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Decoding the Tumor Heterogeneity: Advances and Perspectives of Single-Cell Transcriptomic and Single-Cell Proteomics in Cancer Research

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Abstract. Diverse molecular and phenotypic variations can be observed across tumour-cell populations, manifested as inter-patient discrepancies, spatial intra-tumour diversity and phenotypic shifts triggered by anti-cancer therapies. Conventional analytical workflows including pathological sections, bulk RNA-seq and bulk-level proteomics generate population-averaged read-outs and fail to resolve distinct tumour-cell sub-clones. Single-cell RNA sequencing (scRNA-seq), supported by cell barcodes and Unique Molecular Identifiers, achieves high-resolution transcriptomic profiling for individual tumour cells, as demonstrated in exploratory clinical studies of gastric cancer. Nevertheless, transcriptomic profiles alone cannot reflect actual cellular function owing to extensive post-transcriptional regulatory events. Complementary single-cell proteomic tools, such as phospho-protein flow cytometry, mass-spectrometry-based pipelines and microfluidic single-cell western blotting (scWestern), have been applied to patient-derived leukaemia blasts and circulating tumour cells to map protein-level variation. Despite valuable biological outputs, these techniques are constrained by technical drawbacks, sample-processing-related artifacts, interpretive pitfalls and considerable translational barriers. Multi-omic integration of transcriptomic and proteomic datasets represents a practical research direction. Further technical refinement and validation in large independent clinical cohorts are required to obtain clinically actionable tumour biomarkers.

Keywords: tumor heterogeneity, single-cell RNA sequencing, single-cell proteomics, microfluidic single-cell western blotting, cancer biomarkers

1. Introduction

Tumor heterogeneity is a fundamental feature of malignant tumors and form a barrier to precise cancer therapeutics and accurate prediction assessment. This heterogeneity primarily demonstrates in three key forms: inter-individual heterogeneity among patients with the same tumor type, spatial dimensions reginal differences among tumors and therapy-induced dynamic heterogeneity driven by chemotherapy, radiotherapy and targeted treatment. There are three common conventional analytical methods: Pathological sections, flow cytometric analysis and western blot. These three methods are both feature mature and widely adopted technical systems that enable direct detection of tumor

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biomarkers and qualitative or semi-quantitative analysis of differential molecular expression. However, they all have prominent limitations. Firstly, Pathological sections only obtain local tissue samples from lesions and are susceptible to sampling bias due to tumor heterogeneity, failing to fully reflect the overall cellular lineage characteristics of tumors. Second, flow cytometric analysis only examines dissociated single cells derived from partial tissue. Tumor lesions contain diverse cell subpopulations with distinct genotypes and phenotypes. Local sampling may omit rare, drug-resistant tumor clones, and the measured proportions of cell subsets cannot reflect the overall characteristics of the lesion, leading to biased experimental conclusions. On the same time, Bulk RNA-seq also only captures averaged transcriptional signals from bulk tissue and cannot resolve gene expression profiles of distinct intratumoral subpopulations. Simultaneously, traditional Western blot also fails to reflect protein expression differences among individual cells. Collectively, these conventional approaches only yield population-averaged or macroscopic results, making it difficult to dissect tumor cellular heterogeneity comprehensively. In contrast, emerging single-cell analytical technologies overcome the limitations of bulk detection and provide high-resolution tools for tumor heterogeneity research.

There are three representative single-cell technologies: single-cell RNA sequencing (scRNA-seq), single-cell proteomics (scProteomics), and single-cell Western blotting (scWestern). Among these tools, scRNA-seq is a well-developed transcriptomic method. It mainly collects overall gene expression data from individual tumor cells. This technology works well for finding different cell subgroups and studying transcriptional heterogeneity. Transcriptomic data alone cannot fully show the real conditions of cells. Single-cell proteomics makes up for this limitation. It provides large-scale protein information to show how cells actually function. It fills the information gap between gene activity and cell real-world performance. Unlike the two methods above, single-cell Western blotting does not run large-scale full-sample tests. It focuses on highly sensitive targeted tests. At the single-cell level, it detects and checks key functional proteins and their post-translational modifications. It is a useful tool for studying core signaling molecules. When researchers combine these three technologies, they can carry out multi-dimensional analysis. This analysis covers transcription, whole-proteome data and targeted functional-protein checks. It helps scientists understand tumor heterogeneity, which traditional testing methods cannot describe well.

