**Uncovering tumor-reactive ectopic GC in lung adenocarcinoma using Stereo-XCR-seq**

**Abstract**

T/B cell receptors (T/BCR), coordinating antigen-targeting immune response, plays crucial roles in anti-tumor immune response. Tracking T and B cell clonal evolution in situ at single-cell resolution is essential for understanding adaptive immune responses. To address the lack of tools for in situ single-cell T/BCR (XCR) sequencing tools, we developed Stereo-XCR-seq, an efficient method for retrieving and sequencing TCR and BCR from Stereo-seq cDNA libraries at subcellular resolution. Stereo-XCR-seq enables high-fidelity, unbiased recovery of XCR sequences alongside spatial transcriptomics, facilitating the identification of heterogeneous lymphoid aggregates with distinct clonal activities in situ. Applying Stereo-XCR-seq to 11 lung adenocarcinoma (LUAD) patient specimens, we uncover that IgG+ plasma cell aggregates function as ectopic germinal centers (GCs) to select tumor-reactive clones from immature tertiary lymphoid structures. The presence of these IgG+ plasma cell aggregates in LUAD indicates an improved anti-tumor immune response. Collectively, Stereo-XCR-seq enables in situ single-cell profiling of T and B cell clonal activities and their interactions with local microenvironments, offering a versatile tool for dissecting immune–stromal interactions across human diseases.

**Introduction**

The immune repertoire represents the diverse T-cell receptors (TCRs) and B-cell receptors (BCRs) that enable the immune system to recognize and respond to a wide range of antigens1. This diversity arises during T and B cell maturation through variable, diversity, and joining (VDJ) recombination, producing unique and heritable TCR and BCR sequences2. Upon antigen recognition, T and B cells undergo clonal expansion, a hallmark of immune activation that can be profiled through TCR and BCR sequencing. The composition and organization of the immune repertoire differ significantly across tissue contexts3-6, highlighting the importance of profiling immune repertoires within their spatial environment. In addition, spatial profiling of immune repertoires offers the potential to uncover how antigen-specific immune responses are orchestrated within tissues, providing critical insights into antigen recognition and immune system development7-9. Therefore, the development of spatial immune repertoire technologies is a crucial step toward advancing our understanding of basic immunology and unlocking new clinical applications10.

Various toolkits have been developed to capture spatial immune repertoires *in situ* alongside transcriptomics, with notable examples including Slide-TCR-seq/Slide-tags11, 12, Spatial VDJ13, BCR-MERFISH14 and SPTCR-seq15. Slide-TCR-seq and Slide-tages11, 12 leverages the Slide-seq platform for transcriptomics profiling and employ multiplexed PCR to enrich XCR transcripts. These methods enable the capture of both Coordinate ID (CID) and complementarity-determining region 3 (CDR3) sequences using short- or long-read sequencing. However, its reliance on pre-designed PCR primer panels introduces potential biases stemming from prior knowledge. In contrast, SPTCR-seq15 and Spatial VDJ13 utilize the Visium platform for transcriptomics profiling and incorporate probes targeting the constant (C) region to enrich TCR transcripts through hybridization. While these approaches broaden accessibility to spatial immune repertoire profiling, they suffer from low efficiency, are time-consuming, and rely on hybridization-based enrichment methods that may limit sensitivity. Furthermore, these methods were designed for an earlier generation of the Visium platform with a spatial resolution of 100 µm, which lacks the subcellular precision now achievable with the latest advancements in spatial transcriptomics technologies.

Spatial transcriptomics has made significant strides, with commercial platforms now achieving subcellular resolution and high-throughput gene detection. Sequencing-based spatial transcriptomics technologies, such as Stereo-seq v1.316 and Visium HD17, have achieved resolutions of 0.5 μm and 2 μm, respectively. The rapid progress in these technologies underscores an urgent need to integrate them with spatial immune repertoire sequencing, offering a unique opportunity to gain deeper insights into the immune repertoire landscape and its functional organization within tissues7-9. However, significant technical challenges remain in capturing TCR and BCR sequences *in situ* at single-cell resolution. First, the physical distances between VDJ regions and barcodes within transcripts exceed 1,000 base pairs, making them unsuitable for high-throughput short-read sequencing. Second, TCR and BCR transcripts are extremely rare, representing less than 0.01% of the cDNA library, which poses a challenge for low-throughput single-molecule long-read sequencing.

We recently developed Stereo-seq16, a spatial transcriptomics technology that integrates DNA nanoball (DNB)-patterned arrays for capturing poly-A mRNA at 500nm resolution, along with a rapid and efficient sequencing method called single strain circle DNA PCR (sscirPCR) to retrieve TCR and BCR transcripts from the Stereo-seq cDNA library. This method, which we named Stereo-XCR-seq, enables unbiased immune repertoire profiling at single-cell resolution with paired-chain information in various tissues with robust performance. By applying Stereo-XCR-seq to 11 LUAD specimens, we identified ectopic GCs, structures that are transcriptomically and clonally distinct from canonical tertiary lymphoid structures (TLS) and enriched for tumor-reactive lymphocytes. These ectopic GCs appear to arise in response to antigenic stimulation and are associated with an enhanced antitumor immunity. Integrating spatial transcriptomics with spatial clonal dynamics, we delineated a maturation trajectory from immature TLS (iTLS) to ectopic GCs along the B/plasma cell axis. Notably, this maturation process can be disrupted by terminally differentiated *POSTN*+ fibroblasts. Overall, Stereo-XCR-seq enables high-resolution, *in situ* characterization of T and B cell clonal activities, providing novel insights into the spatial organization of immune responses.

**Results**

**Stereo-XCR-seq achieves high-fidelity spatial immune repertoire mapping with robust performance in disease tissues**

To acquire an unbiased spatial immune repertoire in parallel with subcellular-resolution transcriptomes, we developed Stereo-XCR-seq, a method that integrates Stereo-seq16 with targeted retrieval of full-length V(D)J sequences (**Figure 1a, Methods**). Firstly, double-stranded Stereo-seq cDNA was heat-denatured at 95 °C and rapidly annealed to a splint oligonucleotide, yielding a single-stranded circular intermediate (sscirDNA) in which the barcode resides at the 5′ end (**Figure 1a**, step 1-2). We then sealed the nick using T4 DNA ligase, typically 10–30 % of the input cDNA was converted to circles. Residual linear DNA and excess oligos were removed by exonucleases I and III, eliminating non-specific amplification in subsequent steps (**Figure 1a**, step 2). XCR transcripts were then amplified from the sscirDNA pool by PCR primers directed to the constant (C) region (sscirPCR; **Figure 1a**, step 3). In contrast to multiplex V-gene PCR, this constant-region strategy is repertoire-agnostic and yields unbiased amplicons in which barcodes and V(D)J segments are in the middle and flanked by truncated C regions. These amplicons were re-circularized for paired-end 150-bp (PE150) sequencing: read 1 captures the CDR3, while read 2 contains the coordinate identifier (CID) and unique molecular identifier (UMI) (**Figure 1a**, step 4). The paired sequencing data were then subjected to barcode mapping and clone assembly respectively to examine the spatial distribution of each clone read (**Figure 1a**, step 5). Noteworthy, we also provided an optional fragmentation pipeline for step4 to generate fragmented V(D)J reads, which could be further assembled as full-length V(D)J reads in a UMI-supervised manner (**Supplementary Fig.1a**).

Although long-read platforms recover complete V(D)J-C sequences, their base-level accuracy is limited. Conversely, short reads provide high fidelity but only partial V(D)J coverage. Previous studies have utilized both sequencing methods to mitigate the shortcomings of each method18, while the readouts of the two methods were not directly compared before. In this study, we therefore compared nanopore-based long-read sequencing with DNA nanoball-based PE150 short reads for spatial XCR-seq. Long reads exhibited significantly lower quality (**Supplementary Fig.1c**) and produced 39.9 % non-functional T/BCR sequences versus 6.9 % for short reads (**Supplementary Fig. 1d**). In contrast to the plateaued curve of short-read sequencing, increasing long-read sequencing throughput led to ever-increasing CDR3 clone types per coordinate in saturation test, indicating accumulated false positive readouts by sequencing errors (**Supplementary Fig.1e**). These results strongly suggested short-read sequencing result is imperative to generate high-fidelity clonetype information in spatial immune repertoire. Through the fragmentation pipeline, we reconstructed full-length V(D)J with >90 % coverage of CDR1-FR4 in Stereo-XCR-seq data (**Supplementary Fig. 1e**). Compared to previous studies that utilized the advantages of both sequencing methods18, our method provides a short-read-only strategy that enhances the fidelity of the sequencing results.

Applying sscirPCR to multiple Stereo-seq cDNA libraries, we effectively retrieved XCR reads from multiple spatial cDNA libraries with consistent XCR/total raw reads ratios at 24.66~40.38% (5.3~13.61 log2fold changes over original Stereo-seq cDNA library, **Figure 1b**). Compared to previously released spatial T/BCR retrieving strategy, such as multiplexed PCR12, 19 and probe hybridization15, sscirPCR showed substantially higher efficiencies in XCR reads enrichment (**Figure 1c**). To demonstrate its robust performance, we tested sscirPCR on a wide range of tissue types, including NSCLC, gastric, bladder, renal, colorectal, lymph nodes with metastatic esophageal cancers, autoimmune hepatitis, and rheumatoid arthritis. On average, we assembled 2,145 TCRβ and 4,659 IgH clonetypes per sample library (**Figure 1d**), demonstrating robust performances. Compared with previously disclosed spatial immune repertoire datasets, Stereo-XCR-seq exhibited a strong advantage in the CDR3 clone type counts20 (**Figure 1d**). In addition, the retrieved XCR clone reads exhibited strong consistent spatial distribution along with original spatial transcriptomic T/BCR gene expression (**Figure 1e**). The existence of T cells and plasma cells in highlighted T/BCR-expressing FOVs were further validated by immunofluorescence staining (**Figure 1f**). Importantly, Stereo-XCR-seq method also exhibited efficiencies in FFPE-embedded samples (**Figure 1g-h**). Although the FFPE XCR reads degraded severely, leading to a shorter CDR1-to-CDR3 coverage (**Supplementary Fig.1f-g**), Stereo-XCR-seq could still deliver the CDR3 region for clone definition, thus extending the application in clinical studies using retrospective FFPE-embedded samples. Collectively, the high-fidelity readout, enhanced retrieving efficiencies, unbiased enrichments, and robust performances in different tissues under varied preservation conditions highlights the application potential of Stereo-XCR-seq in immune disorders.

**Stereo-XCR-seq profiles lymphocytes at single-cell level with paired chains**

Effective anti-tumor immunity requires clonal expansion of tumor-infiltrating lymphocytes (TILs) 21, accurate quantification of clone sizes is therefore essential for inferring tumor reactivity. To achieve single-cell resolution, we incorporated ssDNA staining into the Stereo-seq workflow, capturing high-resolution nucleus staining images along with spatial transcriptomes (**Supplementary Fig.2a)**. Using cellbin2 software 22, we performed image-based cell segmentation (**Supplementary Fig.2b**). By manually checking of 30 field of views (FOVs) randomly selected from three LUAD samples (10 FOVs each), we obtained authentic segmentation rates at ~94%, ambiguous segmentation rates at ~6%, and negligible missing cells (**Supplementary Fig.2c-d**), supporting a credible cell segmentation through this pipeline. Existing spatial immune-repertoire methods typically have a resolution at 100 µm (center-to-center distances, eg., Spatial VDJ, SPTCR-seq)23, while spots at this resolution could contain varied number of lymphocytes (**Figure 2a**). Quantifying TCRβ⁺ and IgH⁺ cells revealed a mean of 9.16 TCRβ⁺ and 26.6 IgH⁺ cells per 100µm spot (**Figure 2b**), suggesting a mixture of different clonal types in a coarse-grained resolution. From LUAD P1, we randomly selected 10 pairs of FOVs from the Stereo-XCR-seq section and the serial section stained by CD3E/CD20/CD138. The quantification results of 100µm XCR+ spots and XCR+ cell bins were directly compared with the ground truth in the serial section. As a result, the number of cell bin exhibited similarities with the ground truth, whereas the number of 100µm XCR+ spots showed deviated quantification (**Figure 2c**). The above result suggests that Stereo-XCR-seq provides an authentic clone size counts with single cell resolution.

