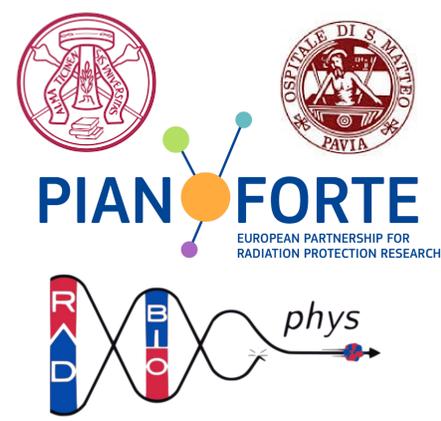# Screening for molecular signatures of radiotherapy and immune system activation in Breast Cancer: a spatial multi-omics approach applied to ex-vivo cancer samples

C. Riani[1], R. Semerano[1], L. Lonati[1], I. Guardamagna[1], A. Mentana[1], G. Gonon[1], A. Previtali[2], M. Maggi[2], C. Scotti[2], G. Baiocco[1]

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Background

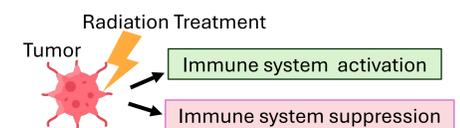### Towards personalized medicine for tumor treatment

Cancers represent **complex ecosystems** comprising tumor cells and a multitude of non-cancerous cells, embedded in an altered extracellular matrix. The composition and functional state of the **Tumor Microenvironment** (TME) varies across patients and strongly influences disease progression and therapy response. **Triple negative Breast Cancer** (TNBC) is associated with a poor prognosis due to its aggressive tumor biology and the absence of targets to direct therapies [1]. Radiotherapy remains a cornerstone of breast cancer treatment, exerting direct cytotoxic effects on tumor cells while also modulating the TME. In particular, tumor irradiation can reshape immune infiltration and activation states, potentially enhancing or suppressing anti-tumor immune responses [2]. Understanding these molecular and spatially organized changes is therefore essential to identify signatures associated with treatment response and immune system activation.