2. Single-cell RNA sequencing (scRNA-seq)

Single-cell RNA sequencing (scRNA-seq) is a high-resolution tool to map gene expression within individual tumour cells. First, intact and viable tumour cells are isolated and sorted to obtain pure single-cell samples. Each cell is confined in an independent microreaction environment. Next, cells are lysed to release mRNA, which is reverse-transcribed into stable cDNA. The cDNA is amplified and processed to build sequencing libraries for high-throughput sequencing. After generating raw sequencing data, researchers carry out strict quality control to remove low-quality reads. Valid sequences are then aligned against gene reference databases to calculate gene expression levels for each cell. For data analysis, dimensionality reduction methods such as PCA, t-SNE and UMAP are used to group cells and identify distinct tumour cell sub-populations. PCA, t-SNE and UMAP are common dimensionality reduction algorithms. They transform high-dimensional gene expression data of single cells into two-dimensional coordinates to visualize cell clustering. PCA is generally used for preliminary data preprocessing. t-SNE focuses on local cellular similarity, while UMAP preserves both local features and global structures, making it the most widely used visualization method in current single-cell analysis. Heatmaps and volcano plots visualize differentiated expressed genes to aid the investigation of tumour heterogeneity. Compared with traditional

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experimental techniques, scRNA-seq has clear advantages. Methods including Western Blot and bulk RNA sequencing only measure average signals from a large mixture of cells. They cannot capture transcriptional differences between individual cells and hide tumour heterogeneity. On the contrary, scRNA-seq enables unbiased, high-throughput transcriptional profiling at single-cell resolution. It reveals subtle differences among tumour cells and supplies precise expression data for tumour research. To accurately track cell sources and reduce bias caused by PCR amplification. Additionally, ScRNA-seq relies on two molecular labeling systems: cell barcode and Unique Molecular Identifier (UMI).

Microbeads modified with barcode sequence is one of the key materials for cell labeling in droplet-based scRNA-seq platforms. Every bead is connected to plenty of oligonucleotide probes. All probes on one bead share the same cell barcode, while different beads carry unique barcode sequences. Within microfluidic devices, one single tumour cell and one barcoded bead are wrapped into a tiny droplet. After cell lysis, released mRNA binds to probes on the bead and gets reverse-transcribed into cDNA labeled with the corresponding cell barcode. After library preparation, cDNA from thousands of cells can be mixed and sequenced together. During data analysis, sequencing reads with identical cell barcodes are grouped and assigned to the same original cell. Researchers can therefore separate transcriptional information from different cells using computational methods. This bead-based labeling approach avoids preparing separate reaction systems for every single cell, greatly improving experimental throughput and supporting large-scale transcriptional analysis of tumour cell populations.

Unique Molecular Identifiers (UMIs) are short random sequences attached to the probes on barcoded microbeads. Each captured original mRNA molecule obtains a unique UMI tag before cDNA amplification. PCR amplification often causes uneven amplification, where some cDNA fragments are copied far more frequently. If researchers directly count sequencing reads, they will overestimate the true abundance of original mRNA transcripts. UMIs solve this problem effectively. All copies amplified from one original mRNA carry the same UMI. In data processing, reads with matching cell barcode, gene information and UMI are counted as one single original RNA molecule. This reduplication process removes repeated sequences generated by PCR and corrects amplification noise, enabling accurate counting of genuine mRNA molecules. Converting read-based expression matrices into UMI-count matrices improves the precision of gene expression quantification and makes results of tumour heterogeneity and differential gene analysis more reliable.