After generating cell masks, we assigned both transcriptomic reads and XCR clone reads into each cell mask to generate paired transcriptomic profile and clonal information at single cell resolution. To validate the coordinates of the retrieved clonal reads, we performed co-localization analysis by checking clonal reads and transcriptomic reads within each cell (**Supplementary Fig.3a**). We noticed that around 73% IgH+ cells were supported by *IGH* gene expression, whereas only 4% of the TCRβ+ cells were supported by *TRBC1/2* gene expression (**Supplementary Fig.3b-c**). We ascribed this discrepancy to the significantly lower original TCR mRNA expression abundances (**Supplementary Fig.3d**). By checking other published spatial transcriptome of LUAD (**E-MTAB-13526**)24, 25, we observed a consistently lower abundances of *TRBC1/2* than *IGH* genes in both data types (**Supplementary Fig.3e**), suggesting that low *TRBC1/2* mRNA abundances might led to signal dropouts in high-throughput sequencing data. These extra TCRβ+ reads could be either authentic signals restored by retrieval or false positive signals from experimental procedures. To examine this, we next checked if TCRβ+*TRBC1/2-* cells could be supported by *TRAC* mRNA, TCRα clone reads and CD3E protein expression. Although lacking *TRBC1/2* expression, TCRβ+*TRBC1/2-* cells showed higher expression of *TRAC* over TCRβ-*TRBC1/2-* cells (**Supplementary Fig.3f**). In addition, around half of the TCRβ+ cells harbored TCRα chains, resulting in 12408 T cells with paired α-β chains (**Supplementary Fig.3g**). Furthermore, we checked CD3E expression at protein level by multi-immunofluorescent staining (mIF) using serial tissue sections. Particularly, we selected 2 types of fields of view (FOV) to assess the CD3E expression, including FOV1 with concordant expression of *TRBC1/2* and TCRβ, and FOV2 with discordant expression of *TRBC1/2* and TCRβ (**Supplementary Fig.3h**). Consistent expression of CD3E in both FOVs (**Supplementary Fig.3i**) corroborated the authenticity of retrieved signals, suggesting an enhancement of TCR signals by Stereo-XCR-seq.

Equipping T cells with chimeric antigen receptor (CAR) or elaborately screened TCR endows T cells with tumor-targeting cytotoxicity in clinical practice26, 27, while this engineering requires information of paired chains. To explore the clinical translational potential of Stereo-XCR-seq, we compared resolutions at single cell level and 100 µm in resolving paired chains. By checking the co-localization of α-β and H-L chains in cell bins and 100 µm spots, we categorized the result to 1. ambiguous pairing with multiple different clone types, 2. unpaired chains with only one type of chains and 3. unique pairing with paired α-β or H-L chains (**Figure 2d**). In comparison to the 100 µm resolution, Stereo-XCR-seq provided significantly higher proportion of unique paired chains but rare ambiguous pairing (**Figure 2e**). We then ran pairing rate test in multiple LUAD specimens. Overall, Stereo-XCR-seq provided α-β pairing rates at 23% and H-L pairing rates at 5% on average (**Figure 2f**), covering 2259 T cells and 3301 B/plasma cells with paired chains per sample (**Figure 2g**). Combined with the compatibility to FFPE specimens (**Figure 1g-h**), Stereo-XCR-seq provides a possibility to acquire paired intratumoral T/BCR receptors in clinical specimens.

**Stereo-XCR-seq profiles B/plasma cells clonal activities along lineage differentiation**

B/plasma lymphocytes undergo somatic hypermutation (SHM) to develop antigen-binding affinity28, class switching recombination (CSR) to secrete effector-competent antibodies, and clonal expansion for massive antibody secretion29. These three clonal activities were imperative for B/plasma cells to achieve immune surveillance. We then sought to evaluate the capability of Stereo-XCR-seq in resolving each clonal activity. For clonal expansion, we counted cells assigned to each clone family to measure the clone sizes (**Figure 2h**). In LUAD P1, 437 IgH clones were found with ≥10 cells (eg., clonal 1352), while 2061 IgH clones were found as non-expanded clones (**Figure 2i**). For CSR, we defined a CSR event by observing 2 or more isoforms in a segmented cell bin (**Figure 2j**). Averagely,11228 CSR events could be identified in 11 LUAD tumors (**Figure 2k**). For SHM, we mapped clone reads to the built-in set of reference provided by MIXCR30 and calculate the mutation frequencies. Overall, we observed a mean mutation percentage at 8.78% in 11 LUAD specimens (**Figure 2l**), a similar percentage to a recent study profiled B/plasma cells at a pan-cancer scale31. Employing spatial density analysis, we identified mutating hotspot supported by similar BCR CDR3 amino acid sequencies in this FOV (**Figure 2m**). Combining the above capacities together, Stereo-XCR-seq could visualize every single clonal activity of interests when tracing the B/plasma cell lineage differentiation (**Figure 2n,** left, orange bold lines). Taking IgH clone family 375 for instance, the clonal expansion of subclone 2 (clone size at 844), mutation from subclone 2 to subclone 15, and the CSR event from subclone 15 to subclone 21 could be inferred within a single spatial plot (**Figure 2n, right**), demonstrating the capability of Stereo-XCR-seq in deciphering B/plasma cells clonal activities at single cell resolution.

With the above advancements, we benchmarked Stereo-XCR-seq against multiple existing methods, including Slide-TCR-seq11, Slide-tags12, Spatial VDJ13, and SPTCR-seq15 and BCR-MERFISH32 (**Supplementary Fig.4**). Stereo-XCR-seq and BCR-MERFISH are the only 2 platforms compatible for FFPE samples and provides single cell resolution based on cell segmentation. Stereo-XCR-seq and Spatial VDJ provides both TCR and BCR clone information, while Stereo-XCR-seq further covers γδT cells. Further, Stereo-XCR-seq and SPTCR seq unbiasedly recover XCR reads from original library. Overall, Stereo-XCR-seq provides single-cell resolution, paired immune receptor chains, unbiased enrichment, improved efficiencies, and high-fidelity readout in studying spatial immune repertoire.

**Stereo-XCR-seq reveals convergence of B/plasma cells from spatially discrete iTLSs in LUAD**

TLSs are ectopic lymphoid aggregates within tumor stroma that orchestrate anti-tumor immunity33, 34. The maturation of TLS is marked by the formation of GCs that generates antibody-secreting plasma cells within the follicular structures 35, 36. The pre-existence of mature TLS (mTLS) shows prognostic value for immunotherapy response, while only a minority of LUAD patients harbour mTLSs37-39. We randomly selected H&E staining images of 100 LUAD samples from The Cancer Imaging Archive40 (**Supplementary table1**). Among these 100 samples, 57 samples were identified with five or more TLSs, denoting an inflamed microenvironment. Yet only 7 of the cases were found with mTLSs (**Figure 3a-b**). The mean frequency of mTLS remained strikingly lower than that of immature TLS (iTLS) across the inflamed cohort (iTLS, mean frequencies: 0.26 vs 10.05, **Figure 3c**), falling well below reported objective response rates to immune checkpoint blockade in LUAD (35.9% 41 and 30% 42). Thus, the prognostic value of mTLS is confined to a minority of LUAD patients.

To dissect the role of iTLSs in LUAD, we applied Stereo-XCR-seq to 11 fresh-frozen LUAD specimens exclusively containing iTLSs (**Supplementary Fig.5a-d, table2**). Among these specimens, 6 tumors (LUAD P1/4/6/8/9/10) harbored multiple iTLSs. In total, we obtained 1098740 segmented single cells, covering 78560 T cells, 482702 B/plasma cells (**Figure 3d**). On average, 103 TCRα clones, 216 TCRβ clones, 2315 IgH clones and 751 IgK/L clones were in each tumor (**Figure 3e**). Unsupervised clustering43 (built in Omicverse44 ) of 25μm x 25μm bin spots (bin50) resolved 7 spatial clusters (**Figure 3f-g, Supplementary Fig.5c-d**): alveolar tissue (*SFTPC*+ *SFTPD*+), lung cilia (*SCGB1A1*+ *CAPS*+), plasma cell aggregates (*JCHAIN*+ *IGKC*+), iTLS (*CXCL13*+ *MS4A1*+ *CD3E*+ *CHST4+ IL33+*), myeloid cell-enriched region (*APOC1*+ *CTSB*+), immune-excluded stroma (*CXCL13- MS4A1- JCHAIN- COL1A1*+ *COL1A2*+) and tumor (*EPCAM+ KRT18+*). We applied Density-Based Spatial Clustering of Applications with Noise (DBSCAN, A density-based algorithm for discovering clusters in large spatial databases with noise)45 to these 6 tumors to partition spatially discrete iTLS clusters. As a result, we identified 3~10 spatially discrete iTLSs clusters in each tumor section (**Figure 3g, Supplementary Fig.5e**). By checking the shared IgH clones by top5 iTLSs in LUAD\_P1, we observed a minor clone sharing ranging from 6.15%~8.82% (**Figure 3h**), implicating a strong spatial heterogeneity of iTLSs. To further validate this observation, we calculated the clone type sharing by different iTLSs in all 6 LUAD tumors with multiple iTLSs and plotted the result as elbow plots (**Figure 3i**). Again, ~70% IgH clones and ~60% TCRβ clones were found unique (appeared in only 1 iTLS) in different iTLSs of each tumor, whereas < 5% IgH clones and TCRβ clones were found shared by 3 or more iTLSs within each tumor (**Figure 3i**). Interestingly, though rare clones were shared by spatially discrete iTLSs, we observed over 90% IgH and TCRβ clones could be also observed outside the iTLSs, suggesting a crucial role of iTLS in supplying tumor-infiltrating lymphocytes (**Figure 3j**).

Next, we integrated the transcriptome and clone information to intepret the migration paths of B/plasma cells in the tumor. Mapping iTLS-associated IgH clones revealed extrafollicular egress and convergence on discrete stromal niches (**Figure 3k**), which was further enhanced by iTLS-associated IgH clones aggregation in all examined multi-iTLS tumors (**Supplementary Fig.5f)**. B cells differentiate into plasma cells to secrete anti-tumor antibody20. To determine whether emigrant B cells complete plasma-cell differentiation at these sites, we transferred cell-type annotations from a public LUAD scRNA-seq atlas (GSE148071) to our in-house generated single-cell resolved spatial data through TACCO46. Marker-gene concordance in both scRNA-seq and cell bins of the spatial transcriptome validated the transfer quality (**Supplementary Fig.6a-c**). While iTLSs harbored dense B and T cells, the emergent niches were overwhelmingly occupied by plasma cells (**Figure 3k**), as evidenced by the intensive expression of *JCHAIN* in the new niches (**Figure 3k**). Given the aggregation of plasma cells in the new niche, we named it as plasma cell zones (PCZ) hereafter. Collectively, the above observation suggested that the fate transition from B cells to antibody-secreting plasma cells might be a process that proceeds asynchronously during migration.

**Stereo-XCR-seq delineates spatiotemporal fate trajectories of B/plasma cells in iTLSs**

To resolve whether B-cell maturation is a spatially dynamic process, we projected *MS4A1*, *CXCL13*, *IGKC*, and *JCHAIN* onto the spatial maps from the 11 LUAD tumors. It turned out iTLSs and PCZs showed mutually exclusive distribution (**Figure 4a-b**). By generating PCZ and TLS gene signature scores (Methods), we found that this observation could be further extended to immune disorder in other tissues, including primary biliary cholangitis (PBC), inflammatory bowel disease (IBD), breast cancer (BC), colorectal cancer (CRC), kidney cancer (KC) and gastric cancer (GC), as presented by distinct spatial expression pattern of TLS scores and PCZ scores and related genes (**Supplementary Fig.7a-c**). Deconvolution of cell-type proportions and CD20/CD138 co-staining on serial tumor sections further confirmed enrichment of B cells in iTLSs and plasma cells in PCZs (**Figure 4c-d, Supplementary Fig.7d**-**h**). Combining the observation of shared IgH clones in iTLSs and PCZs (**Figure 3k**), the distinct intratumoral distributions of B cells and plasma cells implicated that B-to-plasma cell differentiation might be coupled to intratumoral re-location.

