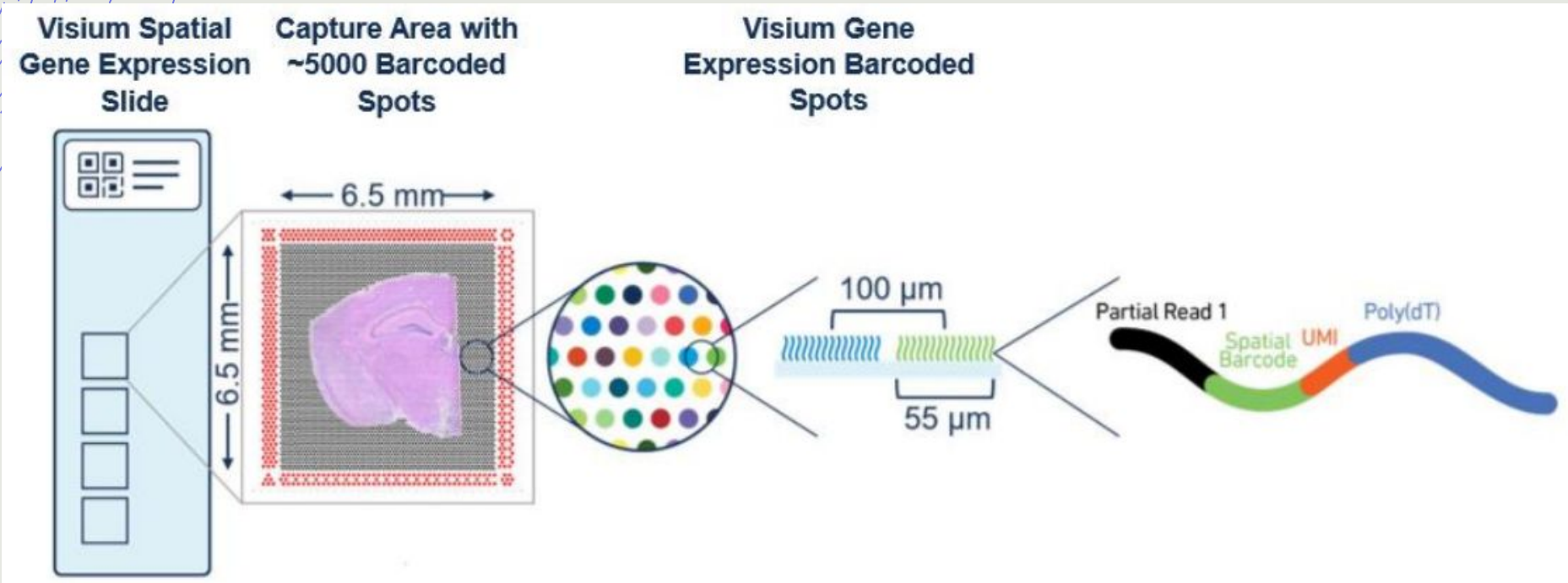
10x Genomics Visium Spatial scRNAseq

A feedback from early experiments @ Gustave Roussy

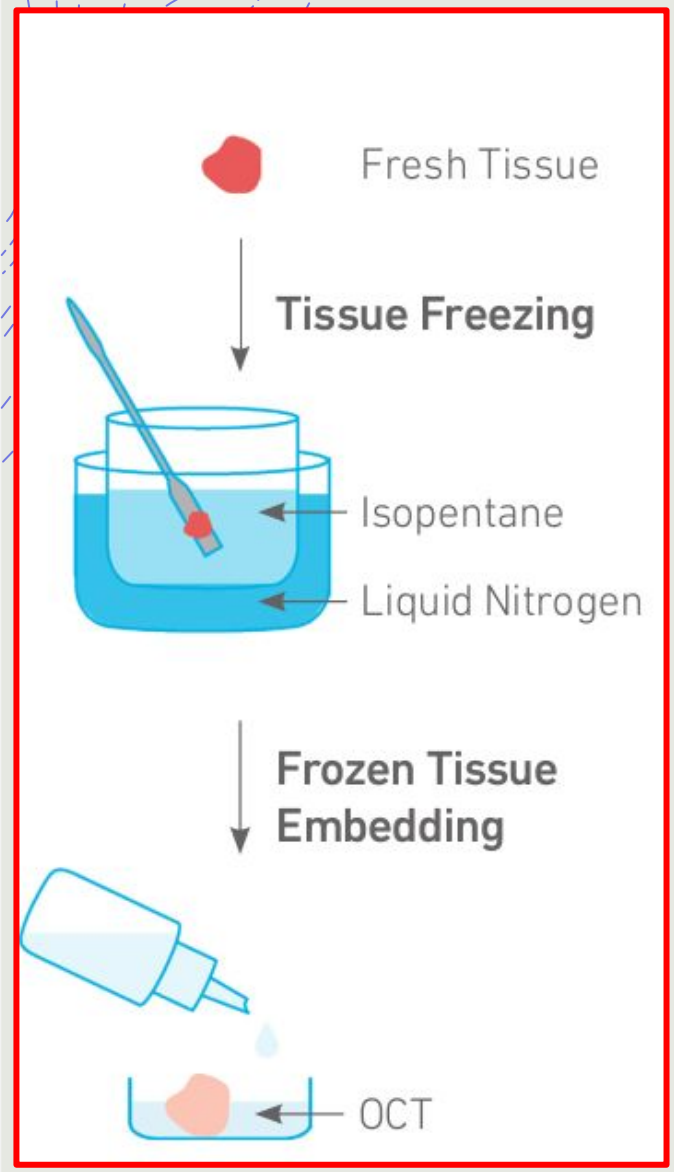
10x Visium Spatial scRNAseq



10x Genomics Visium Spatial Expression Lab Experiment

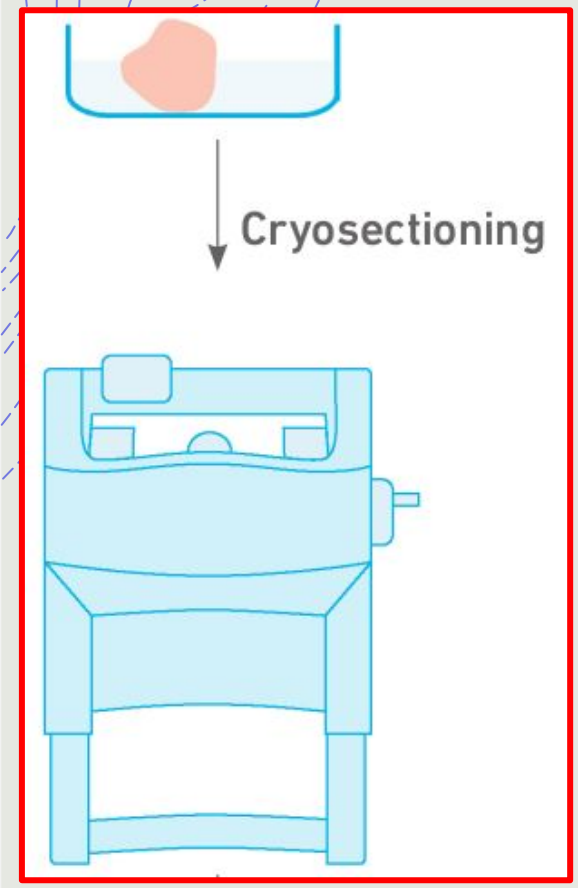
Because data never come from vacuum

Sample snap freeze and embedding

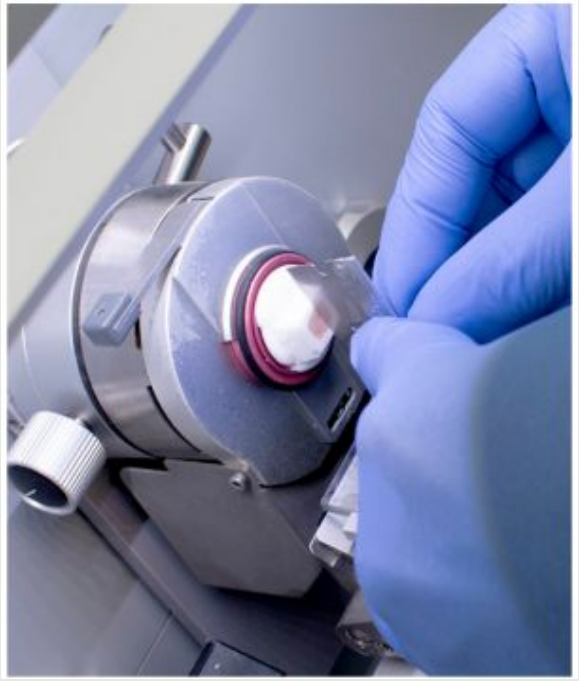


- Isopentane is **mandatory**
- Has to be cooled in liquid nitrogen **in advance**
- **Do not** directly freeze the tissue in nitrogen

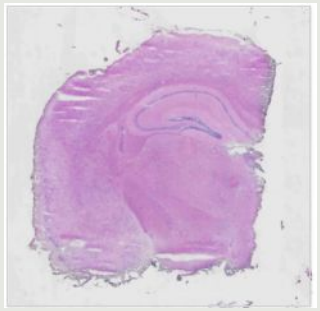
Cryosectioning



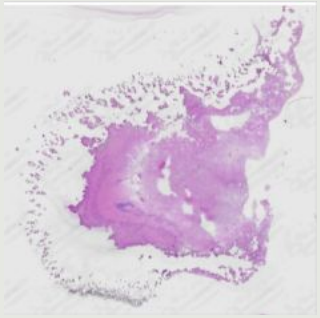
If inclusion is too large, **recut** with a 1mm deep shallow incision