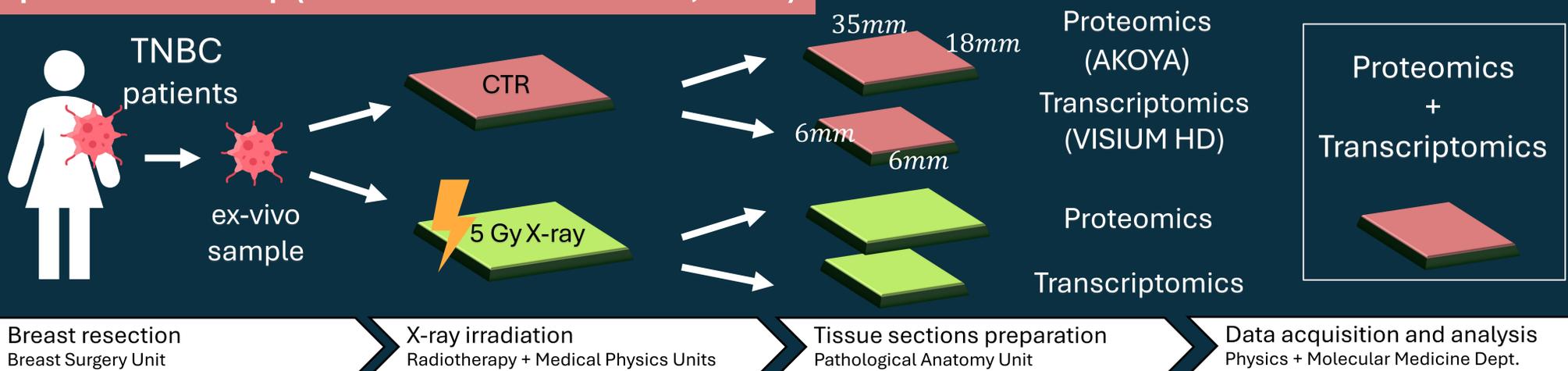### Spatial multi omics technologies

A comprehensive characterization of the TME requires the analysis of multiple molecular layers, as RNA and protein expression provide complementary information on cellular states and functions. **Spatial transcriptomics** (**ST**) and **Spatial Proteomics (SP)** can profile transcriptome-wide gene expression in an unbiased manner and targeted proteins, respectively, without the need for tissue dissociation, thus retaining spatial information. Beyond traditional differential expression analyses, that quantify genes up/down regulated between condition, spatial multi-omics approaches make it possible to investigate how molecular changes vary across tissue niches and conditions while accounting for spatial dependencies [3]. These technologies offer powerful tools to study cellular organization, tumor-immune interactions, and cell-cell communication networks within the TME [4].
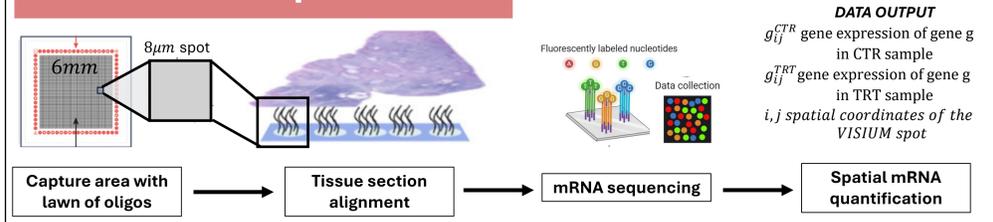
Project aim

The **IMAGEOMICS** Project (Eu-co-funded, PIANOFORTE partnership) aims to improve the benefit/risk ratio of breast cancer (BC) radiotherapy, focusing on immune system activation by radiation. We will leverage spatial multi omics techniques to identify molecular changes in tumor micro-environment after radiotherapy.
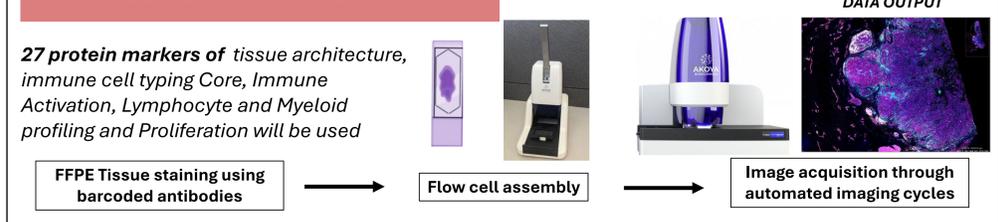
Radiation Treatment
Tumor → Immune system activation
     → Immune system suppression

Experimental set-up (UNIPV+IRCCS San Matteo, Pavia)

TNBC patients → ex-vivo sample

CTR → 35mm 18mm → Proteomics (AKOYA) / Transcriptomics (VISIUM HD)
→ 6mm 6mm

5 Gy X-ray → Proteomics / Transcriptomics

Proteomics + Transcriptomics

| Breast resection Breast Surgery Unit | X-ray irradiation Radiotherapy + Medical Physics Units | Tissue sections preparation Pathological Anatomy Unit | Data acquisition and analysis Physics + Molecular Medicine Dept. |

SP-Transcriptomics

8μm spot
6mm
Fluorescently labeled nucleotides
Data collection

DATA OUTPUT
g_{ij}^{CTR} gene expression of gene g in CTR sample
g_{ij}^{TRT} gene expression of gene g in TRT sample
i, j spatial coordinates of the VISIUM spot

| Capture area with lawn of oligos | Tissue section alignment | mRNA sequencing | Spatial mRNA quantification |

SP-Proteomics

27 protein markers of tissue architecture, immune cell typing Core, Immune Activation, Lymphocyte and Myeloid profiling and Proliferation will be used

DATA OUTPUT

| FFPE Tissue staining using barcoded antibodies | Flow cell assembly | Image acquisition through automated imaging cycles |