We next reconstructed the molecular itinerary underpinning this re-location to elaborate the spatiotemporal fate transition from B cells to plasma cells in LUAD. Firstly, we investigated the B/plasma cells residing niches (**Figure 4e**). We re-clustered the iTLSs and PCZs bin50 spots from 11 LUAD specimens by unsupervised secondary clustering since around 70% all B/plasma cells aggregated in these two structures (~60% in PCZ, ~10% in iTLS, **Supplementary Fig.8a**). In this step, we subdivided these two structures into inner iTLS, outer iTLS, IgM+PCZ and IgG+PCZ (**Figure 4f**). IgM+PCZ and IgG+PCZ exhibited exclusive expression of *IGHM* and *IGHG1/2/3/4* respectively (**Figure 4f**). Inner iTLS and outer iTLS shared expression of *MS4A1*, while inner iTLS was surrounded by outer iTLS (**Figure 4f-g**). As expected, inner iTLSs displayed higher human lymphocyte antigen (HLA) class I (*HLA-A/B*) and class II (*HLA-DQB1/DPA1/DPB1*) expression, indicative of active antigen presentation, whereas outer iTLSs exhibited elevated *JCHAIN*, signifying ongoing plasma-cell commitment (**Figure 4h, Supplementary Fig.8b**). This observation suggested distinct roles of inner iTLSs and outer iTLSs by harboring different biological processes in immune response.

Secondly, we investigated the B/plasma cells from different niches (**Figure 4e**). We plotted the expression of *PAX5, XBP1, IRF4, PRDM1 and JCHAIN*, which represents different B-to-plasma cells differentiation stages47-52, of B/plasma cells in iTLSs and PCZs. *PAX5* exhibited highest expression in inner iTLSs, suggesting B cells residing in the inner iTLSs were at the stage of centrocytes47, 48 (**Figure 4i**). B/plasma cells exhibited progressive transcriptional gradient of *XBP1*, *IRF4*, *PRDM1* and *JCHAIN* from innter iTLSs to IgG+PCZ sequentially, suggesting that B cells gradually established terminally differentiated phenotype when migrating from iTLS to PCZ49-52 (**Figure 4i**). Using slingshot53, we constructed a B/plasma cell-specific pseudotime using B/plasma cells and from iTLS and PCZ and inferred differentiating trajectory. As a result, cells residing in IgG+PCZ and inner iTLS exhibited highest and lowest pseudotime score respectively (**Supplementary Fig.8c & Figure 4j**,left bottom). By setting starting point at inner iTLS, we observed two divergent differentiating paths of B/plasma cells from inner iTLS towards IgM+PCZ and IgG+PCZ (**Figure 4j**). Of note, IgM+PCZ and IgG+PCZ shared a large proportion of the clones (35.3~100%, **Supplementary Fig.8d**). Using Minimum Spanning Tree analysis54, we observed that *MS4A1*+B/plasma cells in outer iTLS could further differentiate into IgM+B/plasma cells or IgG+B/plasma cells (**Figure 4k, Supplementary Fig.8e**), lining with the observation that both differentiating paths went through the outer iTLSs. To infer migratory directionality of B/plasma cells, we calculated quiver fields55, 56 based on the pseudotime of each B/plasma cells. As presented by the vector flows, we noticed emigrating trends from inner iTLSs to the outer iTLSs (**Figure 4l**), coinciding with the elevated B/plasma cell pseudotime at the iTLS margins (**Supplementary Fig.8f**). The outward flux of B/plasma cells likely precludes the establishment GCs within the iTLSs. Further, we plotted the top10 expanded IgH clones of LUAD P6 to examine if the fate transition of B/plasma cells could be linked to intratumoral re-location. As expected, all top 10 IgH clones exhibited higher pseudotime scores in IgG+PCZ over other locations (**Figure 4m-n**), suggesting a spatiotemporal association between B/plasma cell differentiation and migration.

**Stereo-XCR-seq unveils PCZ as ectopic GCs for tumor-reactive lymphoctyes priming in LUAD**

SHM and CSR events are processes typically occurring in GCs to select antigen-specific B/plasma clones to produce antibodies of distinct classes with varied effect functions52, 57, 58. Though GCs were not found in iTLSs in all our tumor sections (**Supplementary Fig.5a**), we observed distal disseminations of the later staged plasma cells and the early staged B cells from the same clone families (**Figure 3k, Figure 4i**). This observation indicated B cells established the antibody-secreting capacity elsewhere. By calculating the Pearson’s correlation between B/plasma cell pseudotime and genes expression, we obtained 10 genes associated with an advanced differentiating stage (**Figure 5a).** The top ranked genesand their non-linear fitting curves indicated strong association with IgG-secretion (**Figure 5a-b**). To uncover where and how B/plasma cells established antibody-secreting capacity in the GC-missing tumors, we firstly defined hypermutated B/plasma cells by IgH chain mutation percentage over 20% (**Figure 5c**) and class-switched B/plasma cells by containing multiple isoforms (**Figure 2j**). Spatial plots of hypermutated B/plasma cells, CSR events and spatial clusters exhibited co-localizations of SHM and CSR activity within PCZs, instead of iTLSs (**Figure 5c-e**). The proportion of hypermutated B/plasma cells (**Figure 5f**) and the proportion of class-switched B/plasma cells (**Figure 5g**) were both significantly higher in PCZs over iTLSs. Further, we calculated the CSR events between each pair of isoforms and normalized them with cell counts in each tumor. Compared to iTLSs, PCZ exhibited uprising normalized CSR frequencies predominantly featuring IgM-IgG and IgA-IgG transition (**Figure 5h**), as presented by spatial plots (**Supplementary Fig.9a**) and box-strip plots (**Supplementary Fig.9b**). As a result of enriched CSR events, PCZ exhibited the highest *IGHG1/3/4* expression over all other spatial clusters (**Figure 5i**), suggesting that CSR occurs primarily within PCZ to generate IgG-secreting plasma cells. Collectively, the co-occurrence of SHM and CSR implicated that PCZ might play a similar role to canonical GC in the tumor microenvironment.

In GC, B/plasma cells undergo SHM in Dark zones (DZs) to generate mutation pools for clone selection and then migrate to light zones (LZs) for CSR alongside T cell priming59. We thus examined if DZs and LZs could be identified through distinct clonal activities in PCZ. By checking the expression of enzymes involved in SHM and CSR, we observed that IgM+PCZ expressed the highest level of activation-induced cytidine deaminase (*AICDA*), an enzyme mediating SHM in dark zones60, 61, lining with the highest mutation frequencies in IgM+PCZs (**Figure 5j**). IgG+PCZ expressed highest level of uracil-DNA glycosylase (*UNG*) and apurinic/apyrimidinic endodeoxyribonuclease 1 (*APEX1*), enzymes mediating CSR process in light zones62, 63, as well as B-cell lymphoma 6 (*BCL6*), marking class-switched B/plasma cells64(**Figure 5j**). Further, we examined the presence of T cell in PCZ. By quantifying T cells in spatial transcriptome, we observed a significantly higher proportion of T cells in IgG+PCZ over IgM+PCZ (**Figure 5k**). This result was further validated by the co-staining of CD3E/CD138 using the adjacent tissue sections; an abundant infiltration of T cells was observed in IgG+PCZ but not in IgM+PCZ (**Figure 5l-m**). The contrast T cell proportions implicated a role played by T cells in IgG+PCZ, but not in IgM+PCZ. To explore this, we partitioned the PCZs using DBSCAN and obtained 113 spatially discrete PCZ clusters (**Supplementary Fig.9c**). T cells proportions in each PCZ cluster exhibited strong Pearson’s correlations with uprising CSR events, IgG secretion and B/plasma cells pseudotime scores (**Figure 5n-p**), but not with the mutation accumulation of IgH chains (**Figure 5q**). T cells participate in B cell fate determination via secreting interleukins or through membrane ligand-receptor interaction65-67. We plotted the potential molecular candidates expressed by T cells to mediate B/plasma cell effect function and differentiation. As shown by the matrix plot, *IL6* concentrated in inner and outer iTLSs, while *CD40LG* were prominent in iTLSs and IgG+PCZs (**Supplementary Fig.9d**). These data suggest that *IL6* signals could be involved at a very early stage of intratumoral B cells fate transition in iTLS, while sustained stimulation via *CD40LG* might drive the terminal differentiation of plasma cells in IgG+PCZ.

Next, we investigated the if the T cells in the IgG+PCZ were primed. According to the presence or absence in IgG+PCZs, we stratified the TCRβ clones into present clones and absent clones. Particularly, the TCRβ clones present in IgG+PCZs exhibited a notable aggregating pattern over the absent counterparts (**Figure 5r**). Moran’s Index measures the spatial autocorrelation of the objects on cartesian coordinate systems, thus quantifies aggregating distribution68. The aggregation of IgG+PCZ-present clones were enhanced by the significantly higher Moran’s Index over IgG+PCZ-asbent clones (**Supplementary Fig.9e**). Antigen-stimulated clone expansion generates larger clone populations to elicit antigen-targeting immune response69. Clone sizes thus indicate the tumor reactivity of each clone70, 71. Compared with IgG+PCZ-asbent clones, IgG+PCZ-present clones exhibited consistently larger clone sizes across all 11 tumors (**Figure 5s**), indicating intensive expansion of these clones. Furthermore, we analyzed the T cells that infiltrated into tumor region. T cell clones absent in IgG+PCZ exhibited naïve-like phenotype as shown by the expression of *LEF1* and *IL7R*, while the T cell clones present in IgG+ PCZ exhibited stronger killing effect and stemness as presented by the tumor necrosis factor (*TNF*), granzyme A (*GZMA*), T cell factor 7 (*TCF7*) and DNA Topoisomerase II Alpha (*TOP2A*), suggesting durable tumor reactivity72, 73 **(Figure 5f**). Collectively, the above observations suggest that IgM+ PCZ harbors SHM whereas IgG+PCZ featured CSR and T cell priming, thus serve as ectopic DZs and LZs respectively and recapitulate canonical GC reaction together.

**Stereo-XCR-seq profiles exclusion of ectopic GCs by terminally differentiated cancer-associated fibroblasts**

Considering the role of ectopic GC in priming tumor-reactive lymphocytes, we next investigated which potential factors could be associated with the B/plasma clone sizes the potential clinical impact of IgG+ PCZs. Firstly, we quantified the top IgH clone sizes within each bin50 spot (**Figure 6a**). As expected, more than 95 % of non-expanded regions resided within tumor tissue, whereas iTLS and PCZ harbored markedly larger clones (**Figure 6b**). Interestingly, we observed that the *IGHM* expression concentrated in the regions with a clone size at 2 and 3, while the expression of *IGHG1/3/4* concentrated in the regions with a clone size over 4 (**Figure 6c**). Consistently, bin50 spots with larger clone sizes exhibited co-localization with IgG+ PCA, implicating an association between intensive B/plasma cells clonal expansion and IgG+ PCZ formation (**Figure 6d-e**). Further, we integrated PCZ, iTLS, myeloid cell region and immune-excluded stroma into a single “stromal” meta-cluster and categorized these stromal spots into non-expanded regions (clone size <2) and expanded regions (clone size ≥ 2, **Figure 6a**). The observation of the distinct distributions of non-expanded stromal regions and expanded stromal regions in the tumor (**Figure 6f**) indicated distinct characteristics of these 2 types of microenvironments. To explore which cells could be possibly involved in the B/plasma cells fate transition, we subdivided the major cell types into 4040 clusters (**Supplementary Fig.10a**). In addition to B cells and plasma cells, we obtained 2 epithelial cell subclusters, 12 fibroblast cell subclusters, 61 endothelial cell subcluster and 7 myeloid cell subclusters and 12 stromal cell subclusters (**Supplementary Fig.10a**). Pairwise cell-cell co-localization analysis at a resolution of 100μm showed that B/plasma cells co-localized with *CXCL13*+ CD8 T cells, macrophage and *INHBA*+ fibroblast (**Figure 6g**), suggesting shared occupancies of the same microenvironment by these cell types. In addition, these 3 cell types exhibited uprising proportion in iTLS, PCZ, immune excluded stroma and myeloid cell region (**Supplementary Fig.10b**). In comparison to IgM+ PCZ, IgG+ PCZ exhibited higher proportions of these 33 cell types, indicating an involvement in later B/plasma cell differentiation stage (**Supplementary Fig.10c**). Previous studies have shown that stromal cells and macrophage can orchestrate either pro- or anti-inflammatory milieus by secreting cytokines or by remodeling the extracellular matrix (ECM)74-78. Lining with these studies, we observed a co-localization of fibroblast and lymphocytes, including T cells and B/plasma cells (**Figure 6h**). Of note, *CXCL13*+ CD8 T cells was enriched in expanded stromal (**Figure 6h,** left), while macrophage and *INHBA*+ fibroblasts were enriched in non-expanded stromal (**Figure 6h,** right).