Keep sample cold to avoid **condensation**

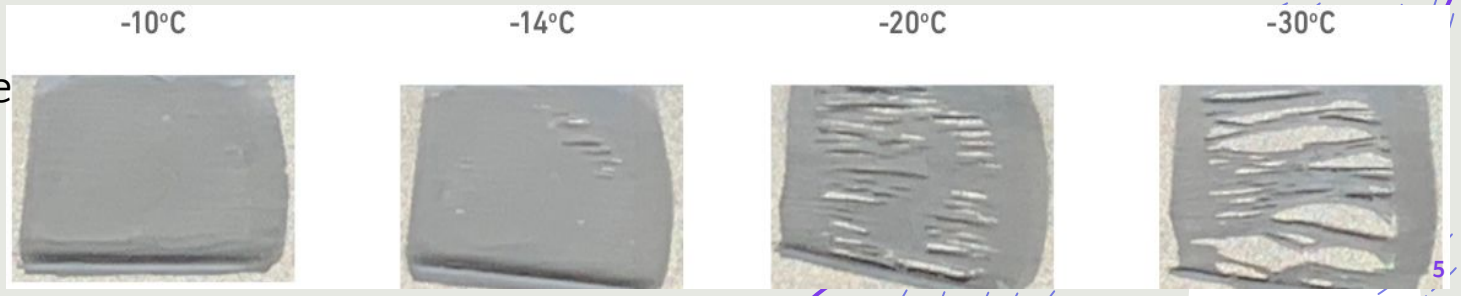


Cold

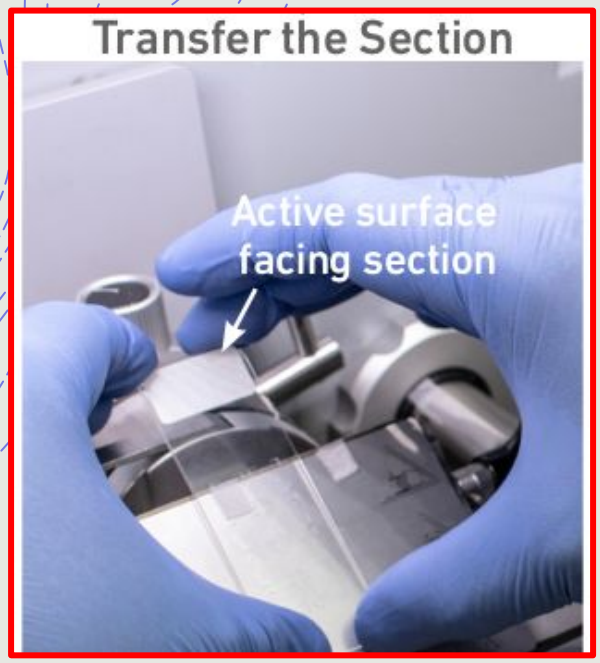


@ RT

Adapt cutting head temperature to tissue and section depth to avoid **tearing**



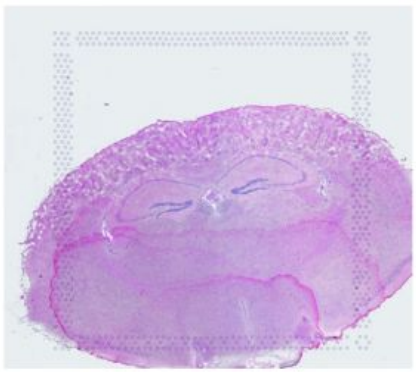
Section transfer



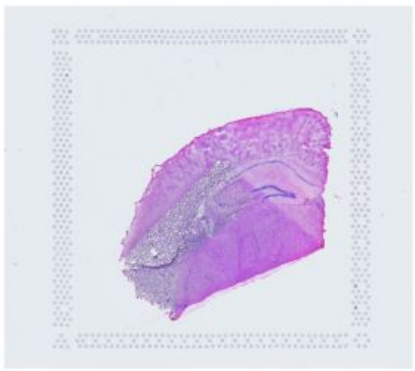
Hard to transfer section with **frozen** fingers !

Active section : 6.5 x 6.5 mm (8 x 8 mm with frame)

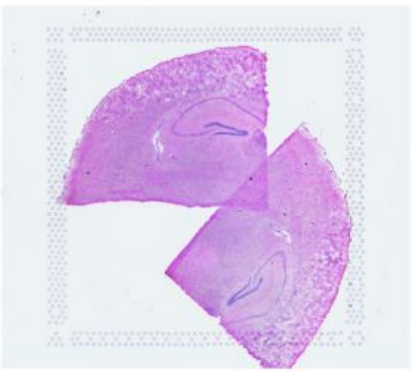
Avoid transfer **imperfections** (folding, overlap, frame covering, surface scratching, ...)



Fiducial frames covered

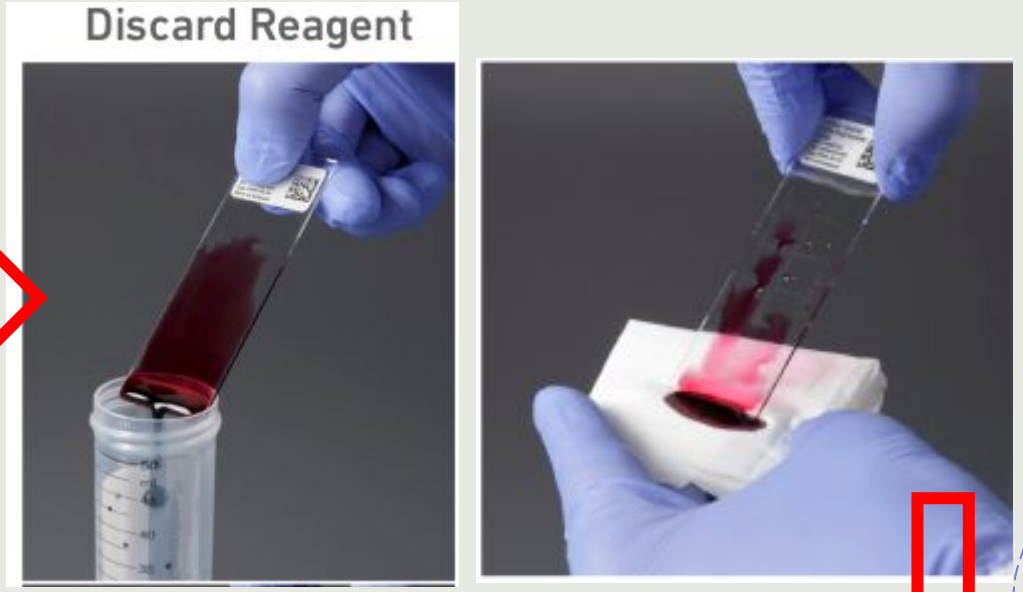


Folded tissue section

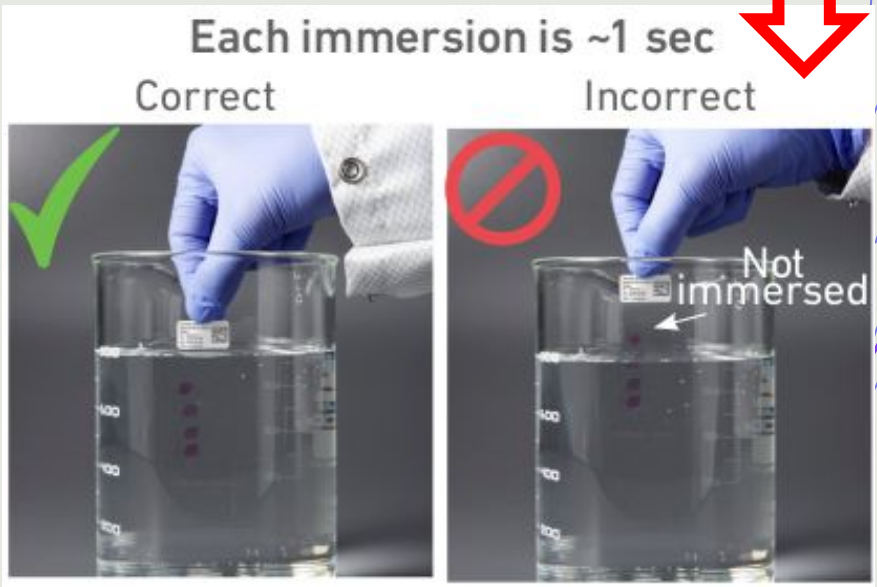


Overlapping sections

Hemoxylin Eosin Staining



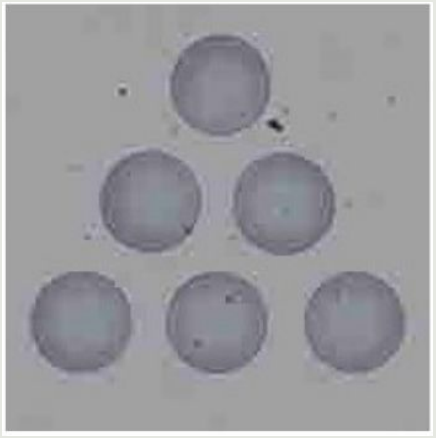
- Avoid reagent **leaking**
- **Multiple** preparations ahead: buffers (Tris – Acetic acid), washing beakers, cooled methanol, warmed thermocycler with a specific adapter, …
- Washing **immersions** :
  - Right **speed** is crucial (section may be washed away)
  - 5 + 15 + 15 + 5 + 15 = **55 rounds** !