Although scRNA-seq provides valuable transcriptomic information, mRNA levels cannot reliably represent protein status inside cells. Genetic information flows from DNA to RNA and finally to functional proteins, yet multiple regulatory gaps exist between RNA and protein. Differences in translation efficiency mean mRNAs are not translated equally. Dynamic protein degradation further decouples RNA and protein abundance. Moreover, post-translational modifications such as phosphorylation and acetylation alter protein activity without changing mRNA quantity, and proper subcellular localization is also required for proteins to exert functions, neither of which can be detected by RNA sequencing. As the real functional executors of cellular activities, proteins directly determine cell phenotypes. Hence, single-cell proteomics complements transcriptome sequencing and enables us to dissect tumour heterogeneity from a functional perspective.

3. Single-cell proteomics

Single-cell proteomics enables quantification of protein abundance at the resolution of individual cells. Undoubtedly, cellular function is ultimately executed by proteins, which means transcriptome data like scRNA-seq cannot completely reflect real cellular phenotypes. Conventional bulk

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proteomics merely yields averaged protein signals from cell mixtures and masks intercellular differences. By contrast, single-cell proteomics resolves cell-specific variations in protein expression, offering unique opportunities to investigate tumour heterogeneity at the functional protein level. Two major technical approaches dominate this research area: antibody-based detection and mass spectrometry-based detection.

Firstly, antibody-targeted single-cell proteomics relies on labelled antibodies to detect predefined protein markers, mainly including flow cytometry and CyTOF (mass cytometry). In flow cytometry, fluorescent-labelled antibodies bind target proteins, and the instrument detects fluorescence signals to quantify protein expression within individual cells. On the other hand, CyTOF employs antibodies labelled with heavy metal isotopes instead of fluorescent dyes. After antibodies bind to target proteins on or inside single cells, samples are analysed by inductively coupled plasma mass spectrometry. It overcomes the spectral overlap limitation of fluorescence detection and simultaneously measures dozens of protein markers in one cell, but its instrument cost is higher and throughput is lower than conventional flow cytometry. Both techniques require pre-selected target proteins and cannot discover unknown molecules, leading to inherent detection bias.

Moreover, bottom-up mass spectrometry-based single-cell proteomics provides untargeted global protein profiling. The workflow starts with accurate isolation of single tumour cells. Intracellular proteins are extracted and digested into peptides, followed by separation via nano-liquid chromatography. The separated peptides undergo ionization and mass spectrometric scanning to produce characteristic spectra. Different peptide fragments are differentiated by their mass-to-charge ratios, and secondary fragmentation data is used to deduce peptide amino acid sequences. In addition, peptide samples are analysed by LC-MS to generate raw data. Subsequent database matching enables protein identification and quantification. This platform can capture thousands of proteins in a single cell, including unknown protein molecules, which overcome the limitation of antibody-targeted single-cell proteomics.

4. Single-cell Western blotting (scWestern)

Single-cell Western blotting (scWestern) enables targeted detection and quantitative verification of key functional proteins within single tumour cells. The whole process starts with fabricating a polyacrylamide gel microfluidic chip. Gel precursor solution is added onto a silicon lithography template patterned with micropillars, then covered and pressed with a silanized glass slide to form an even thin gel layer. After polymerization, the gel forms numerous independent microwells for capturing single cells, and the glass slide is peeled off. Prepared single tumour cells settle into microwells by gravity, followed by UV in-situ lysis to extract intact proteins with natural modifications preserved. In-chip electrophoresis separates proteins by molecular weight under an electric field. After TBST blocking to reduce non-specific binding, chips undergo overnight incubation with specific primary antibodies. Unbound primary antibodies are fully washed away, and fluorescent secondary antibodies are added to bind primary antibodies, with dim-light washing afterwards to avoid fluorescence quenching. Fluorescence scanning produces clear protein bands. Software measures band grey values, and target protein relative expression is quantified via normalization to housekeeping reference proteins. Original scanning images can be viewed directly, and quantitative data are converted into graphs to show protein expression differences among individual tumour cells.