Further, we compared the gene expression profile between non-expanded stromal regions and expanded stromal regions to dissect the stromal programs opposing ectopic GC formation. Genes up-regulated in the non-expanded stromal regions associated with fibroblast activation (*CCL18*)79, collagen production (*COL10A1, COL5A1, COL12A1*)80-82, extracellular matrix (ECM) remodeling (*MMP14,MMP11*)83, 84 and suppressive microenvironment establishment (*IHNBA, TGFBI*)85, 86(**Figure 6i**). In addition to the co-expressing pattern of these genes in the non-expanded region (**Figure 6f & Supplementary Fig.10d**), these genes exhibited a declining trend alongside the increased IgH clone sizes (**Figure 6j**), revealing mutually exclusive localization of IgG+ PCZs and ECM-remodeling stroma. We next sought to identify the major producers of these genes scRNA-seq data attributed these non-expanded stromal genes to *CXCL13*+ CD8 T cells*, INHBA*+ fibroblasts and macrophage (**Figure 6k**), suggesting an ECM-promoting role of these 3 cell types in shaping the IgG+ PCZ-excluding microenvironment. In addition to the higher expression of *TGFBI* and *CCL18* by the macrophage in the non-expanded stromal region, fibroblasts in the non-expanded stroma also exhibited a higher expression of ECM-related genes over their counterparts from the expanded stroma or other spatial cluster (**Supplementary Fig.11a & b**). Because macrophage could activate fibroblast through *CCL18*79, we constructed a diffusion pseudotime to infer the fate transition of the fibroblast87. By setting starting point at *CD34*+ fibroblast, which exhibited distinct stemness among all fibroblast subsets88(**Supplementary Fig.11c**), we observed two terminally differentiated status of the intratumoral fibroblasts (**Supplementary Fig.11d**). In addition, 2 potential fate transition paths constructed by PAGA pointed at the expanded regions (path1) and the non-expanded regions (path2) respectively (**Supplementary Fig.11e**), coinciding with the advanced pseudotime of the fibroblasts in the non-expanded regions (**Supplementary Fig.11f**). Given the terminally differentiated fibroblasts from the non-expanded regions upregulated genes related to extracellular matrix remodeling (**Figure 6f-i**), we ran gene ontology (GO) term analysis using genes significantly upregulated (p-values<0.05) in non-expanded regions and expanded regions respectively. In contrast to the upregulated B cell receptor signaling in expanded regions, the non-expanded regions exhibited enhanced activities of collagen fibril organization and extracellular structure organization (**Supplementary Fig.11g**). These data implicate that *TGFBI*+ macrophage could promote ECM organization via *CCL18*-mediated promoting fibroblast activation in the non-expanded stroma.

Since IgG+ PCZ exhibited tumor-reactive clone selection, we next investigated the clinical impact of IgG+ PCZ formation. We firstly calculated IgG+ PCZ signature scores by Z-scoring *IGHG1/3/4* and IgG+ PCZ-exclusing signature scores by Z-scoring genes upregulated in the non-expanded stroma using bulk RNA-seq expression matrix of the 478 LUAD tumors from TCGA database89, 90. By integrating the survival information, we observed that patients with higher IgG+ PCZ scores exhibited improved overall survival (**Figure 6l,** left), whereas patients with higher IgG+ PCZ-excluding scores exhibited poorer overall survival (**Figure 6l,** right), highlighting the prognostic relevance of IgG +PCZ formation in LUAD patients. Furthermore, we examined gene expression profile of fibroblast, macrophage and B/plasma cells from an anti-PD-1-treated LUAD scRNA-seq dataset (**GSE207422**). In the pre-treatment samples, we noticed that fibroblasts in the stable disease (SD) tumors exhibited higher expression of *MMP11*, *MMP14* and *COL12A1*, macrophages in the SD tumors exhibited higher expression of *TGFBI* and *CCL18*, whereas B/plasma cells in the partial response (PR) tumors exhibited higher expression of *IGHG* genes (**Fig. 6m**). Collectively, the above data indicated that the pre-existence ofECM-mediating macrophage and fibroblasts in LUAD could be prognostically detrimental in immunotherapy response and overall survival. The formation of IgG+ PCZ exhibits tumor-reactive clone expansion, thus indicating an improved immunotherapy response by enhanced local anti-tumor immunity.

**Discussions**

While single-cell analyses of TCR and BCR clonality and transcriptomics have advanced our understanding of inflammatory response, methods to apply this information *in situ* remain limited. Here, we developed Stereo-XCR-seq, enabling immune repertoire profiling at 500 nm resolution with key advancements. Stereo-XCR-seq achieves single-cell resolution and assigns CDR3 sequences to segmented cells, providing a precise definition of clonal expansion, and enabling the exploration of lymphocyte clonal activities in micro-lymphoid aggregates (radius<100μm). To ensure unbiased T/BCR enrichment, we introduced sscirPCR, which targets the constant region instead of the variable region, overcoming primer specificity issues and biases seen in prior methods. This strategy efficiently retrieves T/BCR reads from cDNA libraries of fresh samples and can be extended to FFPE-embedded samples, facilitating immune repertoire analysis from retrospective samples. Additionally, Stereo-XCR-seq uniquely provides paired TCR and BCR chains along with the spatial transcriptome, enabling us to bridge lineage differentiation and transcriptomic profile. Furthermore, by incorporating a short-read sequencing strategy, Stereo-XCR-seq improves cost-efficiency and robustness, making high-throughput immune repertoire analysis more accessible.

Though we have improved the pairing rate of T cell receptors from lower than 10% to around 50%91, the pairing rates of Stereo-XCR-seq are still lower than 5’ends scTCR/BCR-seq92. To address this, we only analyzed IgH and TCRβ chains, a common strategy in immune repertoire study93-95. Increasing sequencing throughput and fine-tuned filtering methods might further improve the pairing rate. Additionally, like other TCR or BCR analysis methods, Stereo-XCR-seq cannot determine the antigen-specificities of clones. Incorporating methods such as fluorochrome-conjugated peptide-MHC (pMHC) staining could help locate antigen-specific T cells and identify their TCRs, as the juxtaposition of antigen, MHC, and TCRs is essential for T cell activation and clonal expansion96. Given extensive clinical studies demonstrating the potential of improving adoptive T cell transfer therapy through antigen-targeting capabilities97-99, we aim to further develop our platform to advance studies on TCR-antigen specificity in the future.

Sequencing-based and image-based spatial transcriptome are two distinct parallel technologies to deliver similar and complementary measurements of gene expression in situ100. Sequencing-based technologies keep the intact mRNA through polyA hybridization and in situ reverse transcription, which is essential to read VDJ sequences. Because of this, recently published tools that retrieved XCR reads to study spatial immune repertoire, including this study, are all based on sequencing-based technologies13, 15, 19, 101. Image-based spatial transcriptome at subcellular resolution relires on complementary hybridization and signal enhancement by either in situ multiplexed hybridization or rolling circle replication102-109. A recently disclosed image-based technology BCR-MERFISH32 utilized probe hybridization to capture BCR information in situ, while this method cannot deliver information of neither SHM nor class switching, two important clonal activities of B cells to develop antigen-binding affinity and effector function. Of note, in situ sequencing exhibits potential application in diverse VDJ sequencing due to its untargeted nature, while this potential is restricted by the short read length ranging from 5 to 30 bases100, 110. Recent improvement by incorporating ex situ sequencing extends the read length to 76 bases in Expansion Sequencing111, still unmet for reading VDJ region. In the near future, further elongation of the read length might substantiate the application of in situ sequencing in spatial immune repertoire investigations.

In the LUAD tumors analyzed in this study, Stereo-XCR-seq enables the identification of the immature TLSs and their ectopic GCs. SHM, CSR and T cell presence demarcates ectopic DZs (IgG+PCZs) and ectopic LZs (IgM+PCZs). In contrast to iTLSs, ectopic GCs exhibit aggregation of terminally differentiated plasma cells and tumor-reactive T lymphocytes. The appearance of ectopic GCs thus indicates an enhanced anti-tumor immunity in LUAD tumors, highlighting the prognostic value in checking co-aggregation of T cells and IgG+plasma cells before anti-PD1 treatment. With the observations in this study, we speculated that the formation of TLS, a commonly seen structure in immune disorders, might be driven by inflammatory chemokine-induced T/B cell aggregation, but not driven by antigens36, 112. Instead, the establishment of GCs in either orthotopic or ectopic forms are driven by antigen-stimulation. In this regard, the ectopic GC serves as the proxy for the generation of tumor-targeting lymphocytes, including both T cells and plasma cells. Hence, the discovery in this study shed lights on the identification of tumor-reactive clones in an antigen-independent manner, exhibiting clinical translational potential in developing T cell-involved immunotherapeutic strategy.

A limitation of this study is that the sample sizes might present the lymphoid structures in LUAD, but not fully reflect the diversity of all kinds of lymphoid structures at pan-cancer scale. The distinct microenvironments originated from different tissue natures might impact on the lymphoid structure formation in other solid tumors. Moving forward, large-scale pan-cancer or pan-disease studies will be essential to expand the definition and stratification of lymphoid structures, thus enhancing the understanding of how tumor microenvironment promotes or excludes the tumor-reactive lymphocytes. Nonetheless, our work demonstrates the potential of Stereo-XCR-seq as a transformative tool to investigate ectopic aggregation and maturation of tumor-reactive clones, providing a framework for categorizing lymphoid structures across tissues and diseases.

We envision that Stereo-XCR-seq could have broad applications in translational studies of immune-related diseases in addition to cancers, particularly those involving abnormal lymphocytes expansion. Disease-associated lymphocyte expansions have been observed in atopic dermatitis113, rheumatoid arthritis114, and lupus nephritis115, highlighting a strong correlation between autoantigen targeting and disease progression. While identifying autologous antigens remains challenging, Stereo-XCR-seq enables the identification of disease-associated T/BCR sequences, creating opportunities for paratope-targeting drug design. By narrowing down potential targets through the detection of aberrantly expressed antibodies, Stereo-XCR-seq can aid structure-based virtual screening in drug discovery. Furthermore, simulating antigen-binding affinity maturation - a natural structure-mimicking process in B cells – might inspire advancements in docking scoring methods, offering a promising path for therapeutic innovation.

**Methods**

***Tissue collection and process***

The LUAD specimens were collected from West China Hospital of Sichuan University. Other human specimens were kindly provided by Peking University People's Hospital. Two human mucosal biopsies samples were kindly provided by Zhejiang University School of Medicine. All samples were collected from donors during either curative surgical resection or enteroscopic examination. The tissues were rinsed twice using precooled PBS and wiped using Kimwipe tissue, then immediately embedded in Tissue-Tec OCT (Sakura, 4583) on dry ice and stored at -80°C. This study was done in accordance with the Declaration of Helsinki. The protocol was reviewed and approved by The Institutional Review Board of BGI Research (BGI-IRB24066, BGI-IRB24012), West China Hospital of Sichuan University (), Peking University (IRB00001052-24061) and Zhejiang University School of Medicine (IR2023396). The clinical information and specimens were collected with written informed consent from all donors.