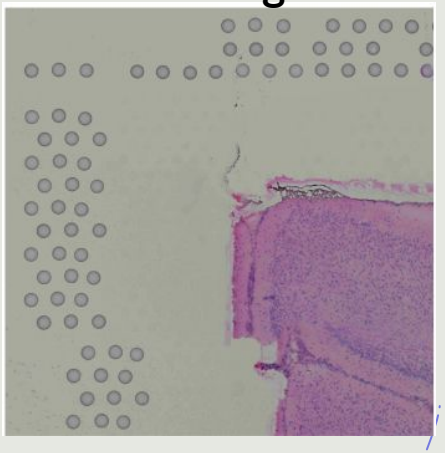
Staining imaging

Bad exposition

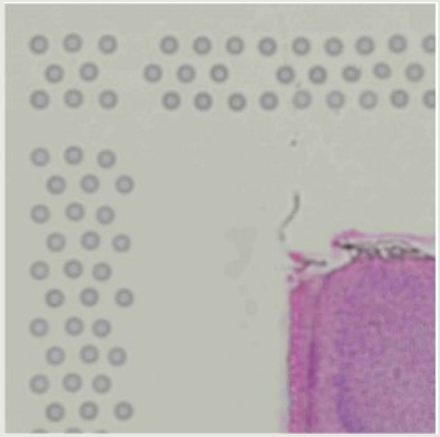
Bad resolution



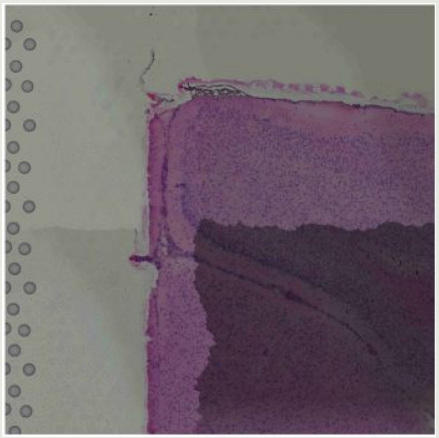
Stitching



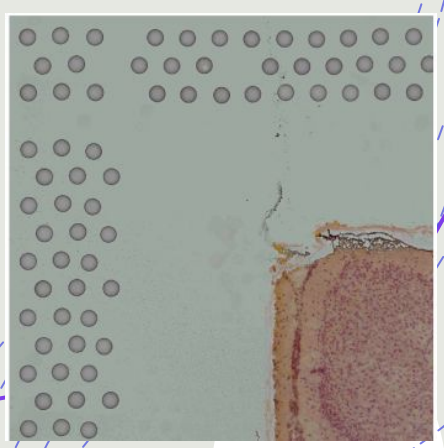
Out of focus



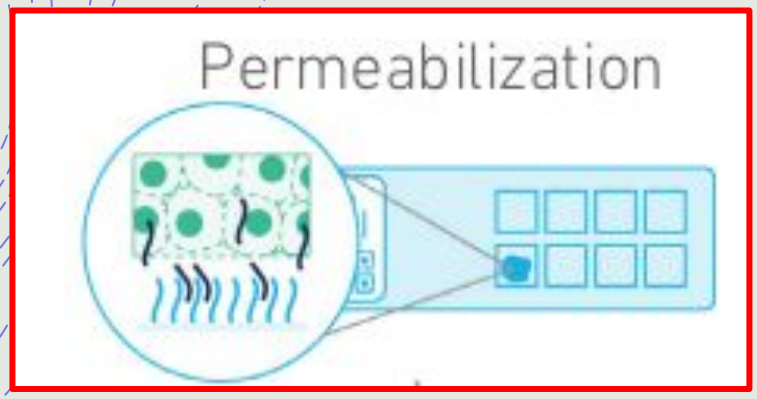
Bad shade correction

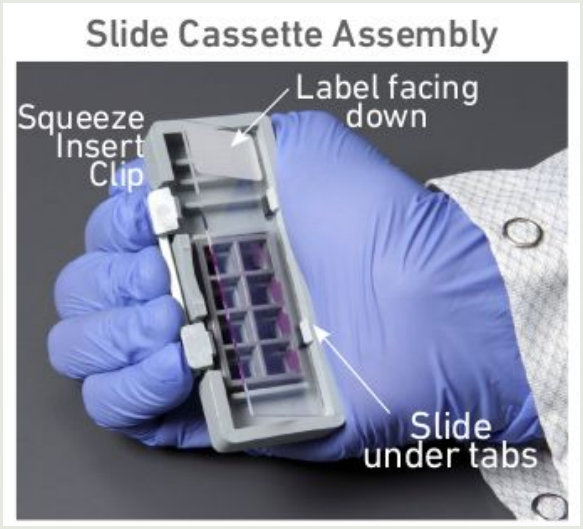


Unbalanced whites



Tissue permeabilization



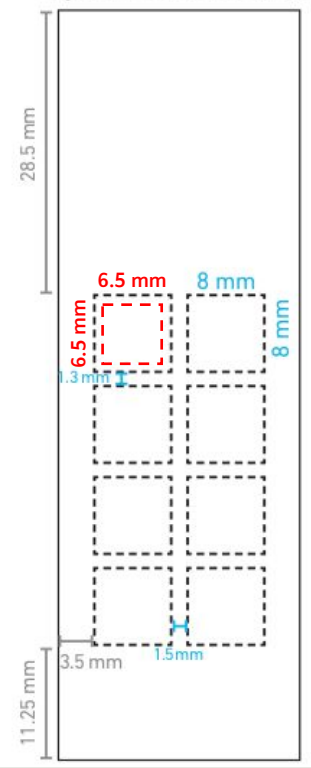


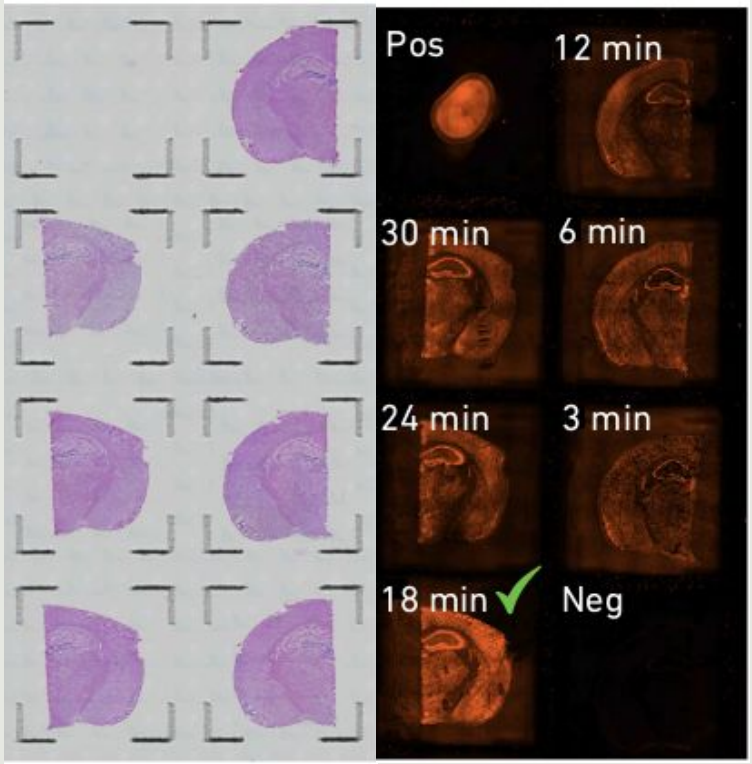
Cassette is a **hard** plastic shell, high risk to **break** the slide

- Wells are **dark** and **deep**
- Be **gentle** while pipetting :
  - Avoid air **bubbles**
  - Gentle **flush** (sections may lose adherence)
  - Avoid **scratching** the surface with your tip

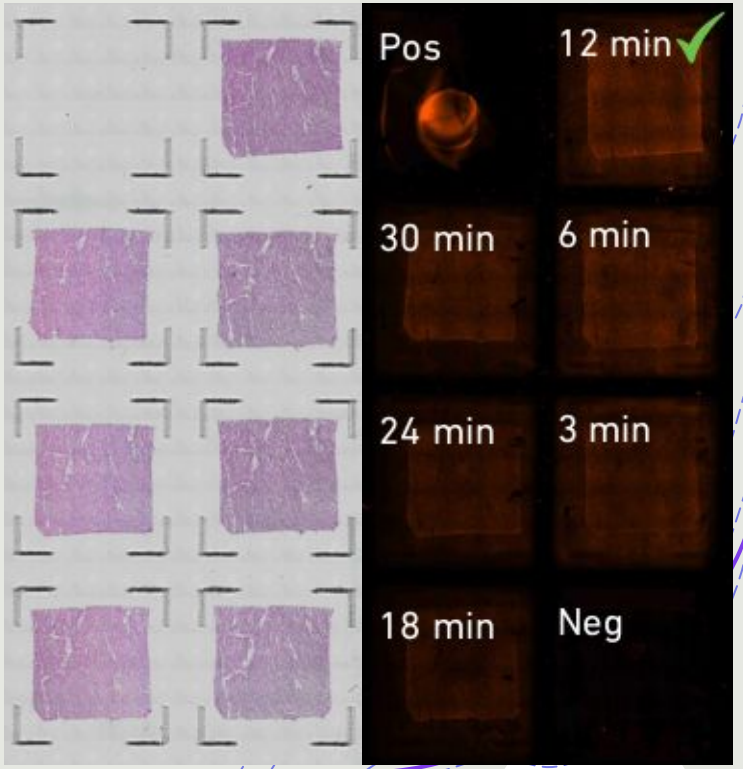
Permeabilization time

- Depends on tissue **type** (guidelines exist)
- **Evaluated** on a time course :
  - Optimization Slide (7 test areas + 1 positive control)
  - Best timepoint = Highest signal level / lowest spread





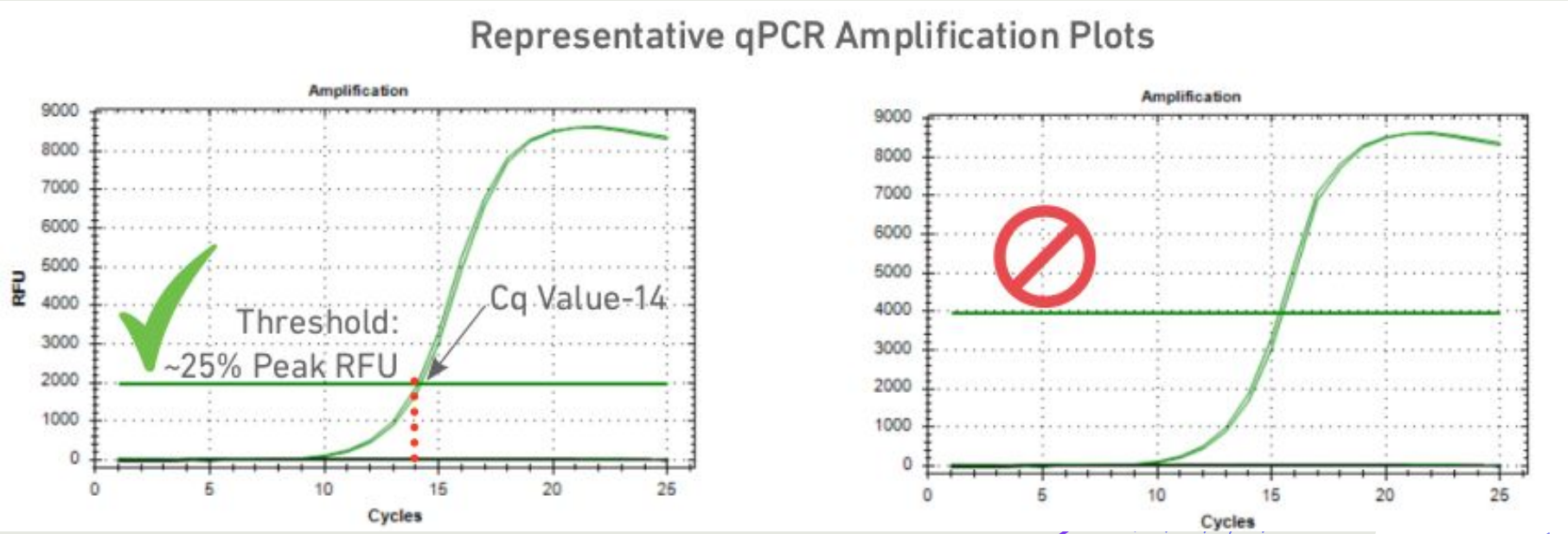
Mouse brain



Human heart

From RNA to amplified cDNA

- In-situ reverse-transcription
- Second DNA strand synthesis
- Denaturation
- cDNA amplification (qPCR) :
  - Identify the **number of cycles to** perform on **the "real" sample**

