   - Atkinson, J., Bezak, E. & Kempson, I. Imaging DNA double-strand breaks — are we there yet?. Nat Rev Mol Cell Biol 2022
5. Mechanistic understanding of biological mechanisms is still in its infancy

Mechanism 1: Oxygen Depletion Hypothesis

- Reactive oxygen species, produced by ionising radiation, cause indirect DNA damage
- Deplete O2 → Fewer ROS → Reduced damage
- Normal tissues - large O2 change
- Tumours - small O2 change





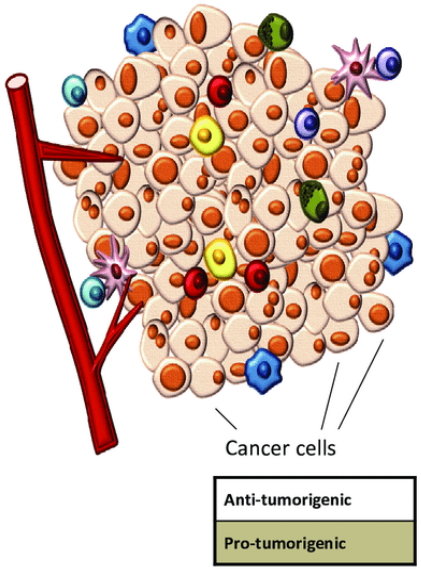
Mechanism 2: DNA Damage and Repair

- Differential effect between normal and cancerous tissues may reside in how cells respond to DNA damage
- Hypothesised that FLASH causes DNA lesions of higher complexity than CONV
- Dysfunction of these pathways is common in cancers - inability for cancers to repair complex DNA lesions responsible?
- Capacity of normal cells to repair complex lesions better than cancerous cells?

Pandey and Raghavan 2017, J Radiat Cancer Res

Mechanism 3: Immune System Modulation

- **Anti-Cancer Effect – Tumour Tissues**
  - Improvement to tumour response and abscopal effect?
  - Improved immune cell influx, reduced vascular collapse in Lewis Cell Carcinoma in mice, Young-Eun Kim et al. (2021)
  - Dendritic, NK, T-Cells – cytotoxic!
- **Anti-Inflammatory Effect – Normal Tissues**
  - Does FLASH spare specific normal cell subpopulations (e.g. stem cells, immune cells)?
  - Proton-FLASH reduces TGFB1 expression (inflammatory marker) in normal cells Buonanno et al. (2019)
  - Simulations predict a reduction in circulating immune cells killed during FLASH (5-10%) vs low dose rate irradiation (90-100%), Jin, J. et al. (2020)



Immune Cell		Roles in cancer
	Dendritic cell (DC)	Release cytotoxic cytokines Antigen presentation to T cells
		Suppress T cell functions Promote tumor growth and progression
	T cell (CD8+, CD4+)	Directly lyse cancer cells Release cytotoxic cytokines
		Release tumor promoting cytokines
	Treg	Restore homeostasis to reduce chronic inflammation
		Suppress anticancer immune responses Stimulate inflammatory cytokine production
	Macrophage	Release cytotoxic cytokines Antigen presentation to T cells
		Promote angiogenesis, tumor proliferation, chemotaxis, invasiveness, and metastasis
	Myeloid derived suppressor cell (MDSC)	Limited
		Suppress T cell functions Recruit immunosuppressive immune cells
	NK cell	Release cytotoxic cytokines Directly cytotoxic to cancer cells
		Limited

Markman and Shiao 2015, J Gastrointest Oncol

Imaging DNA double-strand breaks — are we there yet?

Jake Atkinson[1], *Eva Bezak*[2,3] *and Ivan Kempson*[1]✉

DNA double-strand breaks (DSBs) are fundamental to cell biology, from evolution to the latest gene-editing technologies. Yet, does an assay exist that truly quantitatively visualizes DSBs? Over-reliance on DSB detection by proxies can misguide interpretation of conventional assays, and more faithful DSB representatives await development.