***Preparation and sequencing of Stereo-seq library***

The fresh-frozen tumor tissues were then transferred on dry ice to BGI Research for RNA Integrity examination. Tissue sectioning was performed using Leica Cryostat (CM1950) at -20℃. 10~15 slices (50μm) were collected for RNA extraction and integrity examination and a following 5μm slice was sectioned for H&E staining to determine the morphology of the specimens. Tissues with RIN value over 4.0 and desired FOVs were proceeded to Stereo-seq. After quality control, the specimens were serially sectioned at the thickness of 5μm (slice 1,2,3)-10μm (slice 4)-5μm (slice 5,6,7). Slices 3 were stained using H&E staining kit (Beyotime, C0105S) and scanned using Motic EasyScan System at 10x objective lens. Slices 1,2,5,6,7 were preserved in -80℃ for further use. Slice 4 was flattened and attached to Stereo-seq chip, dried at 37℃ for 5 minutes, and then fixed in absolute methanol at -20℃ for 30 minutes. After fixation, the chip was processed to nucleus staining using Qubit™ ssDNA Assay Kit (ThermoFisher, Q10212). The chip was scanned using Motic EasyScan System at 10x lens to detect signal of ssDNA and washed with 0.1x SSC solution before permeabilization. Tissue was then permeabilized for mRNA precipitation and probe capture at 37℃ for 24 minutes. In situ reverse transcription (45℃, 2 hours) and tissue removal (55℃,10 min) was performed in incubator. After that, CID and UMI co-barcoded cDNA was released, collected, and amplified by PCR using FF-TSO primer (see Table1) for Stereo-seq library construction16. The amplified product was proceeded to Stereo-XCR-seq enrichment. The library of Stereo-seq was sequenced using MGI DNBSEQ-T7 according to the manufacturer’s protocol.

***T and B cell receptors enrichment strategy from Stereo-seq library***

For fresh-frozen (FF) sample to retrive the TCR and BCR transcripts from the amplified stereo-seq library, we added ligating elements (LEs) to stereo-seq library by nested PCR using 2X Platinum HiFi Hotstart ReadyMix (Gcatbio, LS-EZ-K-00009O). 20ng purified dsDNA from the first round was input as the template and amplified using primer LE-F and primer LE-R (see Table1). The program of nested PCR was set as:

1. Denaturation: 95℃ for 5 minutes for denaturation. Heat lid was set to 105 ℃.

2. Amplification: 98℃ for 20 seconds, 50℃ for 20 seconds, 72℃ for 3 minutes. Repeating for 15 cycles.

3. Extension: 72℃ for 5 minutes.

All PCR products were purified using the 0.6x DNA clean Beads (VAHTSTM, N411-03) and quantified by QubitTM dsDNA Assay Kit (Thermo, Q32854), and saved as LE cDNA library.

For FF samples, 400ng LE cDNA library was mixed with 20μM FF-splint oligo1 (see Table1) to generate the sscirDNA. For formalin-fixed, paraffin-embedded (FFPE) samples, the stereo-seq cDNA was directly mixed with 20μM FFPE-splint oligo1 (see Table2) for circularization. The circularization procedure is as followed:

1. Denaturation: 95℃ for 5 minutes. Heat lid was set to 105 ℃.

2. FF/FFPE-splint oligo1 mediated sscirDNA transformation: rapid cooling at -20 ℃ for 5 minutes.

3. Ligation: Adding T4 DNA ligase and ligating buffer (Gcatbio, LS-EZ-E-00008O) according to the instructions. Incubating at 37 ℃ for 1 hour. Heat lid was set to 37 ℃.

4. ssDNA and double-stranded DNA (dsDNA) degradation: Adding exonuclease I and III (Gcatbio, LS-EZ-E-00010P, LS-EZ-E-00011P) and incubate at 37 ℃ for 30 minutes. Heat lid was set to 37 ℃. The restriction-digestion reaction was terminated by adding 0.1 M EDTA (pH 8.0).

Next, the sscirDNA was purified using 1.5X PEG32 beads (BGI, L054) and input as the template and amplified using FF/FFPE T/BCR constant primers for XCR enrichment. The enrichment was achieved by 2 rounds PCR. For the first round, 40 ng sscirDNA was input as the template and amplified using constant primer 1-F and constant primer 1-R at a concentration of 0.5 μM. For the second round, 20ng purified dsDNA from the first round was input as the template and amplified using constant primer 2-F and constant primer 2-R at a concentration of 0.5 μM. Each T/BCR chain was processed respectively, each T/BCR gene requires individual enrichment using its specific constant primer 1 and constant primer 2(see Table1, Table2). The programs of 2 rounds of PCR were both set to:

1. Denaturation: 95℃ for 5 minutes for denaturation. Heat lid was set to 105 ℃.

2. Amplification: 98℃ for 20 seconds, 50℃ for 20 seconds, 72℃ for 3 minutes. Repeating for 15 cycles.

3. Extension: 72℃ for 5 minutes.

For FF samples, all gene PCR products were purified using the 0.6x DNA clean beads (VAHTSTM, N411-03), while for FFPE samples, 1x DNA clean beads (VAHTSTM, N411-03) was used. Quantified by QubitTM dsDNA Assay Kit (Thermo, Q32854).

***Probe hybridization enrichment strategy from Stereo-seq FF library***

We took 1000ng of stereo-seq cDNA library and followed the instrument of MGIEasy Exome Capture V4 Probe Set (MGI, 1000007745) to do probe hybridization as followed:

1. Pre-hybridization: Mixed stereo-seq cDNA with block buffer, 95℃ for 5 minutes for denaturation. Heat lid was set to 65 ℃.

2. Hybridization capture: Prepared a hybridization buffer follow the protocol and add TCR RNA probe (see table3) mixture into cDNA tube, mixed thoroughly and incubated at 65°C for over 24 hours. Heat lid was set to 105 ℃.

3. Elution: Maintained the hybridization mixture at 65°C, measured the remaining volume, and transferred it to the Dynabeads™ MyOne™ Streptavidin T1 beads (thermo, 65601). After incubation at room temperature for 30 minutes, washed beads with Wash Buffer I and II (MGI, 1000007745) and resuspended in nuclease-free water.

4. Probe elution PCR: Prepared RNaseH (NEB, M0297S) digestion system in the PCR tube containing the bead suspension, incubated at 37°C for 20 min. Purified the eluted product using 0.8x DNA clean beads (VAHTSTM, N411-03), washing with 80% ethanol and resuspending in nuclease-free water.

5. PCR amplification: Prepared PCR mix with the eluted product, 95℃ for 5 minutes for denaturation. 98℃ for 20 seconds, 58℃ for 20 seconds, 72℃ for 3 minutes and repeating for 18 cycles. Extension at 72℃ for 5 minutes. Purified the eluted product using 0.8x DNA clean beads (VAHTSTM, N411-03) and collected enriched cDNA products.

***Long-read library construction and sequencing***

The purified enrichment products by constant primer 2 PCR or probe hybridization were used as input into CycloneSEQ and Nanopore library preparation. Each T/BCR chain was proceeded respectively. The starting mass of long read library construction was 1000 ng. We used CycloneSEQ library kit(H940-000001-00) and Nanopore library preparation kit (SQK-LSK109) to carry out end-repaired and sequencing adapter ligation by following the commercialized instructions. The resulting products were purified using 0.6x DNA clean Beads (VAHTSTM, N411-03) and eluted in 30μl elution buffer. The libraries were then sequenced using the CycloneSEQ G100 instrument.

***Optional fragmentation pipelines for full V(D)J construction***

The enriched T/BCR cDNA libraries were fragmented using a modified XCR-Tn5 transposase system, and the full V(D)J sequences were obtained after sequencing and assembly. The XCR-Tn5 transposase workflow includes adapter annealing and embedding, product fragmentation, and PCR amplification as followed.

Adapter annealing and embedding: Annealing buffer was prepared by combining 1 M Tris-HCl (pH 7.8, Beyotime, ST778), 0.5 M EDTA (pH 8.0, Beyotime, ST069), 5 M NaCl (Beyotime, ST348) and Nuclease-free water (TransGen Biotech, GI101-03) to final concentrations of 10 mM, 1 mM and 50 mM, respectively. Tn5 primerA and XCR-Tn5 Read1(see Table1) were separately diluted to 100 µM with annealing buffer, mixture as XCR-adapter. The programs of annealing were set to 95°C for 2 minutes, 75°C for 15 minutes, 60°C for 10 minutes, 50°C for 10 minutes, 40°C for 10 minutes and 25 °C for 30 minutes. The rate of gradient cooling is controlled at 0.1°C/s. Heat lid was set to 105 ℃. The annealed XCR-adapter was mixed with transposase (Gcatbio, LS-EZ-E-00009P) and subjected to the embedding reaction at 25°C for 1 hour. Heat lid was set to 30 ℃. The products were saved as XCR-Tn5 transposase.

Product fragmentation: The enriched T/BCR cDNA libraries (20ng) were fragmented using XCR-Tn5 transposase at 55°C for 10 minutes. Heat lid was set to 75 ℃. Fragmentation was quenched with 5×NT buffer (Gcatbio, 1000007876), 5 minutes at room temperature.

PCR amplification: 20μl fragmented products were amplified by PCR using the Tn5 sequencing adapter F and R primers (see Table1). The amplification program is set to 72 °C for 5 minutes, 95°C for 5 minutes for denaturation. 98°C for 20 seconds, 65°C for 20 seconds, 72°C for 30 seconds, and repeating for 15 cycles to amplification. 72 °C for 5 minutes to extension. All PCR products were purified using the 1x DNA clean Beads (VAHTSTM, N411-03), quantified by QubitTM dsDNA Assay Kit (Thermo, Q32854) and validated on Agilent Qsep100 standard cartridge (Bioptic, C105101).

***Short read library construction and sequencing***

FFPE samples have relatively short fragments and can be directly subjected to short read sequencing. For FF samples, sequencing without fragmentation can also be performed to obtain CDR3 information. The short read library construction without fragmentation is carried out through PCR amplification using sequencing adapter primers. 50ng enriched T/BCR cDNA libraries were input as the template and amplified using sequencing adapter primers (F and R, see table1, table2) at a concentration of 0.8 μM. The program of PCR was set to:

1. Denaturation: 95℃ for 5 minutes for denaturation. Heat lid was set to 105 ℃.

2. Amplification: 98℃ for 20 seconds, 65℃ for 20 seconds, 72℃ for 3 minutes. Repeating for 15 cycles.

3. Extension: 72℃ for 5 minutes.

For FF samples, all gene PCR products were purified using 0.7x DNA clean beads (VAHTSTM, N411-03), while for FFPE samples, 1.2x DNA clean beads (VAHTSTM, N411-03) was used. Quantified by QubitTM dsDNA Assay Kit (Thermo, Q32854).

For each specimen, we generated T/BCR mix for each specimen using equal mass of the enrichment products of each chain. 100ng T/BCR mix was used to generate DNB using DNBSEQ one step make DNB kit (MGI,1000020563) supplemented with 20μM FF/FFPE splint oligo2 (see Table1). The DNB was loaded to sequencing chip of PE150 sequencing kit (MGI,1000012555) and sequenced using MGI DNBSEQ-T7 instrument. The sequencing primers in the kits were displaced by FF/FFPE XCR sequencing primer 1,2 and multiple displacement amplification (MDA) primer (see table1, table2).

***Stereo-seq raw data analysis***

Fastq files were generated using a MGI DNBSEQ-T7 sequencer. Stereo-seq CID and MID are contained in the read 2 (CID: 1-25 bp, MID: 26-35 bp) while the read 1 consist of the cDNA sequences. The complete processing of Stereo-seq raw data was previously described and now packed up as integrative pipelines on DCScloud platform (https://cloud.stomics.tech/#/login). We used Stereo-seq Analysis Workflow V8 to ran Stereo-seq data for all samples for workflow consistency. The gem file, tissue mask, barcode whitelist (h5 file) was downloaded through the platform.