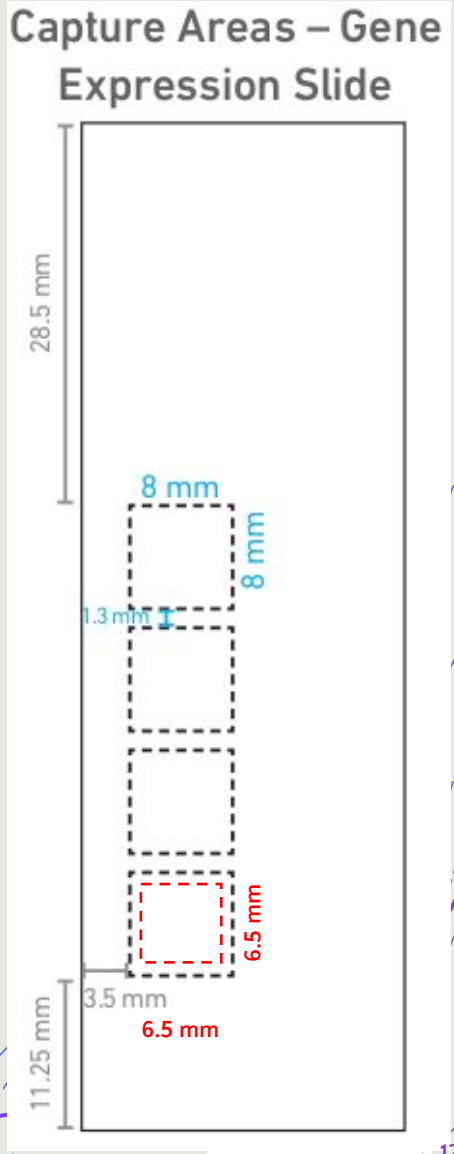


Next : the Gene Expression slide

- Redo **ALL** steps on the expression slide :
  - On other sections from the same sample
  - From the section transfer (without the permeabilization timescale : using the observed best elution time) …
  - … to the cDNA amplification step (using the identified best number of PCR cycles)
- Library construction
  - Analogous to "non-spatial" scRNAseq
- Sequencing
  - Adapt expected generated reads per sample :

    Formula : Occupied area * 5K spots * 50K-100K reads / spot

    Target : 100% * 5K * 100K * 4 samples = **2 billion** reads / run !

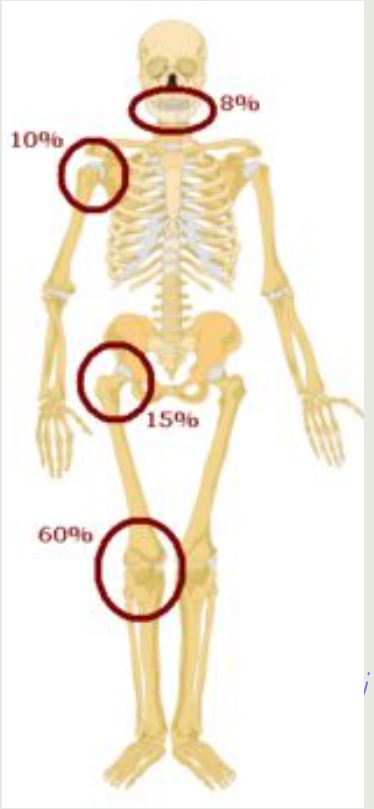


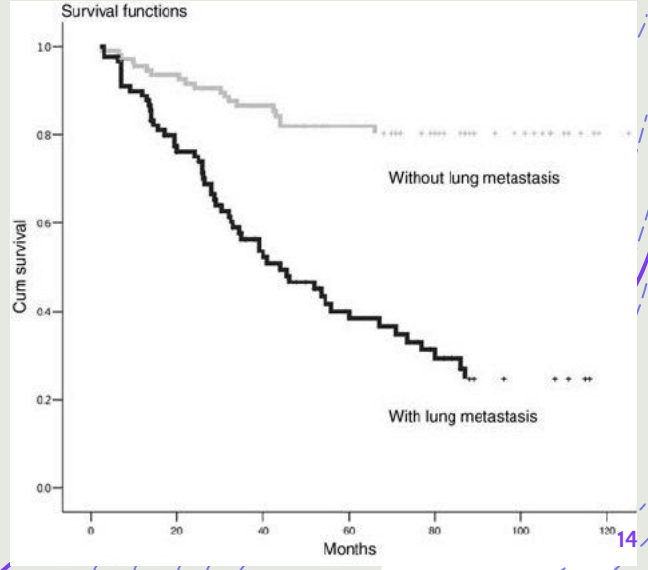
Very first attempts @ Gustave Roussy

Preliminary work on osteosarcoma xenografts

The OS Project

- Osteosarcoma :
  - **Rare** (1/200,000), still **most frequent** malignant bone tumor
  - Affects **children** and young adults
  - Most common treatment : neoadjuvant **chemotherapy** (MTX) + surgical **resection**
  - Still the **lowest survival** rate for pediatric cancers
- The project :
  - Study the spatial **constitution** and **heterogeneity** of OS in murine patient-derived xenografts (PDX)
  - Murine host : tumor **microenvironment**

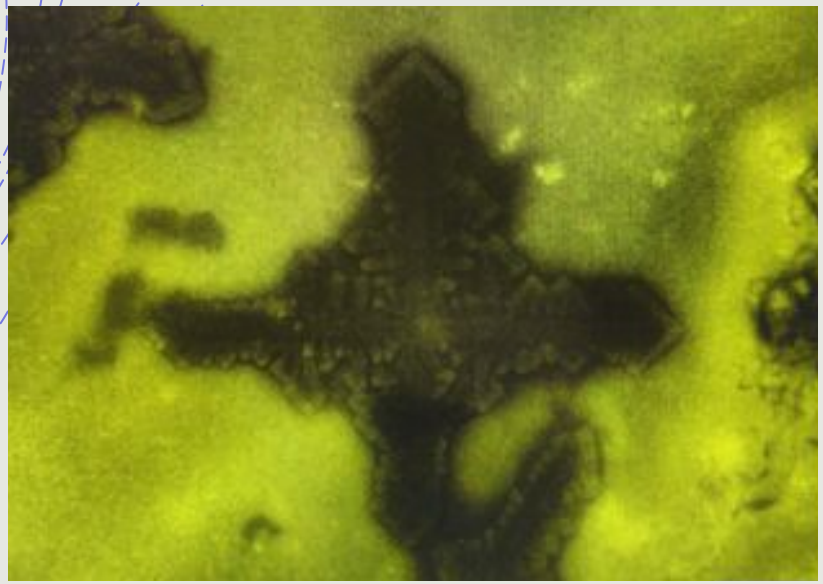


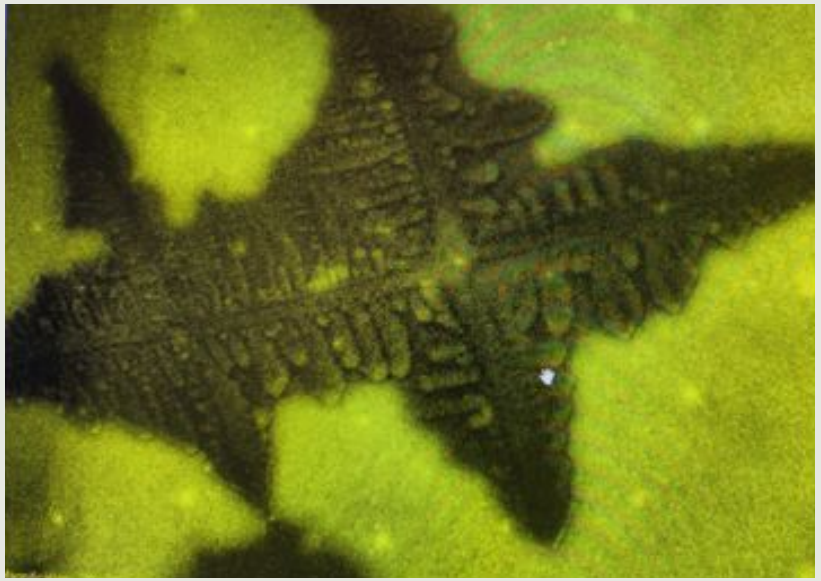


Working on OS : Caveheats

- Sample type
  - Bone = high **calcification** level
  - Tumor = **heterogeneity** = wide different cells behavior = internal variance in permeabilization times
  - PDX = cells from **two** species
- Material
  - **Old** cryostat = inhomogeneous temperature = small and teared sections = < 50% of active space used
- cDNA amplification
  - **Low** RNA quantity (despite good quality) = **25 cycles** (16 max recommended)
- Other :
  - **Problem** with a permeabilization kit
  - **Limited** budget (~100K€ = 8-9 experiments max)

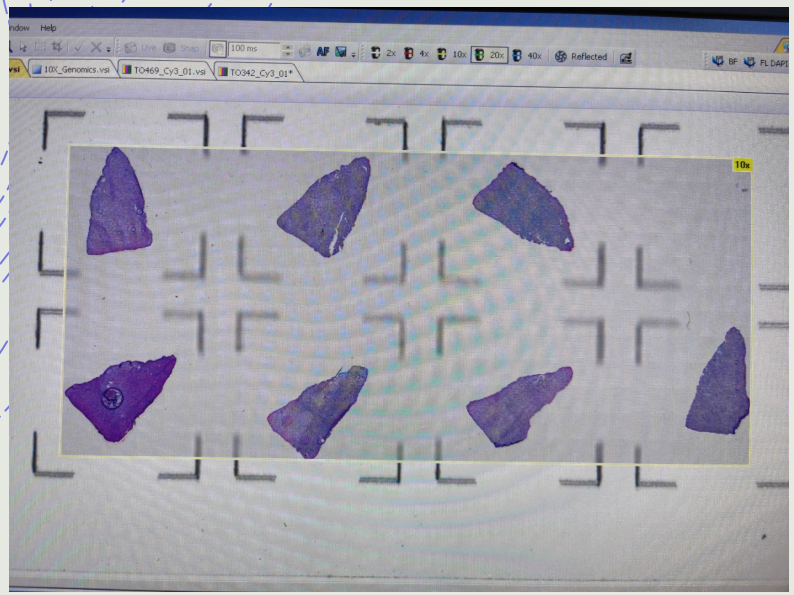
Calcium crystallization





+ Calcium crystals formed during the permeabilization step
+ Could be removed using multiple warm water washings
+ Did not affect the Expression Slide