Compared with antibody-based CyTOF and untargeted mass spectrometry-based single-cell proteomics, scWestern has unique characteristics. Similar to CyTOF, scWestern is a targeted method that only detects predefined proteins and cannot discover unknown molecular targets. Unlike

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CyTOF, it directly separates proteins according to molecular weight and can reliably identify and quantify protein modifications. In contrast to bottom-up mass spectrometry, scWestern does not achieve proteome-wide profiling. Its visualized band signals offer stable, repeatable evidence for validating signalling proteins. However, scWestern suffers from low throughput. It cannot analyse thousands of proteins in one run and is not suitable for large-scale, unbiased exploration of the global molecular landscape of tumour cells.

5. Clinical application

Single-cell RNA sequencing (scRNA-seq) enables the measurement of cell-specific transcriptional variation, and its foundational experimental system was first reported by Tang and colleagues in 2009. Prior single-cell amplification methods only generated short cDNA fragments and failed to capture rare low-expression transcripts efficiently. The team resolved this issue by lengthening the incubation time for reverse transcription and extending the elongation step of PCR, allowing the production of full-length cDNA reaching up to 3 kb. When benchmarked against traditional microarrays that rely on pooled cell populations, sequencing data from just one mouse blastomere recovered an extra 5,270 expressed genes and uncovered 1,753 previously unannotated splice junctions. This experiment clearly illustrated that bulk sequencing conceals widespread transcript heterogeneity between individual cells, laying the groundwork for applying scRNA-seq to human clinical tumour samples. One representative translational study focused on gastric cancer: Wang et al. collected surgically resected primary gastric lesions and matched metastatic lymph node tissues from three patients [1], and carried out Smart-seq2 single-cell transcriptome profiling on these clinical specimens, instead of cultured cell lines or animal tumour models. In line with the core strength of Tang's technique to resolve cell-level differences, unsupervised clustering separated malignant cells into four distinct transcriptional subgroups. Pseudotime trajectory analysis further captured transitional cell states bridging primary and metastatic tumour clones—populations impossible to resolve through bulk RNA sequencing. The research also noted divergent tumour cell transcript profiles across individual patients, alongside consistent microenvironment cell types shared among samples. Genes linked to lymphatic metastasis, such as ERBB2 and CLDN11, as well as core transcription regulators FOS and JUN, were identified from single-cell data, offering transcriptomic evidence to explain the inconsistent metastatic capacity and variable drug responses commonly seen in gastric cancer clinical practice.

While this gastric cancer work demonstrates how Tang's single-cell transcriptome technology can be applied to clinical tumour tissues, the study has several notable constraints that prevent direct use in routine clinical evaluation. To begin with, the patient cohort only includes three sets of matched primary and metastatic samples. Such a small sample size cannot generate molecular signatures with broad statistical reliability, and the metastasis-associated genes identified were not validated using larger independent patient groups to confirm their ability to predict long-term prognosis. Second, the study used the Smart-seq2 platform, a full-length scRNA approach with low cell throughput, which limits comprehensive detection of rare immune and stromal cell subsets within clinical tumour tissue, unlike widely used high-throughput droplet sequencing systems today. Third, this research only measures mRNA levels, a universal limitation inherited from Tang's original single-cell RNA workflow. Transcript quantities do not reliably correspond to intracellular protein abundance, and the sequencing pipeline cannot capture kinase phosphorylation events including activated ERK and mTOR. For this reason, transcriptomic signatures alone cannot fully account for clinical phenotypes such as treatment resistance in gastric cancer subgroups. Finally, the analysis only provides static transcriptional snapshots of resected tumour tissue; it lacks longitudinal single-cell profiling tracking

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tumour evolution during perioperative or postoperative adjuvant therapy, so the detected cell subsets cannot be directly correlated with patients' real treatment responses and survival outcomes. Taken together, this study is merely a preliminary exploratory application of scRNA-seq on gastric cancer clinical material. Larger clinical cohorts and complementary single-cell proteomic and phosphoproteomic platforms are required to translate its observations into clinically usable biomarkers or treatment guidance.