***Short-read XCR data analysis***

Preprocessing and parallelization: Raw paired-end FASTQ reads (R1 and R2) were first split into 20 smaller, manageable chunks using seqkit split2 (v2.8.0) to facilitate parallel processing. The reverse complement of each R2 split was pre-generated using seqtk (v1.4).

Coordinate mapping and read pairing: For each Stereo-seq chip, we performed barcode-to-coordinate mapping in a parallel manner. The reverse-complemented R2 reads were mapped against the corresponding barcodeToPos.h5 file using ST\_BarcodeMap (v0.0.1, https://github.com/STOmics/ST\_BarcodeMap) with a mismatch tolerance of 1 (--mismatch 1). Read IDs from successfully mapped R2 reads were extracted and subsequently used with seqtk subseq to retrieve their corresponding R1 sequences, which contain the transcript information.

VDJ alignment and clonotype assembly: The paired R1 files were processed with MiXCR (v4.6.0) for sequence analysis. Reads were aligned to the reference V, D, and J genes using the `align` command with parameters -p rna-seq, -OallowPartialAlignments=true, and -OvParameters.geneFeatureToAlign="VTranscriptWithout5UTRWithP". Subsequently, clonotypes were assembled based on the CDR3 region using the assemble command with key parameters -OassemblingFeatures='CDR3', -OseparateByJ=true, and -OseparateByV=true. Final clone sequences and alignment details were generated using the exportClones and exportAlignments commands, respectively.

***Long-read XCR data processing***

Long-read XCR sequencing processing procedure has been described previously (reference, BioRxiv). In this study, the long-read sequencing result is only used for benchmarking, not for XCR-meta construction.

***XCR-metadata construction***

To construct a comprehensive spatially-resolved single-cell XCR metadata table, we integrated outputs from MiXCR and spatial barcode mapping. First, we parsed the barcode-mapped FASTQ file to create a dictionary linking each read ID to its spatial coordinates (x, y) and its corresponding CID and UMI. Concurrently, information from the MiXCR clones file, including CDR3 amino acid/nucleotide sequences and isotype data, was loaded into memory.

We then processed the MiXCR alignments file, iterating through each aligned read. For reads successfully associated with both a spatial coordinate and a valid clone ID, we merged the alignment details (V/D/J/C gene hits), clone properties (CDR3 sequences, isotype), and spatial information (CID, UMI, x, y coordinates). This integrated information was written to a temporary file. Finally, this intermediate file was loaded into a pandas DataFrame. We further processed the data by parsing CDR3 start/end positions from the MiXCR refPoints field and classified clones as 'Functional' or 'Non-functional' based on the presence of stop codons or frameshifts in the CDR3 amino acid sequence. The resulting table, containing detailed annotations for each TCR/BCR read, was saved as the final metadata file.

***Cell segmentation and manual check***

Cell segmentation: Single-cell segmentation was performed using the cellbin2 pipeline developed by STOmics (https://github.com/STOmics/cellbin2). Following the initial segmentation, the boundary of each identified cell was expanded by 10 pixels using a morphological dilation operation to ensure the capture of the complete cellular area.

Manual check: For quality control, the performance of the segmentation was manually validated. We randomly selected ten fields of view (FOVs) from the segmented ssDNA image of the target sample. The segmentation accuracy within these FOVs was then qualitatively assessed by visual inspection to confirm the effectiveness of the cell delineation.

***XCR clone reads allocation***

High-resolution cell-segmentation polygons were stored in an STRtree spatial index (Shapely v2.0.6) and intersected with XCR reads coordinates to assign each read to a single cell. Cells were retained if they contained ≥4 functional receptor UMIs. For every TCR (TRA, TRB) or BCR (IGH, IGK, IGL) chain, candidate UMIs were first collapsed into CDR3 amino-acid sequences. When ≤10 distinct CDR3 variants were observed, UMIs were de-duplicated by majority vote, and the most abundant CDR3 was adopted as the chain-level consensus. Cells exhibiting more than one productive chain per locus were resolved to a single clonotype by the same majority rule. The resulting clonotype identifiers were appended to the single-cell metadata for downstream spatial analyses.

***Spatial plot visualization***

***kde plot***

we computed a two-dimensional kernel-density estimate (KDE) using weighted bivariate Gaussian kernels. Each retained cell i with valid Cartesian coordinates (xᵢ, yᵢ) and ≥1 high-confidence mutation call was assigned a weight equal to its mutation-percentage (mutated reads / total reads). Cells without detectable variants were excluded. Bandwidth selection followed Scott’s rule and was further scaled by a factor of 0.9 to balance smoothing against preservation of local spatial structure; kernels were truncated at 0.2 standard deviations (cut = 0.2) to constrain density estimation within the tissue boundary. The resulting probability density surface was visualized as unfilled contour lines using the perceptually uniform ‘inferno’ colormap and overlaid on a dark-background style sheet to maximize contrast for print reproduction.

***tissue outline calculation***

The physical tissue outline was reconstructed from 50-μm binned spatial coordinates. Original (x, y) positions were rounded to the nearest bin center and cast onto a binary occupancy image of dimensions (max(Y)+1, max(X)+1), assigning a value of 255 to pixels containing ≥1 cell and 0 otherwise. Canny（CV2 v4.8.0） edge detection was applied to this binary mask to extract the outermost tissue contour. Detected edge pixels were subsequently re-mapped to micron-scale coordinates and exported as a polygonal boundary file for downstream spatial analyses.

***cellbin polygon plot***

High-resolution cell segmentation masks were converted into polygonal vertices and smoothed with a periodic cubic spline (smoothness = 0.5) to eliminate imaging artefacts while retaining biologically relevant morphology. Polygons comprising fewer than three vertices were discarded. The resulting paths were rendered as 0.1-pt white outlines with zero fill using Matplotlib（v3.7.1） PathPatch objects and aggregated into a PatchCollection for GPU-accelerated display.

***Hypermutation calculation, clone family definition and lineage tree construction***

***Hypermutation calculation***

Clonal B-cell clusters were first defined with the DefineClones workflow (SHazaM v1.1.0)116. Pairwise nucleotide distances between full-length IGH heavy-chain sequences were computed using distToNearest with length-normalised Hamming distances to account for junction-length variability. The bimodal distance-to-nearest distribution was interrogated with findThreshold to obtain an empirical cut-off separating bona-fide clonal relatives from singletons; DefineClones.py was then executed with this threshold to assign each cell a unique clone\_family\_id. Next, clone annotations were mapped onto the spatial transcriptome by linking clone\_family\_id to corresponding cellIDs. Cells lacking clone assignments were excluded. For each clone family comprising >1 distinct IGH CDR3α amino-acid sequence, Euclidean distances between constituent cells were calculated. Cells separated by ≤50 μm were classified as spatially aggregated hypermutating clones (shm = ‘yes’). all others were designated non-aggregated (shm = ‘no’).

***clone family definition***

IGH clonotypes were delineated using the DefineClones workflow. Pairwise nucleotide distances between full-length IGH sequences were first calculated with the distToNearest function, employing length-normalised Hamming distances to account for variable junction lengths. The resulting bimodal distance-to-nearest distribution was analysed via findThreshold to determine the empirical cut-off maximising the separation between clonally related cells and singletons. Cells were subsequently assigned to clone\_family\_id groups by executing DefineClones.py with this threshold.

***lineage tree construction***

BCR sequences derived from Stereo-seq slides were processed with Change-O (v1.2.0)116. Only full-length, in-frame CDR3 regions lacking insertions or deletions were retained. Putative germline V segments were reconstructed by reverting observed point mutations to the IMGT reference and re-introducing alignment gaps required for phylogenetic reconstruction. Cells exhibiting identical V-D-J rearrangements were grouped into clone families. The twenty largest IgG-switched families were selected for downstream analysis. For each clone, a ChangeoClone object was instantiated in alakazam v1.2.0, and a maximum-parsimony tree was inferred with PHYLIP dnapars (v3.697) using the germline sequence as the outgroup root. Internal and terminal nodes were annotated with DFS traversal order and isotype status (IGHM, IGHG, IGE, IGA, IGHD). Node size was scaled by UMI count, node color encoded isotype, and BFS depth was adopted as an evolutionary distance metric. Cell barcodes were mapped to 50-μm Cartesian bins to overlay lineage depth onto tissue sections. Annotated phylogenetic trees and corresponding metadata tables were exported in Newick and CSV formats, respectively.

***CSR definition and normalization***

Constant-region expression values for nine immunoglobulin heavy-chain genes (IGHG1/2/3/4, IGHA1/2, IGHM, IGHD, IGHE) were extracted from the filtered gene-by-cell matrix stored in the Anndata object. Transcript counts were binarised: ≥1 UMI was scored as “1” (presence) and 0 UMI as “0” (absence). Composite IGHG and IGHA categories were considered positive if any constituent gene was detected. Each cell was then assigned a CSR status: “NA” (no isotype detected), “no CSR” (exactly one isotype), or “CSR” (≥2 distinct isotypes). For CSR-positive cells, pairwise co-occurrence frequencies of all isotype combinations were tabulated into a symmetric contingency matrix. Matrices were generated independently for LA-PCA (n = X cells) and LA-TLS (n = Y cells) sub-populations, row-normalised to the respective cell counts, and concatenated for comparative visualisation. Heatmaps were rendered with seaborn v0.13.2 using the RdBu\_r colormap and lower-triangle masking to eliminate redundancy.

***Quiver map construction***

Pseudotime inference and directional velocity mapping for B-to-plasma-cell transition

Eleven Stereo-seq datasets were concatenated in Scanpy (v1.9.8)117, barcode-deduplicated, and annotated with spatial coordinates. Transcriptional pseudotime was calculated on a per-cell basis as pseudotime = log2[(XBP1 + JCHAIN + IRF4 + PRDM1 + 1) / (PAX5 + 1)], where counts are library-size-normalised. Slide D06047A2 was subset to TLS cluster 3 (annotated B/plasma cells). A 50-nearest-neighbour graph was constructed with edge weights = 1 / Euclidean distance. Velocity vectors were derived from the pseudotime gradient on this graph, projected onto a uniform 50 × 50 spatial grid via bilinear interpolation, and rendered as streamlines with Matplotlib v3.7.1.

***TLS and PCA gene signature score calculation***

TLS and PCA gene-signature scores were computed in Scanpy v1.9.8: plasma-cell abundance (PCA) was quantified with IGKC, IGHG4, IGHG1 and IGLC1–7 (≥5 % spot prevalence); TLS activity with TRBC1/2, TRAC, CD3E/D/G, MS4A1, CXCL13 and FDCSP. Log-normalised expression was scored with sc.tl.score\_genes and the continuous values stored in adata.obs for downstream spatial analyses.

***B/plasma cell pseudotime construction***

leven Stereo-seq datasets (D06047A2–D06050E4, D06053D2) were imported into Omicverse (v1.7.1)44, concatenated, and batch-corrected using Harmony. Spatial bins were annotated by left-joining coordinates with the expert-curated LA\_bin50\_meta.csv. After library-size normalisation to 10,000 counts per bin and log-transformation, a B-to-plasma-cell pseudotime score was calculated as log2[(XBP1 + JCHAIN + IRF4 + PRDM1 + 1)/(PAX5 + 1)]. B-lineage cells located within lymphoid aggregates (LA-TLS or LA-PCA; n = 65 812) were retained. Pearson correlations between each gene’s expression and the pseudotime score identified the 20 most strongly associated genes; their trajectories were visualised by third-order polynomial regression.

***iTLS clustering using Density-Based Spatial Clustering***

Spatial coordinates of all DNBs annotated “LA-TLS” at 50-µm resolution were compiled into matrix X ∈ R^(n×2). DBSCAN was applied to X to delineate contiguous TLS foci while excluding background. To mitigate scale-dependent artefacts, we performed a grid search across linear scaling factors 0.10, 0.11 and 0.12. At each scale DBSCAN was run with ε = 30 µm and min\_samples = 100, parameters chosen empirically to maximise sensitivity without over-fragmentation. The solution obtained with factor 0.12 yielded the most biologically coherent domains and was retained. DNBs labelled noise (−1) were discarded, and each remaining cluster was assigned a unique integer ID. This annotation was propagated to the single-cell AnnData object via a left-join on the composite key “DNB\_x\_y”, generating the categorical variable `TLScluster` for downstream analysis.