Permeabilization time



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- Could be removed using multiple warm water washings
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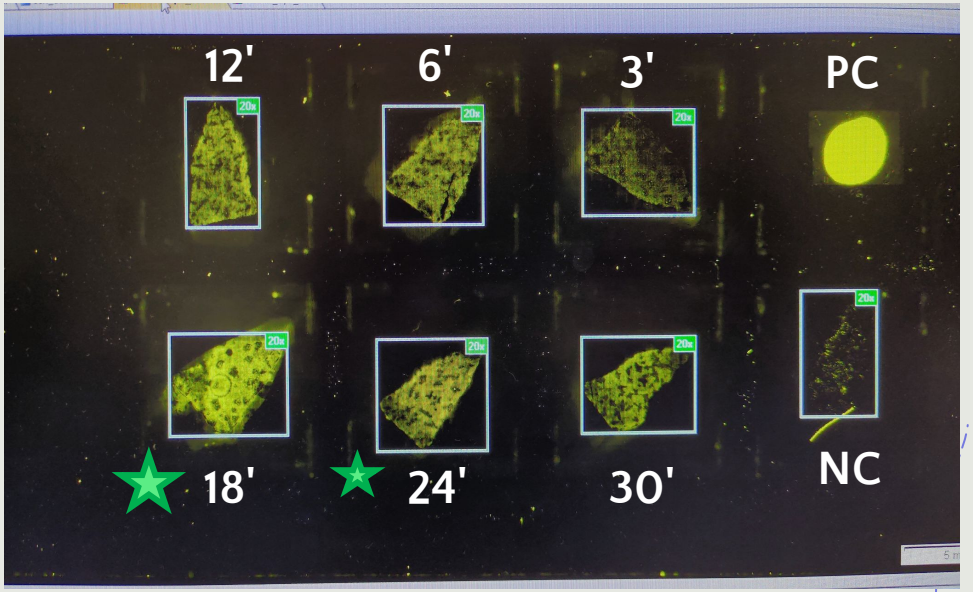
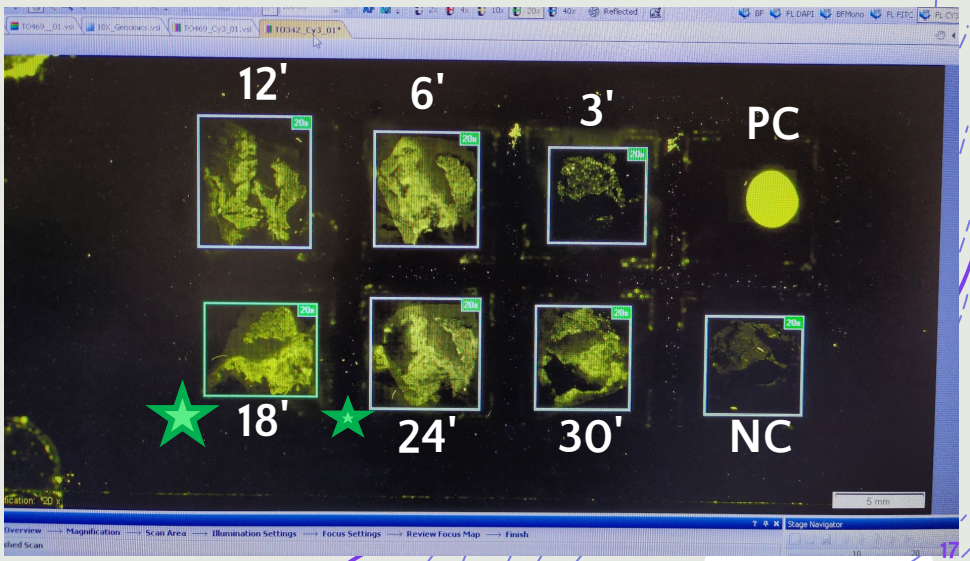
PDX1_OPT
10% calcification

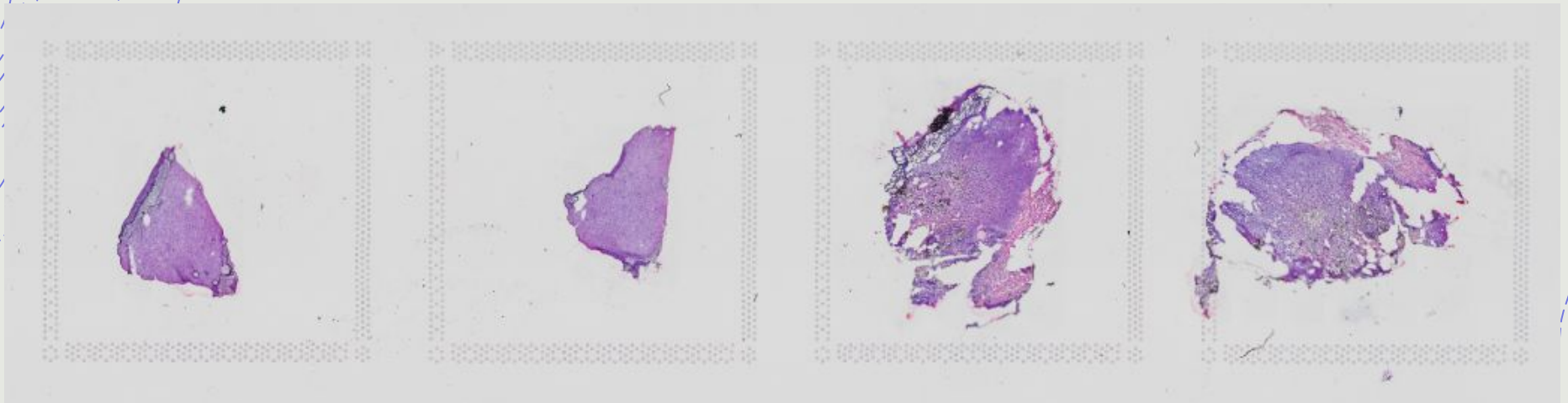


PDX2_OPT
65% calcification



Gene Expression slide

+ Performed on two sections for each PDX sample



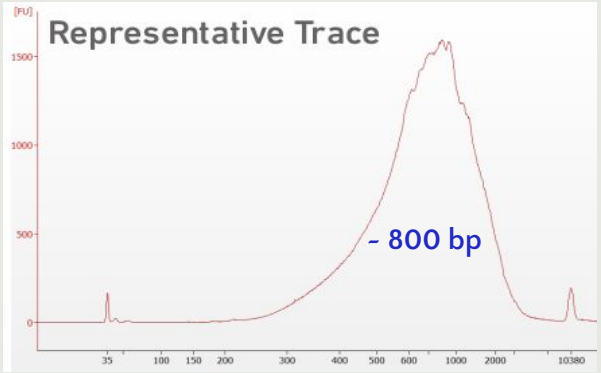
PDX1_A PDX1_B PDX2_A PDX2_B

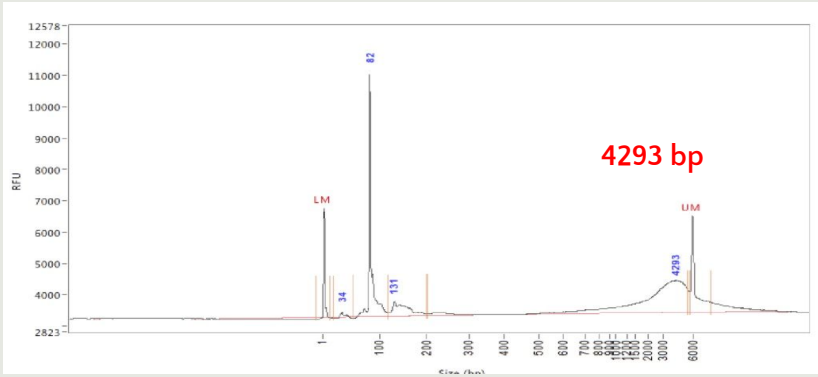
Sample	PDX1 (A,B)	PDX2 (A,B)
Calcification	10%	**65%**
Permeabilization time	21 min	21 min
Used area	**~15%**	~33%