Bioinformatic analysis framework

1. Deep learning-based cell and nucleus segmentation for Spatial Proteomics

Segmentation is performed using *Stardist* or *CellPose* algorithms

■ Ki67 (proliferating cells/tumor)
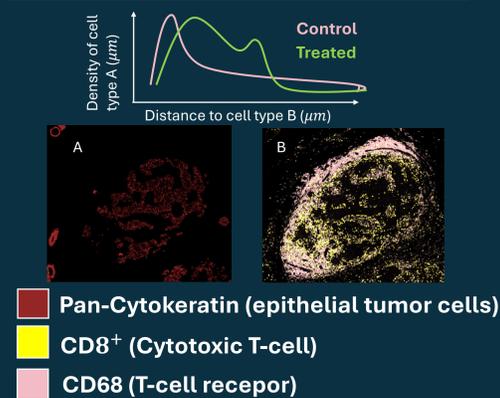■ CD68 (macrophages/phagocytic cell)
■ DAPI (nucleus)
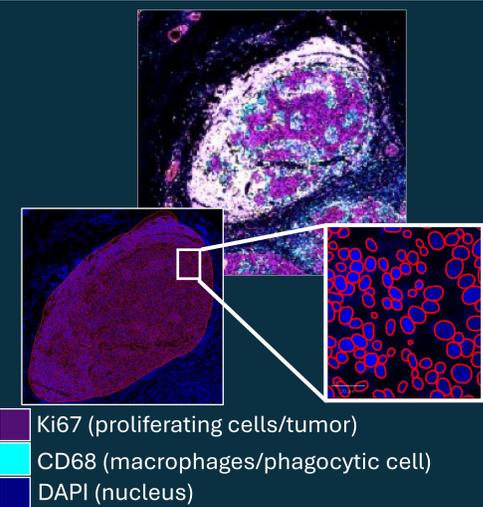
2. Identification of spatial structures through annotation and clustering

Tissue niches are identified through a combination of unsupervised clustering and manual annotation.

• ROI selection and extraction of nuclear and cellular mean/median MFI per marker

• Protein marker expression (MFI) per cell is reduced in dimensionality using PCA, then visualized with t-SNE or UMAP
• Unsupervised clustering (Leiden)

• Integration of spatial coordinates with cluster annotations to map cell types across tissue regions.

### 3. Spatial Distribution of Cells and cell-cell interactions

The generated cell type maps are used to quantify the density, localization, and proximity of immune cells (e.g., CD8+ T cells) to tumor cells. Spatial statistics, such as nearest-neighbor analysis, Ripley's K function, or cell interaction networks, can be applied to infer communication patterns and immunological niches.

Density of cell type A (μm) vs Distance to cell type A (μm)
Control / Treated

A / B

■ Pan-Cytokeratin (epithelial tumor cells)
■ CD8+ (Cytotoxic T-cell)
■ CD68 (T-cell recepor)

4. Differential gene expression (DGE) analysis

DGE will be performed using two strategies: pseudo-bulk aggregation with DESeq2 or dedicated spatial differential expression methods
• Within-condition: DGE between tumor regions (e.g., core vs immune-infiltrated).
• Between-condition: DGE in the same region before vs after 5 Gy radiation.
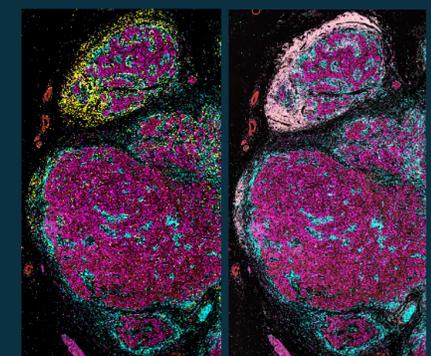
Conclusions and future perspectives

• The IMAGEOMICS approach establishes a **spatially resolved computational framework** to investigate how radiotherapy reshapes the breast cancer immune microenvironment.
• The identification of **spatial biomarkers of response** may support patient stratification and guide **personalized radiotherapy immunotherapy combinations**.
• Notably, **spatial multi-omics studies of ex vivo irradiated TNBC remain scarce**, highlighting the relevance and novelty of this approach.
• Future work will integrate **spatial transcriptomics and proteomics** to achieve a comprehensive multi-modal characterization of tumor–immune interactions.

[1] Aysola K, Desai A, Welch C, Xu J, Qin Y, Reddy V, Matthews R, Owens C, Okoli J, Beech DJ, Piyathilake CJ, Reddy SP, Rao VN. Triple Negative Breast Cancer - An Overview. Hereditary Genet.
[2] Zhang, Z., Liu, X., Chen, D. et al. Radiotherapy combined with immunotherapy: the dawn of cancer treatment. Sig Transduct Target Ther 7, 258 (2022)
[3] Longqi Liu, Ao Chen, Yuxiang Li, Jan Mulder, Holger Heyn, Xun Xu, Spatiotemporal omics for biology and medicine, Cell, Volume 187, Issue 17, 2024
[4]Palla, G., Fischer, D.S., Regev, A. et al. Spatial components of molecular tissue biology. Nat Biotechnol 40, 308–318 (2022).