Single-cell proteomic approaches fall into two major technical categories: antibody-based phospho-protein flow cytometry and mass-spectrometry. Antibody-based single-cell phospho-protein flow cytometry enables functional measurement of stimulus-driven signalling activity at single-cell resolution, as demonstrated in profiling primary acute myeloid leukaemia (AML) patient blasts [2]. Conventional bulk-oriented molecular assessments largely relied on static basal phosphorylation status or fixed genetic lesions such as Flt3-ITD, yet these read outs could not reliably predict patient chemotherapy outcomes. Irish and co-workers addressed this limitation by performing ex-vivo cytokine stimulation prior to phospho-specific flow cytometry measurement, focusing on cellular signalling potential rather than only resting-state protein modification [2]. Compared with conventional genotyping, this single-cell functional profiling demonstrated that Flt3-mutant AML blasts did not necessarily show elevated basal Stat5 phosphorylation; instead, these malignant cells exhibited markedly enhanced Stat3 and Stat5 phosphorylation upon exposure to myeloid cytokines. Unsupervised clustering built from phosphoprotein-response signatures separated AML patients into discrete signalling clusters [2]. Among these subgroups, the SC-P2 cluster, defined by potentiated Stat3/5 activity together with blunted Stat1 signalling, was strongly associated with chemotherapeutic resistance and early disease relapse. This functional single-cell assay uncovered malignant cell sub-populations masked within bulk tumour measurements and provided phenotypic evidence explaining divergent treatment responses observed among patients carrying identical driver mutations [2]. Even so, this antibody-dependent workflow is restricted to pre-selected phosphoprotein targets. It also requires intact viable primary leukaemia blasts for ex-vivo cytokine perturbation [2]. Although this strategy supports functional risk-stratification for AML patient samples, large-scale multi-centre clinical validation remains essential before routine clinical implementation.

While antibody-based flow cytometry delivers targeted read outs for predefined signalling nodes, mass-spectrometry based single-cell proteomics allows large-scale unbiased protein quantification without pre-defined antibody panels [3]. Conventional bulk-level proteomic mass-spectrometry averages signals across large cell populations and conceals intra-tumour cellular heterogeneity [3]. SCoPE-MS integrates mechanical single-cell lysis, TMT isobaric labelling and carrier-cell-assisted peptide identification to quantify more than one thousand proteins within individual mammalian cells. Side-by-side comparisons between single-cell proteomes and transcriptomes obtained from differentiating mouse embryonic stem cells revealed substantial discordance between mRNA-level covariation and corresponding protein-level covariation for development-associated genes [3]. This observation points to widespread post-transcriptional regulatory events that cannot be captured solely through single-cell RNA sequencing. Nevertheless, multiple practical bottlenecks prevent direct clinical translation of SCoPE-MS and related mass-spectrometry-based single-cell proteomic workflows. Firstly, inherent technical sensitivity constraints allow robust detection mainly for highly abundant proteins, leaving many low-copy signalling regulators and tumour-relevant markers undetected [3]. Secondly, the technique has relatively high per-cell cost, low experimental throughput and complicated sample-handling workflows that are difficult to standardise across independent laboratories [3]. Thirdly, unlike perturbation-capable phospho-flow cytometry assays

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described above, most existing single-cell MS-proteomic pipelines measure static steady-state protein abundance and are poorly suited for capturing dynamic stimul-triggered signalling responses [3]. Collectively, this class of technology remains largely confined to exploratory basic-science research. Expanded clinical sample cohorts, improved detection sensitivity, streamlined experimental pipelines and rigorous independent validation are all indispensable prerequisites before unbiased single-cell mass-spectrometry proteomics can generate clinically actionable protein-level biomarkers or inform therapeutic decision-making for human tumour specimens.