***Annotation of bin50 spots and cell bins***

***Bin50***

Stereo-seq bin50 matrices were imported into Scanpy. Mitochondrial reads were flagged by “MT-” prefixes and QC metrics inspected without global filtering because of high capture efficiency and low mitochondrial leakage. After log-normalisation and z-scaling of the top 3,000 highly variable genes (Omicverse v1.7.1 “pearson|pearson”), PCA retained the first 50 PCs (>90 % variance). A k-NN graph (k = 15) and Leiden clustering (resolution 1.2) delineated transcriptional communities visualised in UMAP (Omicverse palette 14–28). Cluster-specific markers were then identified with Wilcoxon, t-test and COSG, and the top 3–5 genes per cluster visualised in dot plots for manual cell-type annotation.

***Cellbin***

Stereo-seq cell bins were imported into Scanpy as raw counts and gene symbols were de-duplicated. LUAD scRNA-seq (GSE148071; n = 57,218 cells)118 served as the reference, annotated with primary (`anno`) and refined sub-type (`anno2`) labels. In a two-step strategy, TACCO (v0.4.0)46 first mapped each bin to the full reference to assign the highest-probability major cell type (`anno`), then re-mapped each bin within its major class against the corresponding reference subset to obtain sub-types (`anno2`).

***Deconvolution spatial bin50 spots***

single-cell data were converted to an integer count matrix, filtered for genes detected in ≥3 cells, and used to build a spacexr(v2.2.1)119 Reference object with harmonised subtype labels. Stereo-seq cell-bin counts were imported as a sparse dgTMatrix, assigned unique “DNB\_x\_y” identifiers from x–y coordinates, and combined with position data in a SpatialRNA object. RCTD (doublet\_mode = "full", 4 cores) deconvolved each bin into cell-type proportions, and the resulting model was saved as RDS for downstream analyses.

***Moran’s Index Calculation***

Spatial autocorrelation of TCR specificity in the eleven Stereo-seq tissue slices was quantified as follows. First, the integrated dataset was subset by batch identifier to derive a sample-specific list of unique TRB CDR3 amino-acid sequences (TRBcdr3aa) that were non-missing and annotated as PCA\_IGHG/A plasma cells. Each 50-µm spatial bin in the corresponding sample-level AnnData object was then encoded with two mutually exclusive binary variables: (i) PCA\_TRBcdr3aa\_counts = 1 if the bin’s TRBcdr3aa exactly matched any sequence in the PCA\_IGHG/A reference list, otherwise 0; (ii) not\_PCA\_TRBcdr3aa\_counts = 1 if the bin contained a non-missing TRBcdr3aa absent from the reference list, otherwise 0. A grid-based adjacency graph (coord\_type = 'grid', radius = l bins, eight-neighbor connectivity) was constructed with Squidpy (v1.2.2.)120 Moran’s Index was computed for each binary variable using sq.gr.spatial\_autocorr (mode = 'moran', attr = 'obs', use\_raw = False, n\_jobs = 1). Positive Moran’s Index values indicate significant spatial clustering of identical TCR specificities, values near zero suggest a random spatial distribution, and negative values imply dispersion.

***Immunofluorescence staining***

Human fresh-frozen tissue sections were collected using Leica Cryostat (CM1950) at -20℃, and 5μm slices were fixed in 4% paraformaldehyde fix solution (Sangon, E672002-0500) for 30 minutes at room temperature. The tissues were blocked in 20% BSA (Sangon, A600332-0025) for 1 hour at room temperature. And then incubated with the primary antibody incubation solution, containing CD20, CD3 and CD138 in 2% BSA(Sangon, A600332-0025) and 5% Human TruStain FcX™ (BIOLEGEND, 422301) at 4°C for 12 hours. Washed 3X in PBS (Sangon, E607008-0500) after incubation. The secondary fluorescence antibody incubation solution was applied and incubated at room temperature for 1 hour, followed by 3X washes with PBS. SlowFade™ Diamond Antifade Mountant with DAPI (thermos, S36968) was used as

nuclear counterstain. The signal was examined by Motic EasyScan System at 20x objective lens.

The antibodies for mIF staining are listed as follow:

Reagent and resource

antibodies Dilution resource identifier

Anti-CD20 rat mAb 1:100 Abcam ab279300

Anti-CD3 rabbit rAb 1:100 Gcatbio LS-PA-02008O

Anti-CD138 mouse mAb 1:100 Zhongshan Golden Bridge ZA-0584

YSFluor™ 488 Donkey Anti-Rat IgG (H+L) 1:100 Yeasen 34406ES60

AF555 Donkey Anti- Rabbit IgG (H+L) 1:200 Beyotime A0453

AF647 Goat Anti-Mouse Recombinant Antibody 1:100 Gcatbio LS-PA-03011P

***Diffusion pseudotime and trajectory analysis***

Fibroblasts were first isolated from the D06053D2 Stereo-seq slice by selecting cells annotated as “fibroblast” in the high-resolution cell-bin object. B-cell clonal expansion status was assigned at the bin level by merging the spatial object with an external TSV file enumerating the number of B cells per expanded clone (≥2 cells). Bins located within annotated stromal regions (Stroma, LA-PCA or LA-TLS) were classified as “yes” (≥2 expanded B cells), “no” (<2 cells), or “other region” (outside stromal structures). After quality control, the fibroblast subset was pre-processed with Omicverse: Pearson-residual normalisation was applied, the 3 000 most variable genes were retained, data were log-normalised and scaled, and principal-component analysis was performed retaining the first 50 PCs. A k-nearest-neighbour graph (k = 15) was constructed on this reduced space and visualised with UMAP. Diffusion pseudotime was then calculated using diffusion maps implemented in Omicverse, with the “other region” population defined as the trajectory root. Partition-based graph abstraction (PAGA) was subsequently performed with edges weighted by diffusion pseudotime to summarise transitions among the three B-cell expansion states.

***Gene ontology analysis***

we integrated the data of 11 samples with B-cell clonal size information. B-cell clonal sizes (number of cells per B-cell clone) were estimated from matched B-cell receptor (BCR) sequencing data and merged with the cellbin object using genomic coordinates (‘loc’) as keys, yielding a new metadata variable B\_clone\_expansion\_n\_cells.Next, stromal regions of sample D06053D2—including stroma, lymphoid aggregate–proximal cancer-associated (LA-PCA), and tertiary lymphoid structures (LA-TLS)—were subset for downstream analysis. B-cell clonal expansion was dichotomized: clones with ≥2 cells were labeled “yes”, otherwise “no”. Gene expression matrices were normalized (10,000 counts per cell) and log1p-transformed. Differentially expressed genes (DEGs) between expansion and non-expansion groups were identified using the Wilcoxon rank-sum test (scanpy.tl.rank\_genes\_groups), applying thresholds of log2-fold change >0 and Benjamini–Hochberg adjusted P < 0.05.GO enrichment was performed on up-regulated genes using g:Profiler’s Enrichr API (GO\_Biological\_Process\_2023, human background), with a significance cutoff of FDR <0.1. Resulting GO terms were visualized as dot plots (gene set enrichment significance versus term fold enrichment) using the gseapy plotting module. Dots were overlaid on horizontal lines to emphasize hierarchical relationships, and figure aesthetics (marker size, legend scaling) were customized in Matplotlib. Analyses were conducted independently for genes up-regulated in B-cell expansion (“yes”) and non-expansion (“no”) phenotypes, yielding Figure 6J (right) and Figure 6N (left), respectively.

***Data and code availability***

The raw sequencing FASTQ files of the stereo-seq and stereo-XCR-seq data could be accessed on Genome Sequence Archive (accession number:, https://ngdc.cncb.ac.cn/gsa-human). The external single cell RNA-seq data could be downloaded through the following links: E-MTAB-13526 (https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13526)24, GSE189357 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189357)25, GSE148071(https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148071)118, 121. The external tumor stage expression analysis is based on the TCGA lung adenocarcinoma (LUAD) cohort89, and the stage plot and Survival curve plot was generated using the GEPIA2 online tool (http://gepia.cancer-pku.cn/)122. Code used in this study has been uploaded to github (https://github.com/fengyu9481) for the reproducibility of this study. For any further inquiries for the use of the code or for the data accession, please send correspondence to fengyu1@genomics.cn.

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**Figures and figure legends:**

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**Figure 1. *Stereo-XCR-seq*** ***achieves high-fidelity spatial immune repertoire mapping with robustness performance in disease tissues.***

**a.** Schematic graph shows 5 key steps of Stereo-XCR-seq. **b**. Paired box-strip plot shows the comparison of pre-retrieval (Stereo-seq) and post-retrieval (Stereo-XCR-seq) target reads/total reads ratios in fresh frozen tissues. Each dot represents an independent experimental replicate. Boxes are presented as mean±25% percentile and colored by pre-retrieval and post-retrieval quantification. Error bar indicates extreme dots. N number of replicates has been labelled on the graph. **c**. Paired strip plot shows the comparison of post-retrieval target reads/total reads ratio. Each line indicates an cDNA library. 3 cDNA libraries were tested using different retrieving strategies. Dots are colored by retrieving strategies.**d**. Bar chart shows the CDR3 clone types of each tissue section. For Stereo-XCR-seq, dots are colored by different tissue origins. Data are presented as mean±SEM. \*\*, p-value<0.01; \*\*\*, p-value<0.001. **e**. Spatial plots show the density and distribution of XCR transcriptomic reads and clone reads at a resolution of bin50 (25μm x 25μm). For data presentation, *IGHA/D/E/M/G* are integrated into *IGH* gene and TRBC1/2 are integrated into *TRBC* gene. Clone reads are presented using color map Oranges, whereas transcriptomic reads are presented using color map Greens. Both colors are shaded by UMI counts. FOV selected for immunofluorescence staining is highlighted in white frame. **f**. mIF staining of CD3e and CD138 using adjacent 5μm tissue section. FOVs corresponding to panel E are shown. **g**. Spatial plots show the tissue sections from breast cancer. Each dot represents an assembled T/BCR clone. Pre-retrieval dots are enlarged for data presentation. **h**. Paired box-strip plot shows the comparison of pre-retrieval (Stereo-seq) and post-retrieval (Stereo-XCR-seq) target reads/total reads ratio in FFPE tissue sections. Each dot represents an independent experimental replicate. Boxes are presented as mean±25% percentile and colored by pre-retrieval and post-retrieval quantification. Error bar indicates extreme dots. N number of replicates has been labelled on the graph.

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**Figure 2. *Stereo-XCR-seq profiles clonal activities at single cell level with enhanced resolution.***

**a.** Spatial plot shows the distribution of IgH clone family 1322 at 2 different resolution, 100μm and single cell resolution. FOV is highlighted in white frame. Bin200 spot (100μm) is plotted as pink frame. Single cells are plotted in pink polygons with white cell border. **b**. Violin-box plots show the TCRβ and IgH+ cell counts in each bin200 spot. Boxes are presented as mean±25% percentile. **c**. Spatial plot shows the distribution of IgH clone reads (red), IGH genes (blue), TCRβ clone (yellow) reads and TRBC1/2 genes (green) in each cell bin. Cell borders are plotted in white. FOVs are selected to present BCR zone (FOV1) and TCR zone (FOV2). **d**. Venn plots show the detection of IGH genes and TRBC1/2 genes in IgH+cells and TCRβ +cells. The circles are colored as indicated below. **e**. Box plot shows the supporting ratio of IgH (red) and TCRβ reads by transcriptomic reads. Each dot represents an independent experimental replicate. Data are presented as mean±25% percentile, with error bar indicating extreme value. **f**. Dot plot shows the expression of TRAC, TRBC1 and TRBC2 by different cell types. Dots are colored by mean expression and sized by expression fraction.

g. Bar charts show the TCR α-β pairing rate (left) and counts of the α-β paired cells. Each dot represents an independent experimental replicate. N=4. Data are presented as mean±SEM. **h**. Spatial plots show the distribution of TRBC1/2 transcriptomic reads and TCRβ clone reads at a resolution of bin1 (500nm). Two FOVs are selected to present the similar (FOV1 and the discrepant (FOV2) pattern of TRBC1/2 transcriptomic reads and TCRβ clone reads. **i**. Immunofluorescence staining of CD3E using adjacent 5μm tissue section. FOVs corresponding to panel h are shown. **j-l**. Spatial plots showing the capacity of Stereo-XCR-seq in visualization of clone expansion (j), class switching recombination (k) and somatic hyermutation (l). FOVs are elaborately selected for visualization according to location of each the clonal activities. For panel l, each hollow dot represents a cell, colored by mutation frequencies

m. Lineage tree plot and spatial plot shows the clone family 375 and subclones 2/15/21 in different FOVs. Only subclones 2/15/21 are shown in spatial plot. In lineage tree, dots are sized by clone size. In spatial plot, each dot/polygon represent a cell, colored by subclone definition.

