Q&A Hybrid Day 3 (online)

Talk 1 - Exploring Host Factors in Metastatic Progression of Early-Onset CRC: A Spatial Perspective - Raquel Pérez Mateo

Q: In the single cells to inject in mice, do you check if there are additional mutations from the ones you would expect?

A: No, we do not check for additional mutations on the injected tumor cells. Additional mutations are of course possible to gather through prolonged culture. But even if that's the case, it shouldn't influence the age-dependent perspective as the same cells are injected into both young and old mice.

Q: When checking for the immune cells in the metastasis, did you check any kind of ratio (metastasis vs surrounding tissue)?

A: We have relative abundances for metastasis and surrounding tissue, so in case of interest one can compare the abundances between ages, and between ROIs

Q: Does your panel include cancer stem cell markers? If so, did you evaluate the number and activity of those cells in the young compared to the old ones?

A: We evaluated the stem cell number via IHC, with no visible differences between ages.

Q: You showed similar metastasis in organoids between young and old. Would it be possible to use this setup and test some of these metabolic pathways (e.g. supplementation)?

A: One could do that! But we believe if you keep the organoids for long times in vitro they lose their phenotypic differences. We are currently trying to do some metabolic experiments with cells that have just been excised from the metastases, as we believe they will keep their phenotype for a few hours.

Q: In the organoid experiment, you measured the size and concluded that there is no difference. However, from your pictures it looks you may have much lower organoid forming efficiency in the old?

A: Yes! We need to repeat that experiment to make sure it is not a false positive, but we do see lower forming efficiency in the old - which interestingly is not related to proliferation capacity of these cells, nor to stem cell proportions.

Talk 2 - Using Spatial Transcriptomics to Determine the Memory Signature - Camin Dean/Hanna Dubrovska

Q: Could you explain, what's synapse weakening? What's the difference between weak and strong synapses? How do you measure the strength of synapses? What causes synapse weakening?

A: In the hippocampus synapses need to be very plastic (able to adjust between strong and weak) to encode memories and also to forget irrelevant information depending on experience, so there is active weakening of synapses (often by internalization of glutamate receptors) in addition to active strengthening. Synapse weakening can also be induced, by a low frequency train of stimuli, which causes LTD (long-term depression) of synapse strength. This can be measured electrophysiologically by giving a short stimulation and recording the response to see if it is smaller after LTD induction, for example.

Q: There is evidence regarding the role of Tau transcripts/protein in cytoplasmic cell condensates in Alzheimer disease. Are you focusing only on nuclei transcripts or are you also considering this RNA/protein enriched content inside this kind of membraneless organelles which control limited proteolysis among other putative processes?

A: At the moment we are focusing on nuclei, just because neurons have long fragile processes and we were afraid we would lose single cells, or lose particularly fragile cell types preferentially. But this is a super-interesting question, also because there can be local translation of mRNAs in neuronal processes and synapses during synapse strengthening that we would like to detect. One could maybe purify cellular compartments and sequences to compare to data from nuclei, for example... I'm not sure if you might also be able to see differences with high definition spatial sequencing in some cases? Although this could be complicated by where the non-nuclear RNA is and if it can be separated from being near a cell.

Q: You mentioned that the hippocampi are incubated with the CSF. Did you have a look at whether there are also changes in the CSF? I was wondering if this model could also be used to analyze the CSF in AD.

A: We did not look yet at the biomarkers present in ACSF, but that would be definitely interesting to look if any of them are present there, as well for the biomarkers present in patient CSF.

Q: Will the spatial images be available publicly? Also where can i get project specific images (say for training CNN algorithm) apart from 10x Genomics (or any other popular sources)?

A: Yes! We will make sure to upload everything we have on public access platforms once we publish the related data.