DNA double-strand breaks (DSBs) can arise spontaneously from nuclease activities or through insults from reactive species, radiation and cytotoxic agents. DSBs are the most deleterious form of DNA damage, causing genome instability and chromosomal aberrations; DSB repair responses are thus rapid and stringently regulated[1]. By contrast, prescribed cleavage of DNA at targeted loci facilitates gene editing technologies such as CRISPR–Cas.

Quantifying DSBs is essential for identifying relationships between DNA cleavage and repair, which are pertinent to ageing, mutagenesis, genotoxicity and therapeutics, both established (chemo- and radio-therapies) and emerging (gene editing, particle and FLASH radiotherapy, and so on). Currently, our most powerful tools to spatially observe DSBs rely on immunofluorescence labelling of DNA damage response (DDR) proteins or histone modifications such as 53BP1 and γH2AX, respectively. These well-established markers typify the DDR[2]. However, interpretation of these downstream proxies is often extended upstream, through a range of assumptions, to DSB formation in a directly proportional, quantitative manner. These interpretations can be problematic: repair can be partial, thereby skewing protein is 53BP1, which recruits additional DSB repair proteins and regulates repair-pathway choice. Through immunofluorescence, 53BP1 and γH2AX are widely used, often dogmatically, as DSB markers, as they form discrete foci at or in proximity to DSBs.

Biases of current assays

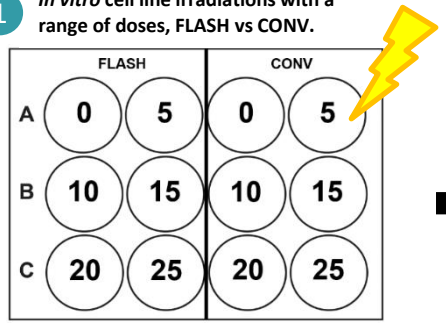
The only conventional method for in situ DSB detection is terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL), but it does not quantify discrete DSB foci. Consequently, other markers are used, but considering many reports in the literature, it is necessary to remember that DDR markers generally reflect repair, not DSBs. An excellent example is perturbation of the DDR with a TGFβ inhibitor prior to DNA insult. Consequently, fewer γH2AX foci are detected compared with control, which correlates with decreasing clonogenicity, that is, repair is impaired, leading to reduced proliferative capacity[5]. This highlights the crux of assays based on DDR factors: they are ambiguous. Do foci correlate with DSB accumulation, increased repair capacity and/or efficiency, or expression of DDR factors? Other misconceptions when using DDR proteins as DSB markers may arise.

Nature Comment

- ‘Mini review’ accepted into Nature Reviews Molecular Cell Biology: Impact Factor = 113.9
- Imaging DSBs is useful for understanding the impact of cytotoxic agents i.e. radiotherapy
  - Primary mode of tumour cell death
- Current detection techniques lack target specificity – they are **proxies**: proteins that signal *breakage repair*, but **not the breaks themselves** (γH2AX)
- Paper delves into why current assays are biased, novel alternatives, and advantages/disadvantages
- Desire for DSB-specific techniques
  - dSTRIDE – **currently optimising this technique** to compare its effectiveness versus “conventional” DSB detectors

Future Experiments – In Vitro

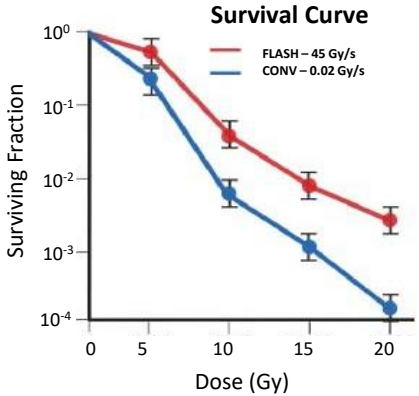
1 *In vitro* cell line irradiations with a range of doses, FLASH vs CONV.