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**Figure 3. *Stereo-XCR-seq reveals*** ***pronounced spatial heterogeneity of immature TLS in LUAD.***

**a.** Pie chart shows the percentage of mTLS and iTLS appearance in LUAD H&E staining images. **b**. Box plot shows the frequencies of mTLS and iTLS in 57 inflamed LUAD H&E staining images. Data are presented as mean±25% percentile, with error bar indicating extreme value. \*\*\*\*, p-value<0.00-1. **c**. Spatial plot shows iTLS in LUAD P1. FOVs presents the region of interests, along with the CXCL13, MS4A1, CD3E expression in this FOV. Each dot represents a bin50 spot, colored by cluster or expression level respectively. **d**. Dot plot shows the expression of gene of interests by different spatial clusters (bin50 spots). Dots are colored by mean expression and sized by expression fraction.**e**. Spatial plot shows spatially discrete iTLS clusters in LUAD P1. Each dot represents a bin50 spot, colored by clusters. The cluster numbers are labeled nearby each cluster. **f**. Bar chart shows the proportion of iTLS-emigrating TCRβ clones and IgH clones. Each dot represents an iTLS cluster corresponding to panel d. Data are presented as mean±SEM. N=10. **g**. Venn plots show the shared IgH clones by the top 5 iTLS clusters. iTLSs 7/3/8/0 are compared with iTLS 6 respectively. **h**. Elbow plots show the sharing of IgH clones (left) and TCRβ clones (right) by different iTLS clusters in each tumor. The sharing clones are plotted as absolute counts (up) and percentage (below) respectively. Each line represents a tumor, colored by patient number as indicated below. **i**. Spatial plots show the distribution of iTLS-emigrating IgH clones. Each plot presents one iTLS-associated clones as labeled above. The iTLS cluster of interest is colored in blue and highlighted by red border. The emigrating distributions are presented as kernel density estimate (KDE) plot, with the most enriched niche highlighted by green borders. The KDE plots are colored by densities. **j-k**. Spatial plots show the representative distribution of cells of interests (**j**) and related gene expression (**k**) in 2 LUAD tumors. Each dot represents a cell, colored by either cell type (**j**) or genes (**k**).

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**Figure 4. *Stereo-XCR-seq delineates spatiotemporal fate trajectories of B/plasma cells in iTLSs.***

**a**. Spatial plot shows the spatial expression of *MS4A1* (green), *CXCL13* (red), *IGKC* (purple) and *JCHAIN* (blue) in LUAD tumor. Each dot represents a bin20 spot, colored by genes and hued by UMI counts. Tissue borders are presented as white outlines. 2 FOVs are selected to present the co-expression of *MS4A1/CXCL13* (green frame) or *IGKC/JCHAIN* (purple frame). Scale bar is set at 1mm. **b**. FOVs selected from pane **a** toshow the co-expression of *MS4A1/CXCL13* (green frame) or *IGKC/JCHAIN* (purple frame). Each dot represents a bin20 spot, colored by genes and hued by UMI counts.

**c**. UMAP plots show the re-clustering of lymphoid aggregates (left up) and gene expression by each sub-clusters (other plots). Each dot represents a bin50 spot, colored by either clusters (left-up) or UMI counts (other plots). **d**. Spatial plot shows representative view of inner and outer iTLSs in LUAD P4 and P6. Each dot represents a bin50 spot, colored by clusters. **e**. Spatial plot shows the gene expression in the representative view of inner and outer iTLSs in LUAD P4 and P6. Each dot represents a bin50 spot, colored by UMI counts of each gene. **f**. Dot plot shows the expression of geneof interests by inner iTLSs and outer iTLSs (bin50 spots). Dots are colored by mean expression and sized by expression fraction. **g**. Dot plot shows the expression of geneof interests by B/plasma cells in different spatial clusters. Dots are colored by mean expression and sized by expression fraction. The cell stages corresponding to the gene expression are labelled in the parentheses. **h**. Boxen plot shows the B/plasma cell pseudotime of B/plasma cells in different spatial sub-clusters. Pairwise significances are assessed by Welch’s t-test: \*\*\*, p < 0.001. **i**. Spatial quiver maps show the migrating paths of B/plasma cells in iTLS clusters. Each dot represents a B/plasma cell, colored by spatial clusters. White vectors represent the migrating path. FOVs are selected to present the B/plasma cells egress. **j**. Spatial plot shows the B/plasma pseudotime in the representative view of inner and outer iTLSs in LUAD P4 and P6. Each dot represents a bin50 spot, colored by pseudotime score. **k**. Dot plot showing the Pearson’s correlation between gene expression and pseudotime scores. Only top genes (r>0.1) are plotted. Each dot represents a gene, colored and sized by p-value as indicated on the right. Gene of interests are listed above the plot. **l**. Multiple curve plots show the nonlinear quartic fitting of normalized UMI counts of each highlighted genes and pseudotime score in B/plasma cells. Curves are colored by genes.

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**Figure 5. *Stereo-XCR-seq*** ***unveils IgG+PCZ as ectopic GCs for tumor-reactive lymphoctyes priming in LUAD.***

**a**. Spatial plot shows the cells in PCZ and iTLS. FOVs are selected to show the distribution of PCZ and iTLS. iTLS in P10 FOV is highlighted in black. Each dote represent a cell, colored by spatial clusters. **b**. Spatial KDE plot shows the CSR event densities in LUAD tumors. FOVs are selected corresponding to panel **a**. iTLS in P10 FOV is highlighted in black to show the missing CSR events in the iTLS. **c**. Paired Box-strip plot shows the comparison of CSR event percentage in iTLS and PCZ of 11 tumors. Paired t-tests, \*\*, p-value<0.01; \*\*\*,p-value<0.001. Boxes are presented as mean±25 percentile, with error bar indicating extreme values. **d**. Heatmap shows the normalized CSR frequencies of B/plasma cells in iTLS and PCZ. Each square indicates a type of switching class indicated by the Y labels and X labels, colored by average CSR frequencies shown as numbers. Arrows indicate the uprising events. **e**. Spatial plot shows the IgA-IgG and IgM-IgG events in PCZ and iTLS. FOVs are selected corresponding to panel **a**. Each dote represent a cell, colored by CSR types. **f**. Paired Box-strip plots show the comparison of different CSR event frequencies in iTLS and PCZ of 11 tumors. Paired t-tests, \*\*\*, p-value<0.001; \*\*\*\*, p-value<0.0001. Boxes are presented as mean±25 percentile, with error bar indicating extreme values. **g**. Dot plot shows the expression of geneof interests in different spatial clusters (bin50 spots). Dots are colored by mean expression and sized by expression fraction. **h**. Paired Box-strip plots show the comparison of T cell infiltration in IgG+PCZ and IgM+PCZ of 11 tumors. T cells were counted by using spatial transcriptome data. Paired t-tests, \*\*, p-value<0.01. Boxes are presented as mean±25 percentile, with error bar indicating extreme values. **i**. Spatial plot (above) shows the cells in PCZ and iTLS. FOVs are selected to show the distribution of IgG+PCZ and IgM+PCZ. Each dote represent a cell, colored by spatial clusters. Multiplexed immunofluorescence staining using the adjacent tissue sections show the CD20+, CD3E+ and CD138+ cells repectively. FOVs are selected according to the spatial plots above to show the T cells infiltration in IgG+PCZ. **j**. Paired Box-strip plots show the comparison of T cell infiltration in IgG+PCZ and IgM+PCZ of 11 tumors. T cells were counted by using mIF staining data. Paired t-tests, \*\*, p-value<0.01. Boxes are presented as mean±25 percentile, with error bar indicating extreme values. **k**. Spatial plot shows the whole slide distribution of T cell clones present in IgG+PCZ (up)or absent in IgG+PCZ (below). Each dote represent a cell. Spatial KDE plot of T cell clones presents in IgG+PCZ is overlayed to show the aggregation of these clones. **l**. Paired Box-strip plots show the Moran’s Index of T cell present or absent in IgG+PCZ in 11 tumors. Each dot represents a tumor. Paired t-tests, \*\*, p-value<0.01. Boxes are presented as mean±25 percentile, with error bar indicating extreme values. **m**. Grouped box-whisker plots show the clone sizes of each T cell clone present or absent in IgG+PCZ in 11 tumors. Unaired t-tests, ns, p-value>0.05; \*\*\*\*, p-value<0.0001. Boxes are presented as mean±25 percentile, with whisker bar set to Tukey. **n**. Dot plot shows the expression of geneof interests by cells in tumor. Cells in tumor are categorized into 3 clusters, T cell clones present in IgG+PCZ, T cell clones absent in IgG+PCZ and other non-T cells as negative control. Dots are colored by mean expression and sized by expression fraction. The functional annotation of each gene is labelled in the parentheses.

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**Figure 6. *Stereo-XCR-seq profiles exclusion of ectopic GCs by terminally differentiated cancer-associated fibroblasts*.**

**a**. Graph shows the definition of non-expanded stroma and expanded stroma. **b**. Stacked bar chart shows the frequencies of spatial spots with indicated clone sizes in different clusters. Bars are colored by spatial clusters as indicated above. **c**. Spatial plot shows clone sizes of each stromal spots (left). Each dot represents a bin50 spot, colored by clone sizes. Spatial KDE plot shows the distributive density of non-expanded stroma (middle) and expanded stroma (right). **d**. Spatial KDE plot shows the distributive density of IgG+PCZ in LUAD P1. **e**. Dot plot shows the expression of geneof interests in stroma spots at different clone sizes. Dots are colored by mean expression and sized by expression fraction. **f**. Dot plot showing the log10 fold change of the genes upregulated in expanded stroma spots. Genes are ranked by log10 fold changes. Each dot represents a gene, colored and sized by p-value as indicated on the right. Gene of interests are listed above the plot. **g**. Spatial KDE plot shows the expression density of gene of interests in LUAD P1. **h**. Heatmap shows the expression of gene of interests in stroma spots. **i**. Dot plot shows the expression of geneof interests by different cell clusters in scRNA-seq (GSE148071) data. Dots are colored by mean expression and sized by expression fraction. **j**. Dot plot shows the expression of geneof interests by fibroblasts in single cell resolved spatial transcriptome of LUAD P1. Dots are colored by mean expression and sized by expression fraction. **k**. UMAP KDE plot shows the expression density *CD34* in fibroblasts. **l**. UMAP plot shows the diffusion pseudotime in fibroblasts. **m**. UMAP KDE plot shows the distribution of fibroblasts in expanded stroma (left) and non-expanded stroma (middle). Vectors on UMAP show the 2 differentiation paths of fibroblasts. Line width indicates the transcriptional similarities. **n**. Spatial KDE plot shows the pseudotime of fibroblast in the tissue section. **o**. Dot plot shows the GO term analysis using genes upregulated in expanded stroma (left) and non-expanded stroma (right). Dots are colored by combined scores and sized by percentage of genes in each gene set.

**Reference**

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