Spatial transcriptomics – a new frontier in atherosclerosis research?

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Atherogenesis involves a complex interplay between multiple immune and stromal cell subsets and cell states situated within specific tissue regions[1]. The composition of atherosclerotic lesions is crucial in determining whether and how an atherosclerotic plaque causes manifestations of disease[2-6]. An increasing appreciation of tissue and cellular heterogeneity has opened the way to a much more granular way of thinking about culprit lesions. As such, there has been an explosion in studies using single cell biology techniques such as cytometry by time of flight (CyTOF) mass cytometry and single-cell RNA sequencing (scRNAseq) to reveal the true intricacy of the cellular heterogeneity of human atheroma[2-6]. Whilst these techniques have significantly advanced our understanding of the cell populations present in atherosclerotic lesions, they rely on tissue dissociation and information on the relationship between cellular states and location is lost.

The advent of spatial 'omics' technologies has provided the opportunity to examine the heterogeneity of atherosclerotic plaques in unprecedented detail. Numerous different spatial 'omic' platforms have been developed (reviewed in [7]) including the use of metal-tagged antibodies against proteins of interest such as imaging mass cytometry and multiplexed ion beam imaging (MIBI) or spatial transcriptomics such as GeoMx, CosMx, Visium and Xenium. All these techniques allow both tissue structure and cell phenotype to be simultaneously examined with varying degrees of phenotyping, sequencing depth and cellular resolution.

In this issue of Arteriosclerosis, Thrombosis and Vascular Biology, Gastanadui *et al.,* report spatially resolved transcriptional analysis of human coronary atherosclerotic plaques[8]. Using the GeoMx digital spatial profiler (DSP) platform, the authors performed a number of comparisons between different regions of the plaque (tunica intima, tunica media, atheroma) and cell types (defined by immunofluorescent staining with antibodies against smooth muscle myosin heavy chain 11 and CD68) in autopsy-derived "stable" and "unstable" human coronary atherosclerotic plaques to reveal spatial and cell-specific transcriptional profiles (Figure 1). Coronary lesions were categorised as "stable" and "unstable" on the basis of morphological post-mortem data. In total, the authors imaged 61 regions of interest (ROI). Initially the authors compared the 25 ROI from stable plaques to the 35 ROI from unstable plaques to investigate global changes between the disease states. In total 107 genes were differentially regulated between the two plaque types. Pathway analysis revealed the importance of inflammation in unstable plaques with pathways associated with IFNg, TNFa and cytokine signalling all upregulated.

One cellular focus of the authors was smooth muscle cells (SMC), that are known to proliferate, migrate and undergo phenotypic transformation during atherogenesis (reviewed in [9]). Gastanadui *et al*. identify spatially-resolved smooth muscle cell changes between "stable" and "unstable" patients. They observed modest changes in gene expression in medial SMC from unstable versus stable plaques, with upregulation of

genes including *KLF4* (SMC phenotype switching), *MYDGF* (cell proliferation) and *HSPH1, SCO2 and TXN2* (cell stress responses). A more marked shift in transcriptional phenotype was observed in intimal SMCs. A total of 428 genes were upregulated, including those associated with pro-inflammatory, prothrombotic and cell stress pathways, in intimal SMC from unstable compared to stable plaques. In contrast these cells exhibited lower expression of genes such as *ACTA2* and *TAGLN* which are more associated with contractile SMC. These results highlight the plasticity of intimal smooth muscle cells and their role in unstable atherosclerosis.

The authors went on to examine 'macrophage-like' cells. CD68+ cells were identified to either display a classic myeloid phenotype or a 'hybrid' phenotype with shared transcriptional signals with myeloid, SMC and endothelial cell types. Interestingly, these two cell types were traceable to two distinct plaque regions: the myeloid CD68+ cells located peripherally (presumably in the shoulder region) and the 'hybrid' CD68+ cells located in the plaque lipidic/necrotic core. Furthermore, the calcification status of plaques was shown to significantly alter the transcriptional profile of SMCs and CD68-positive cells. These cell types are known to be important in atherosclerotic plaque calcification[10-12].