Q:I was wondering if you have experience with PFA fixed frozen brain/hippocampus sections to be processed with RNAseq as you did. I would be interested in conducting more or less the same technique for genes of interest in a mouse model, but all my mouse samples are embedded in OCT. Did you try it and was it not good enough? Or is there another reason you proceeded with FFPE sections? (I am referring to the experiment with the punches and the LTP)

A: We are in the process of doing single cell RNAseq on nuclei purified from PFA fixed brain/hippocampus using the 10x Flex system (where we could perfuse a mouse brain after a memory task and use half the brain for spatial seq and half for single cell seq, for example). So far the nuclei look good but we're awaiting test sequencing runs. We've also purified nuclei from PFA-fixed, paraffin-embedded brain slices (by de-paraffinizing them first) and this also yields nice nuclei, so maybe purifying from OCT would work too?

Q: To study Alzheimer's Disease (AD), you only need to study Microglia cells in scRNA, is this true?

A: There's increasing evidence that microglia play a large role in AD, so this is an intense area of research (and one of the reasons we would definitely like to have human microglia in our network for cognitive drug screening). But it's still unclear when and where AD starts precisely, in terms of which cell-type is driving versus reacting to the disease, so ideally one would examine multiple cell types (both for cause and effect).

Q: Do you notice that there appear to be two distinct types of granule cells? For instance, could it be that one type expresses c-Fos while the other expresses NPAS4?

A: We actually haven't looked in detail at this yet, but we will! There's also some evidence in the engram field that Npas4 is a marker of mouse activity (since some people only see it in enriched environments where mice are actively moving or in cages with running wheels). So it's certainly possible that different populations of cells have different immediate early gene transcriptomes.

Talk 3 - Combining unbiased single-cell RNA sequencing and spatial transcriptomics to study zebrafish brain regeneration - Juan Sebastian Eguiguren Palacios

Q: I Was wondering, you showed new cell types are coming up upon lesions. Do you also see cell types disappearing?

A: It is difficult to conclude that a cell type is truly disappearing based on the single cell dataset from the lesion timepoints. This is because the cell type might still be present in the brain but not survive the tissue dissociation and FACS protocol. Additionally, what I observe in my dataset is that the cell types often remain but show very different transcriptomic profiles, so they appear in a different region of the UMAP as a distinct cell state rather than disappearing completely.

Q: Regarding one of your last points, Where you would like to transfer data from single cell to spatial. Were you able to benchmark or try several algorithms? And do you have any idea which one might work best?

A: I did not benchmark different algorithms because it was challenging to define a clear way to compare their results or performance. For this initial label transfer approach, I followed a method similar to what was used in the mouse transcriptomic and spatial atlas from Yao et al. 2023 (https://doi.org/10.1038/s41586-023-06812-z).

Follow Up Q: I was asking because a lot of algorithms actually are conceptually doing the same thing as you just mentioned. You can do it with harmony, sorato is doing the same. Tangrum is a bit different. That is one of my questions, have you ever tried that?

A: Yes, I used Harmony for integrating the two datasets and then applied a simple k-nearest neighbors approach for label transfer from the single-cell to the spatial data. I am also currently testing Tangram but do not have results yet.

Q: When you overlay the in-situ and single cell data, did you look at the number of cells in the cluster and check the proportions between the technologies? If you lost some cells in the single cell, it could affect your data.

A: I have not checked the proportions yet. The Xenium panel was designed based on the single cell data, so I can only detect in space the cell types and states that were already identified in the single cell dataset. It's indeed a good point to keep in mind.

Q: This question comes from never having explored such datasets. For integration of single cell with spatial, does one have a probability of a cell in spatial being a part of a single cell cluster? or is it a yes/no kind of thing? If it's probabilistic, does this have any weight when performing gene expression imputation?

A: It does have weight when performing gene expression imputation. Since the spatial dataset measures only a limited number of genes per cell, the imputation relies on the probability of a spatial cell belonging to a single cell cluster. The Xenium panel was designed using the top marker genes for each cell type and state identified in the single cell data, so I

thought that these should be sufficient to represent the single cell clusters in the spatial context.

Talk 4 - Spatial transcriptomics: strengths and weaknesses of in situ and sequencing-based platforms - Eduard Porta

Q: With Xenium resolution, could we detect transcripts in the cytoplasm or would we need an expansion microscopy combination?