Sequencing

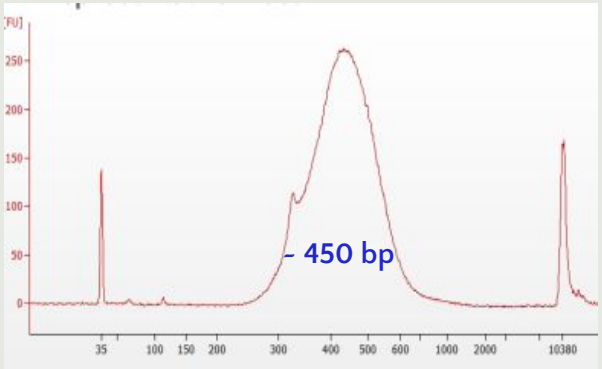
Example for PDX1_A

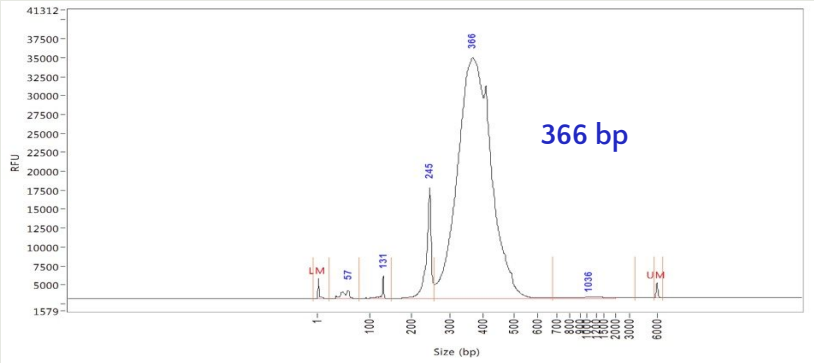
cDNA QC





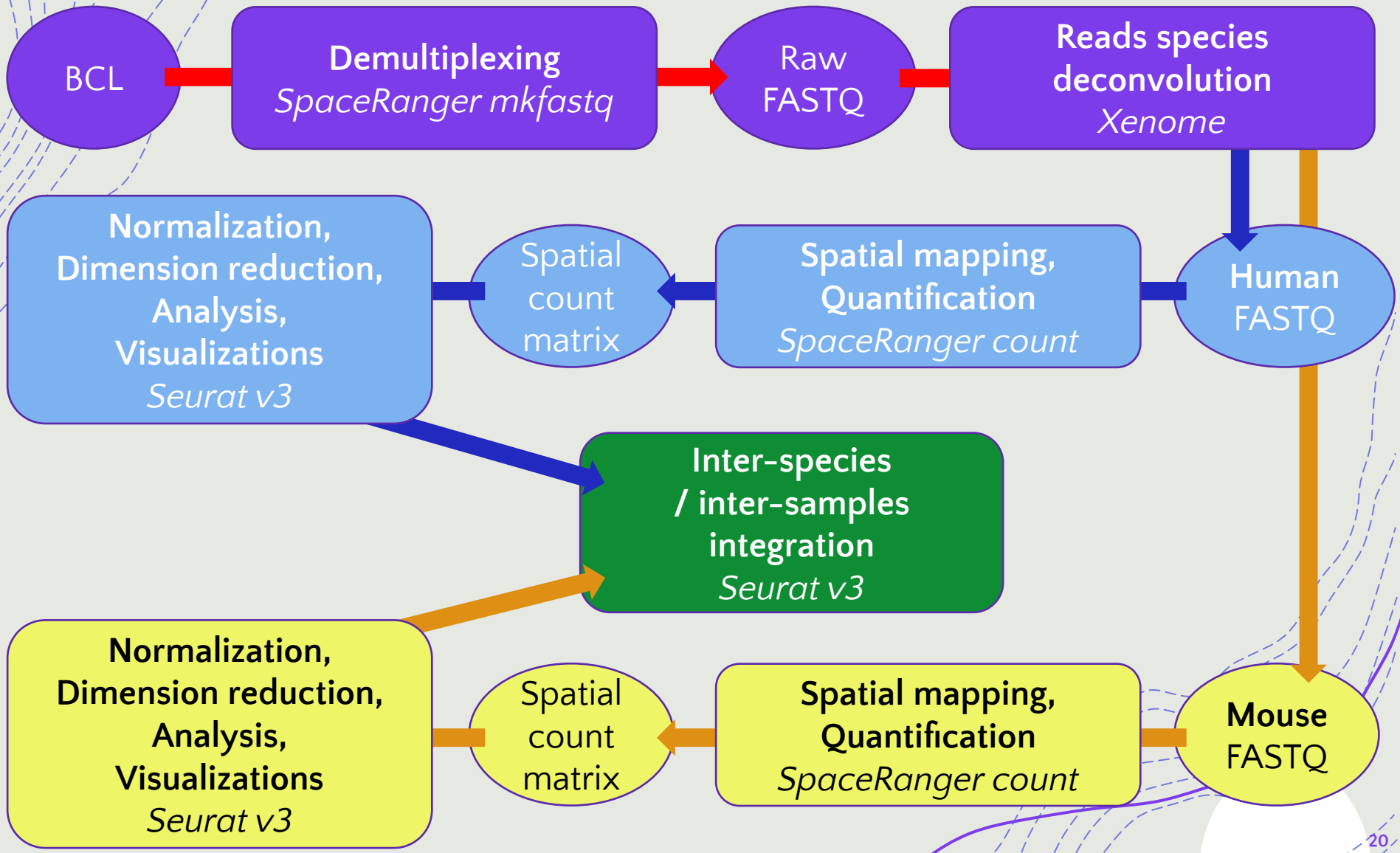
Library QC





- Performed on an Illumina NovaSeq S1 flowcell (up to 2.5 billion reads) :
  - PDX1_A = 534.50 million reads
  - PDX1_B = 788.02 million reads
  - PDX2_A = 593.95 million reads
  - PDX2_B = 575.29 million reads

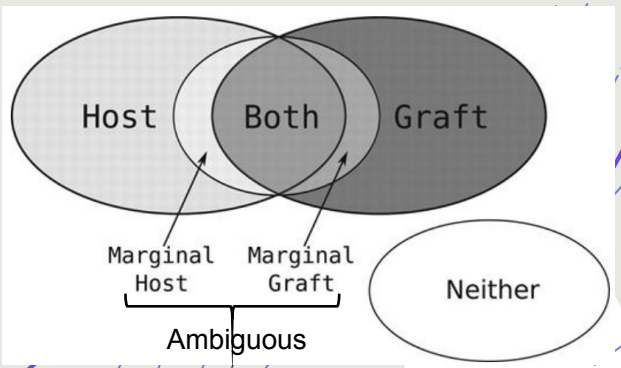
Analysis workflow



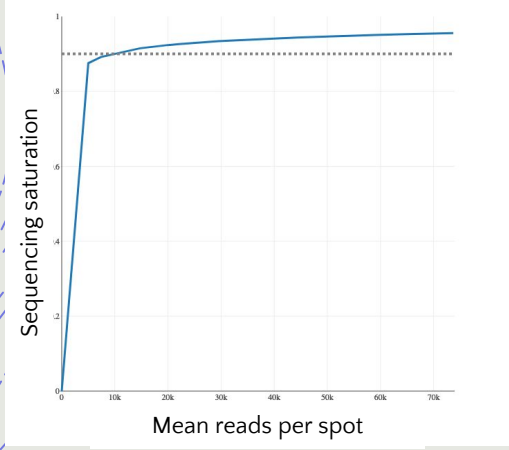
Xenome mapping rates

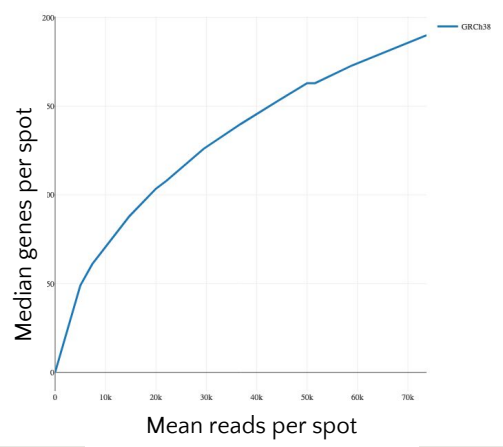
Read address	PDX1_GE_A	PDX1_GE_B	PDX2_GE_A	PDX2_GE_B
Human (graft)	**39.0%**	**42.8%**	**30.7%**	**35.1%**
Mouse (host)	13.8%	13.7%	14.8%	15.3%
Both	16.3%	18.0%	39.9%	36.3%
Ambiguous	30.1%	24.8%	13.7%	12.9%
Neither	0.7%	0.6%	0.9%	0.5%

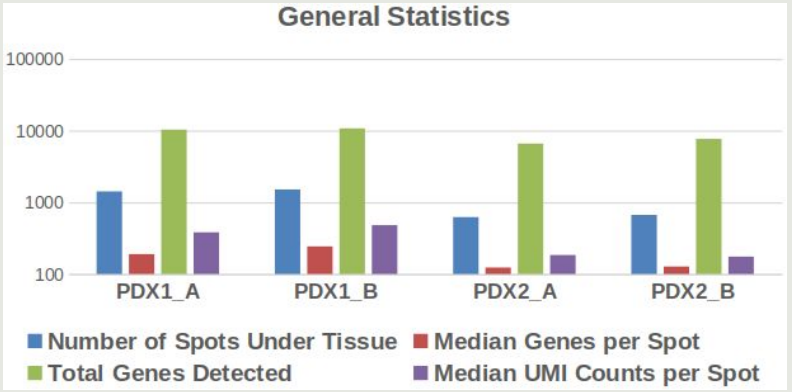
- Both + Ambiguous reads account for **40-50%** of total reads
- Hypothesis : Half of captured reads belong to conserved (housekeeping ?) genes

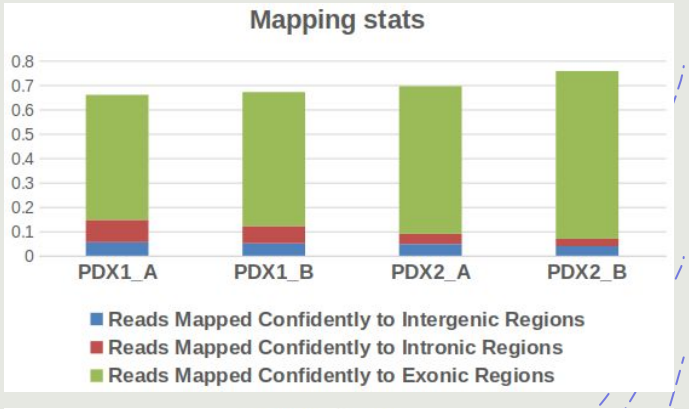


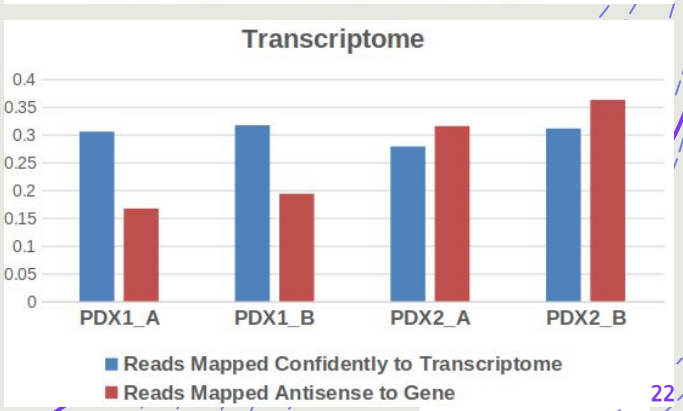
Mapping stats on human







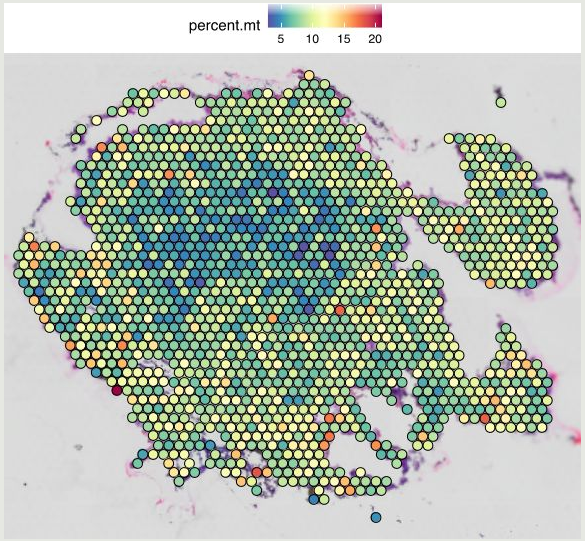




- 15 ~ 30% of spots covered by tissue
- ~ 30% of reads confidently mapped to transcriptome (most mapped to exons) …
- … but 16 ~ 36% of reads mapped antisense to gene ?!
- ~ 150 genes / spot (median)
- ~ 300 UMIs / spot (median)
  - Libraries complexity is low
  - Few genes mapped, with high read depth