Single cell RNA sequencing has identified a multitude of different cellular subsets within atherosclerotic plaques, revolutionising our views on the disease. It has additionally revealed the importance of accurate cell annotation. The study by Gastanadui *et al.,* signals a first step in the right direction, by mapping specific cell programming to specific intraplaque locations. While we often focus on cellular transformation in the fibrous cap, this work attracts attention towards the plaque core as a site of intense cellular transformation with an overlap of macrophage, smooth muscle cell and endothelial cell gene expression in a hybrid population. The authors acknowledge that their study relies on cellular markers that are non-exclusive (CD68 is not a specific macrophage marker and SMCs that have downregulated smooth muscle myosin heavy chain may be missing from their analysis). As a result, we lack information on the true identity of these cell communities, and we have no information on their lineage and their origin. Lineage tracing options in humans are limited and thus we are heavily reliant on experimental models.

The study by Gastanadui *et al*., exemplifies some of the challenges the cardiovascular field is facing in the adoption of these new spatial transcriptomic technologies and highlights the limitations on the type of information that can be captured by these technologies. Nanostring's GeoMx DSP system examines the transcriptome in selected regions of interest in conjunction with standard histological stains or immunofluorescent/*in situ* hybridisation staining. Combined with the deep whole transcriptome profiling afforded by this technique, GeoMx is well suited to focussed, hypothesis testing experiments such as Gastanadui *et al*., have performed, comparing different tissue areas and disease types. Similarly, Visium from 10x, allows whole genomic transcripts to be captured[13]. Neither GeoMx nor Visium allow resolution of the transcriptome at the single cell level. Nanostring and 10x have advanced their technologies with CosMx and Xenium that can profile whole sections at a single cell/sub cellular level but they can only interrogate a more limited transcriptome of

around 1000 transcripts compared to the 18,000 of whole transcriptome profiling. Visium HD is following, combining whole transcriptome and single cell resolution.

In the field of cardiovascular disease there are additional challenges which, although not unique to spatial transcriptomics per se, may hinder the potential of the technology. Fresh human coronary arteries are hard to obtain and thus most studies, including the one by Gastanadui *et al*., discussed here, rely on post-mortem samples which may suffer some protein/transcript degradation before the samples are processed in the lab. It is likely that each technique will give us a different picture of atherosclerotic plaques. scRNASeq and CyTOF Helios, based on tissue dissociation, revealed for the first time the true abundance of T cells in human atheroma, often underestimated using traditional immunohistochemistry[2-4] . Spatial transcriptomics analysis like in this study may give us a different estimation of the cellular composition.

Atheroma are eccentric and non-uniform and they are likely to be even more heterogeneous than we think. Revealing and understanding this heterogeneity will aid the identification of patients that are at risk to the acute complications of the disease. Spatial transcriptomics in cardiovascular disease is in its infancy and time will tell if it can truly deliver enough data about the composition of atherosclerotic plaques to silence our questions. However, it may just allow the "needle in the haystack" to be found.

Figure Legend

Figure 1: GeoMx reveals spatial differences in the transcriptome of cells in atherosclerotic plaques. Comparisons between regions of interest from stable and unstable human coronary artery atherosclerotic plaques revealed increased expression of proinflammatory genes including *CXCL4, IFNGR2,* and *CCL5* and reduced *IGFBP6* expression (associated with healthy smooth muscle cells) in unstable plaques. Medial and intimal smooth muscle cells (SMC) also exhibit different transcriptomes depending on whether they derive from stable or unstable atheroma with cells from plaques more vulnerable to rupture expressing higher amounts of pro-inflammatory, proliferation and cell stress response associated genes and reduced markers of contractile SMCs. Within unstable plaques, 'peripheral' CD68-expressing cells were associated with a myeloid transcriptome and gene set enrichment analysis linked these cells with the innate immune system, TNFa signalling and signalling by interleukins. In contrast, CD68-expressing cells in the core of the plaque displayed a 'hybrid' transcriptional phenotype with a transcriptional phenotype overlapping with myeloid, SMC and endothelial cells and pathway analysis accordingly revealed these cells are associated with pathways such as extracellular matrix reorganisation and endothelial-mesenchymal transition. Figure created in BioRender.com

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