Tumorigenic cell lines:
- 4T1 – Breast Tissue
- K7, K7M2 – Osteosarcoma

Normal cell lines:
- BALB/c Primary Lung Fibroblasts
- BALB/c Mouse Primary Bone Marrow-Derived Endothelial Cells

2 Clonogenic assay to determine cell survival as a function of dose for each dose rate.



To determine extent of breaks 'clustering', foci will be quantified in MATLAB.

3 γH2AX/53BP1 + dSTRIDE immunofluorescence to compare DSB detection techniques + "quantify" DSBs…

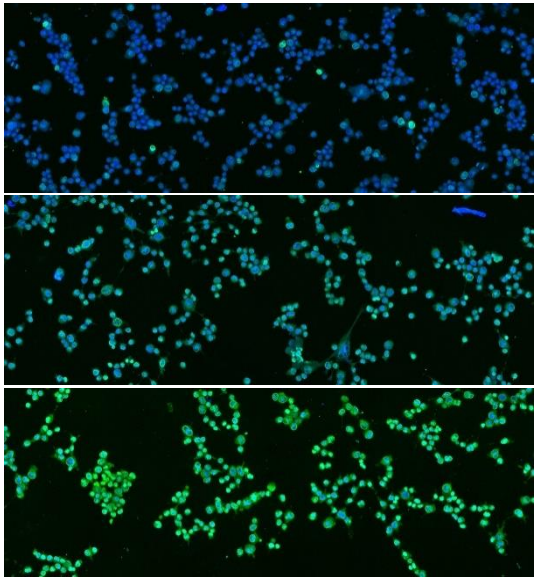


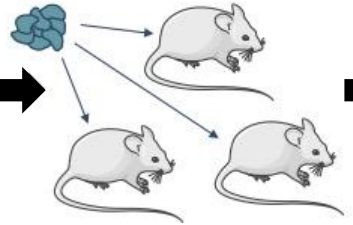
Figure: 4T1 cells vs varying concentrations of neocarzinostatin

Future Experiments – In Vivo

1. *In vivo* tumour from inbred strain BALB/c. Syngeneic model, <u>immunocompetent.</u>

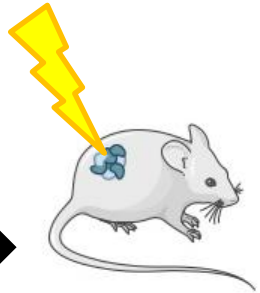
2. *In vitro* growth of tumour cells (4T1 and K7M2).

3. Subcutaneous injection of tumour cells (into hind leg) of mice from same inbred strain.



4. Irradiation of mice with either FLASH (45Gy/s) or CONV (<0.02Gy/s) protons. Tumour and comparative normal tissue irradiations will be performed.

Normal skin irradiations will also be scored qualitatively to record dermatitis severity i.e. erythema, desquamation, necrosis.



5. Excision of tumour and normal tissues and analysis via…

1. Hematoxylin and Eosin (H&E), Masson Trichrome, TUNEL Staining
   - Tissue structure and morphology
   - Immune cell infiltration
   - Apoptotic cells
2. qPCR, Western Blot
   - Aim to quantify markers of inflammation including:
   - TGF- β
   - IL-1, IL-6
   - TNF-α
   - NF-κB
   - MAPK, JAK-STAT pathways
3. Flow Cytometry
   - Immune cell population
   - Tumour and normal cell microenvironment modulation

Acknowledgements

- UniSA, Future Industries Institute
- Research Training Program (RTP) Domestic Stipend
- Royal Adelaide Hospital Commitment – Dr. Hien Le
- MITRU, SAHMRI
- Cyclotron Engineer - Johan Asp
- PhD Supervisors – Ivan Kempson, Eva Bezak and Hien Le
- Members of Kempson and Bezak Research Groups

Thank you for listening!