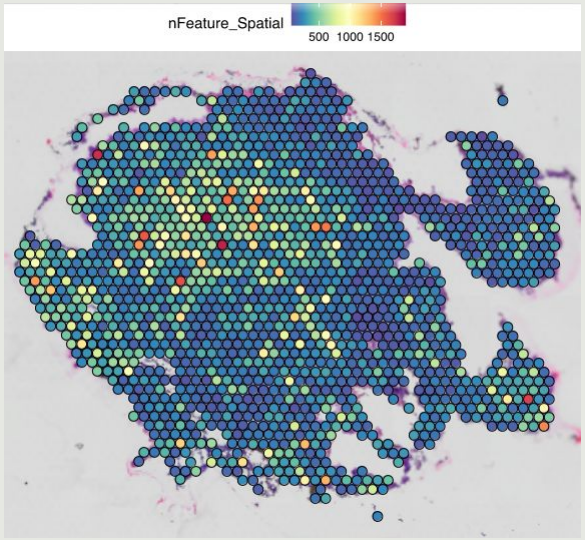
Mapped SC QCs

% mito

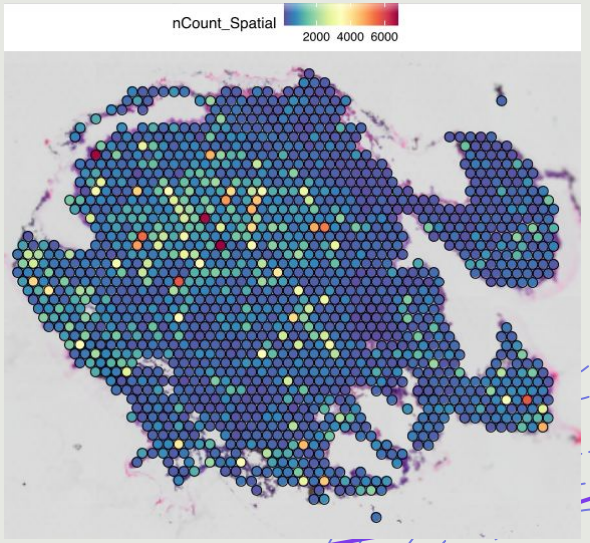


Example for PDX2_B

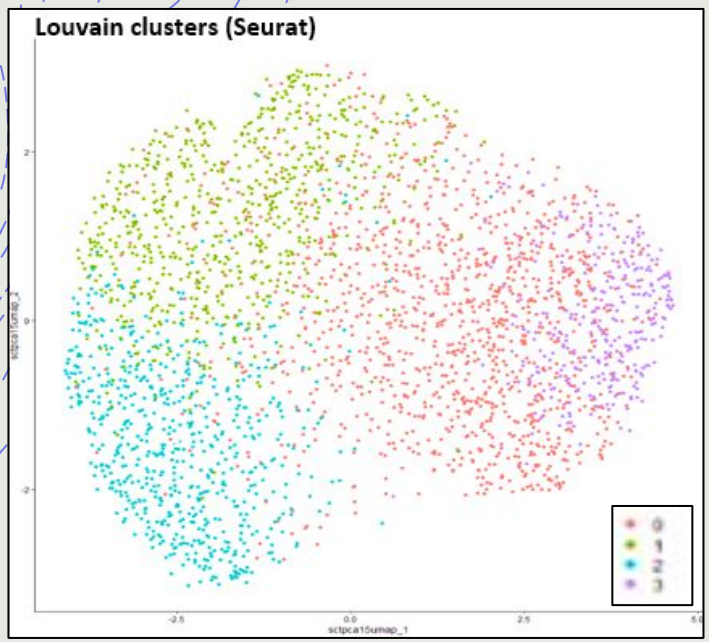
% ribo

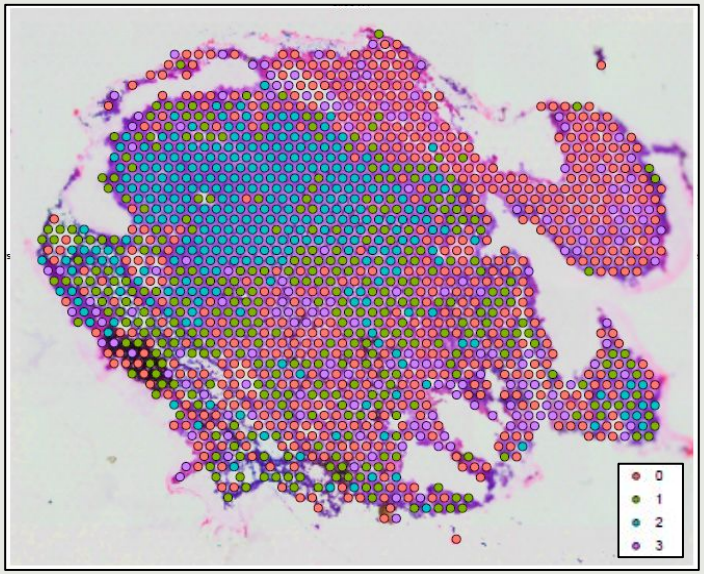


UMIs / spot

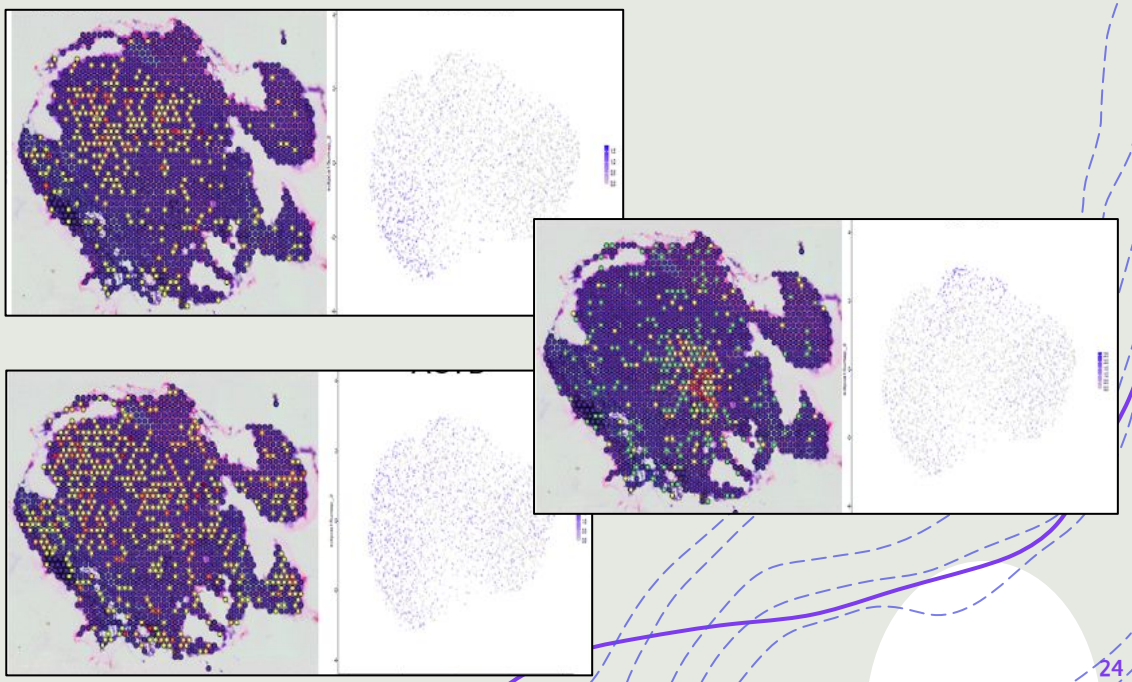


Clustering and visualization



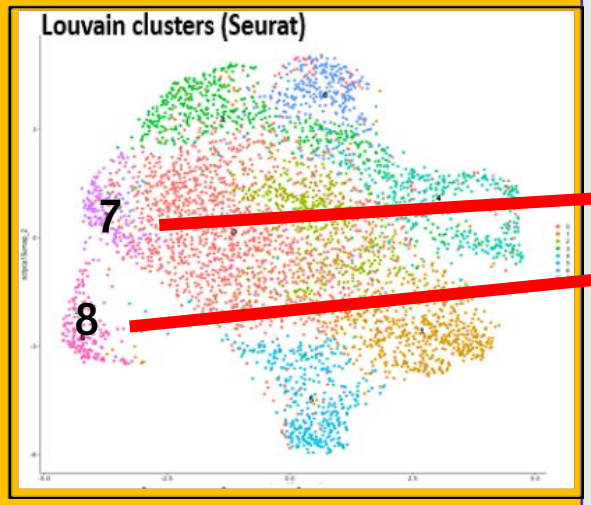


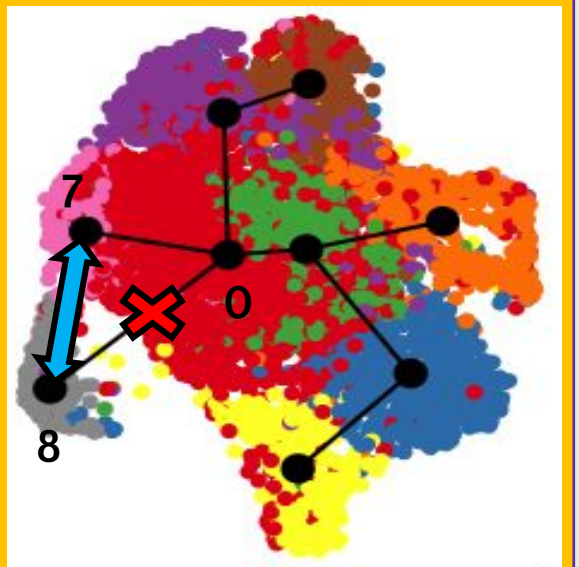
- 4 clusters identified by Louvain
- Nice spatial mapping of clusters
- Spatial mapping of some markers (below) sounds interesting, but poorly correlated with clusters