A: Yes, it has nm resolution so you can quantify the distance between transcripts and different parts of the cell

Q: When looking at the distance of epithelial cells to immune cells, did you look at some markers of immune activation? I would assume immune cells next to cancer cells would be more active but, I may be wrong.

A: It's a very good point! We looked at some markers, but this was the multi-tissue panel (n = 377 genes), so we could not look at this question in depth.

Q: In the different epithelial neighbourhoods, was there any measured difference in the cancer cells themselves across the neighbourhoods?

A: That's a good question that we have not looked at! We'll try it now, thanks!

Q: In the neighbourhood analysis, specifically when comparing "richness" in cell types, do you think it would be interesting to apply concepts & approaches that exist in ecology (e.g. measures of diversity, evenness, etc.)

A: Absolutely! Look at Carlo Maley's papers!

Talk 7 - Multiscale topology for analysing spatial transcriptomics - Heather Harrington

Q: I have a question regarding cell segmentation. All of what we have heard is about cell segmentation for Visium HD. Is there a cell segmentation package/framework for Visium 10x v1?

A: Most of the time people think of deconvolution for Visium, however some tools can try to map the signal to single cell level. Tangram was one of the first that I remember, and then later some ideas that try to infer super-resolution of Visium emerged. Xfuse was an early

one, but there are now a few more. I can't remember exactly, but perhaps also check out iStar, Hist2Map for more examples.

Talk 8 - The problems of cell segmentation and how to perform cell segmentation free analysis - Naveed Ishaque

Q: Is Sainsc suitable for Visium 10x v1?

A: Unfortunately not. Sainsc was made for 'high' resolution methods. The lowest resolutions we have worked with is Visium HD (2 um)

Q: Xenium uses DAPI + nuclear expansion + density-based segmentation,while CosMx uses DAPI + membrane markers + cell type-specific markers + algo segmentation. Using cell type-specific markers for segmentation can increase precision, but it also risks missing other cell types. Given this trade-off, why aren't we moving more toward cell type-specific marker based segmentation?

A: Good (tough) question. Disclaimer - I am not a segmentation expert.
My feeling is that there probably isn't a pan-cell type membrane stain (... otherwise we would have found it by now). Including cell type specific membrane stains requires prior knowledge, which I don't think that we have right now. Please don't quote me on this.
.... cell-segmentation-free doesn't have this issue

Q: The segmentation results after improvement can be still not perfect for some cells. What would you recommend for such cells which still did not separate well from each other? Does it make sense just to exclude them from subsequent analysis? And are there any approaches of their detection similar to that which exclude doublets from droplet-seq data?

A: Again, a good (tough) question. Honestly, I don't know. I generally tell people in my group to annotate everything (including QC metrics), do not filter first, cluster, then see how QC issues affect clustering, and then investigate which QC metrics confound clustering/other analysis. The limitation with this advice is that I don't think we actually know what constitutes good or bad quality - for example, we have kinda ignored 3D overlaps until recently. If you check out the overly biorxiv (Tiesmeyer et al 2024, 2D or not 2D...) our last supplemental figure (Fig S16?) shows that if you remove cells with high potential overlap score you get a much cleaner UMAP. A paper from Peter Karchenkos groups also investigate this from the 2d perspective (I can't remember the name, but it was out on biorxiv in Jan 2025, titled something like `segmentation errors...`) investigates this, and provides a couple of Jupyter Notebooks to allow users to investigate this in their own data.

Talk 9 - Spatially Aware Omics Data Alignment and Integration using VoltRon - Artür Manukyan

Q: Could we do image registration with an atlas image to define some regions in the brain?! / maybe allen reference / IF image associated with Visium v1.

A: This would of course depend on whether the two assays that you are trying to align are from the same tissue section or adjacent sections. That is, it doesn't matter if the data is either Visium (spot-based) or Xenium, MERFISH, CosMx (imaging, FISH-based). Thus, any combination of DAPI and H&E image would do. However, I am not sure if samples in Allen atlas include either DAPI or H&E images.