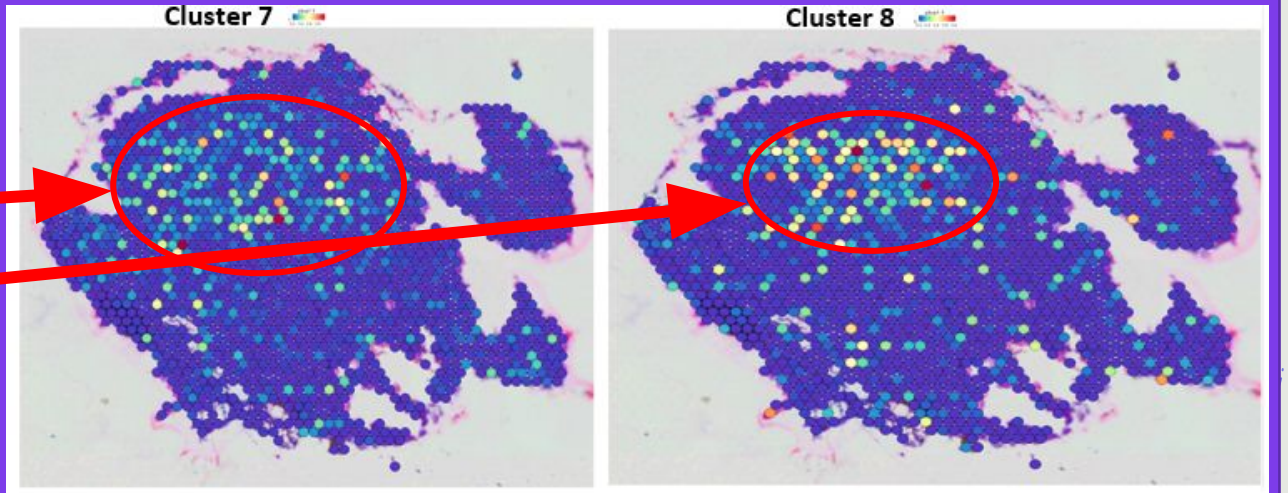
Interaction with non-spatial SC

Non-spatial scRNAseq





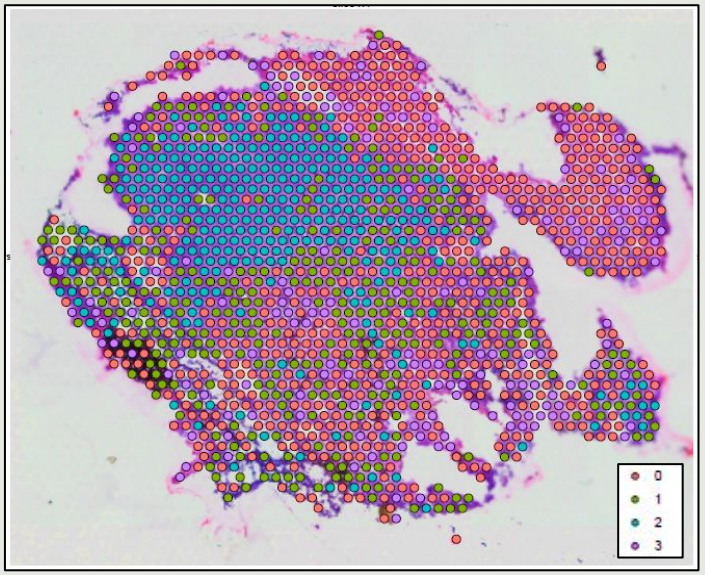
Spatial scRNAseq

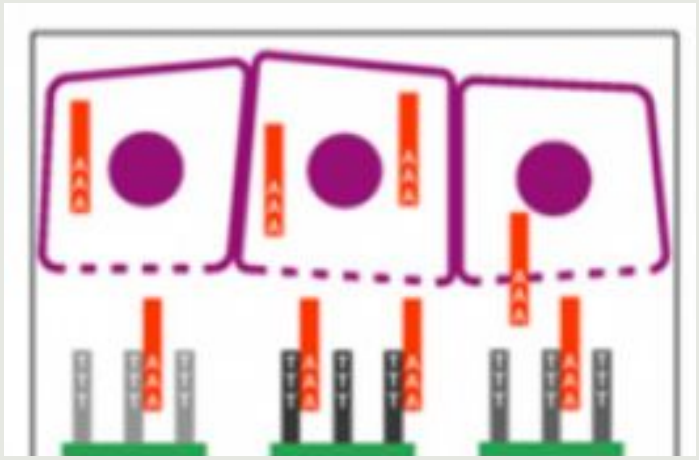


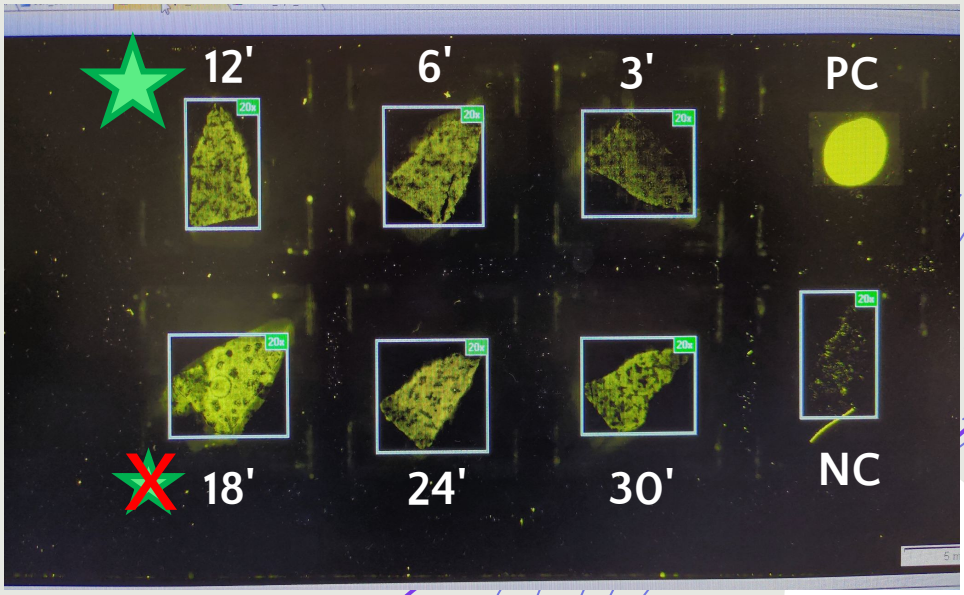
+ Non-spatial scRNAseq already **available** for PDX2_B
+ Slingshot trajectory : builds a path from cluster **0 to 7**, and **0 to 8**
+ **Concentric** location of clusters 7 and 8 in spatial (7 surrounding 8): correction of the trajectory with a path **from 7 to 8**

Update in late 2021

- TBH results were disappointing
  - high spread on uMAP
  - low distance between clusters
  - clusters mapping on spatial *very* noisy
- Why ?
  - Optimal elution time ***IS NOT OPTIMAL*** :
  - **RNA spread** for long times







Update in late 2021

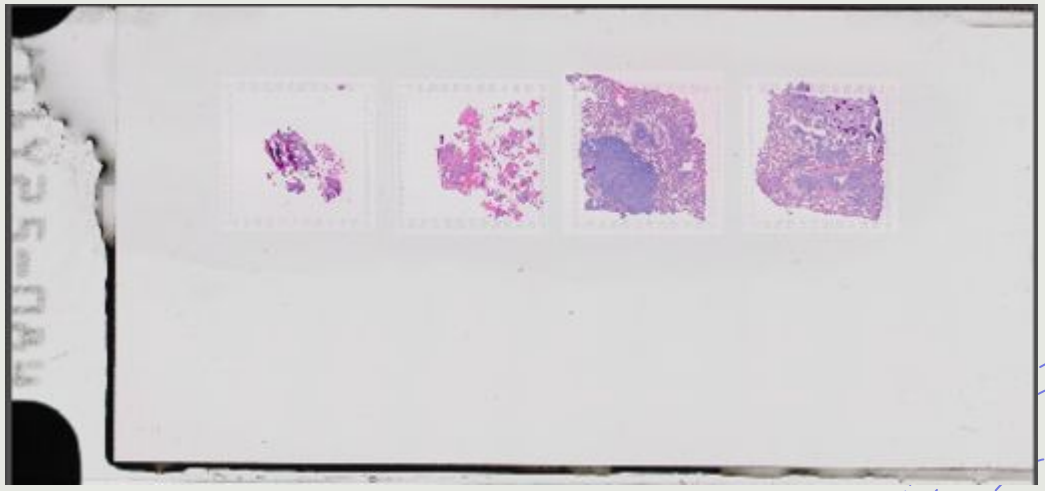
- **Improvements**
  - Lower elution time
  - Out of protocol step removed : usage of *Sybrgreen* for RNA quantification involving a damaging 90°C heating step
  - PCR bubbles observed in BioAnalyzer profile due to too many cycles (not sequenced)
- **Problems solved !**
  - First good results on 2 PDX !
  - First successful tumor !
  - Successful shift to 2 new tumor types :
    - Glioma
    - Rhabdomyosarcoma
- **Results are under NDA ;)**

Update in late 2021

- **New problem emerged**
  - early isopentane bath induce a heavy deformation of cells on HES (can’t identify osteoblasts from osteoclasts!)
- **Looking for a solution**
  - Use an alternative embedding that does not alter cells for staining
  - 10x Genomics released **Visium FFPE** !

10x Genomics Visium *FFPE* Spatial Expression Lab Experiment

Because on frozen samples, it was way too easy ...



A look at CG000408 protocol from 10X

FFPE-specific difficulties

- Samples quality unlinked to the age of FFPE block, but to the quality of their processing (reagents used, wait time before inclusion) and conservation (T°C variations, number of rehydratations, ...). You can't know this by ocular inspection...
- Grey plastic cassette does not handle well a heavy heating step : deformation, leakage !
- Frozen Visium = 1 full day of sample prep, can freeze the slide for long periods
- FFPE Visium = 2 full days, can store the slide in dehydrated container for 2 days max

FFPE+OS-specific difficulties

- Included biopsies are really small (~ 1 mm wide)
- Included samples are already de calcified, most often using an acidic protocol (faster than EDTA), incompatible with Visium FFPE
- None of our samples did adhere to the test slide (but all did on the real one!)
- Many washing steps during deparaffinization (after HES) : sections moved (can't be mapped to image !)

Latest news :

After 4 attempts, first library ready for sequencing !!

Acknowledgements

+ Nathalie Gaspar, MD, PhD (Pediatric oncologist)
+ Antonin Marchais, PhD (Bioinformatics project manager)
+ Doris Lebeherec (PETRA)
+ Nicolas Signolles (PETRA)
+ Maria Eugenia Marques da Costa, PhD (Biologist)
+ Maela Francillette (Genomics facility)
+ Hanane Boudhouche, Graduate student (Bioinformatics facility)

A message from Antonin :

WE ARE HIRING !

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Credits

- Lab experiments illustrations : 10x Genomics manuals :
  - CG000239_revA
  - CG000240_revB
  - CG000241_revB
- Osteosarcoma analysis : Gustave Roussy Pediatrics Bioinfo Team
- OS skeleton image : Wikipedia
- OS survival curve : ResearchGate
- Xenome Venn : Xenome publication (https://doi.org/10.1093/bioinformatics/bts236)