Unlike conventional antibody-based single-cell proteomic tools such as flow cytometry and mass cytometry, which rely solely on antibody-antigen binding for target identification, microfluidic single-cell western blotting (scWestern) introduces gel-electrophoretic separation by molecular weight before immunodetection to mitigate off-target antibody cross-reactivity [4]. This targeted proteomic workflow was developed for profiling rare circulating tumour cells (CTCs) isolated from oestrogen-receptor-positive metastatic breast-cancer patient blood. CTCs are difficult to analyse via standard cytometry owing to substantial cell loss during sample processing. The assay combines microwell-array single-cell capture, in-situ cell lysis, PAGE protein separation, UV-triggered covalent protein photo-immobilisation, and iterative antibody stripping-reprobing to achieve multiplexed protein readouts. Validated with cancer-cell lines spiked into healthy-donor blood, scWestern enables detection of 8-12 distinct protein markers within individual CTCs, covering oncoproteins, intracellular signalling mediators and house-keeping proteins. Studies on patient-derived primary CTCs revealed a unique lysis-resistant biophysical phenotype absent in cultured cancer-cell lines, demonstrating notable phenotypic divergence between clinical primary tumour material and laboratory cell-line models. Moreover, scWestern captured substantial inter-cell protein heterogeneity and two statistically distinct GAPDH-expressing CTC subpopulations, features completely concealed by bulk-level population measurements. Even so, several practical hurdles restrict its direct translation into routine clinical workflows. As a targeted technique, scWestern is dependent on high-quality antibodies and can only interrogate predefined protein panels, without capacity for unbiased global proteome discovery. The experimental pipeline remains labour-intensive: manual micromanipulator-assisted cell placement into microwells is required, leading to relatively low sample throughput. Although UV-covalently fixed protein samples can be archived for later re-probing, the platform demands intact rare primary CTC material, and large-scale multi-patient clinical cohort evaluations have not yet been reported. Collectively, microfluidic scWestern serves as a valuable complementary targeted-proteomic approach for studying rare circulating tumour-cell populations [4]. Simplified sample-handling protocols and large independent clinical cohorts will be required before this technology can generate clinically actionable protein biomarkers to guide oncological therapeutic decisions.

6. Conclusion

Single-cell RNA-seq, underpinned by cell barcodes and unique molecular identifiers (UMIs), enables high-dimensional transcriptional profiling of individual tumour cells and uncovers tumour-cell subpopulations concealed in bulk measurements [1, 5]. By applying dimensionality-reduction algorithms such as PCA, t-SNE and UMAP, researchers can resolve intratumour heterogeneity and identify differentially expressed genes linked to tumour progression and metastasis [1]. Nevertheless, as described in the technical overview of this work, mRNA abundance provides incomplete information about cellular functional phenotypes. Discrepancies originate from variable translation efficiency, protein turnover, post-translational modifications including phosphorylation and acetylation, as well as protein subcellular localisation, all of which cannot be captured by

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transcriptome sequencing alone [1]. This fundamental gap has driven the rapid development of single-cell proteomic approaches, broadly categorised into antibody-dependent detection strategies and mass-spectrometry-based untargeted workflows, alongside the specialised microfluidic single-cell western blotting (scWestern) platform.

Antibody-based phospho-protein flow cytometry, SCoPE-MS-type mass-spectrometry proteomics and scWestern each possess distinct technical merits that have been demonstrated in patient-derived clinical tumour material [2, 3, 6]. Phospho-protein flow cytometry can conduct ex-vivo cytokine perturbation to probe signalling potential at single-cell resolution. In primary AML patient blasts, this functional read-out revealed that Flt3-ITD mutation-associated chemoresistance was reflected by augmented cytokine-triggered Stat3/Stat5 signalling rather than elevated basal phosphorylation, offering phenotype-level evidence for heterogeneous therapeutic responses among patients carrying identical driver mutations [2]. Even so, this perturbation-based assay is subject to a critical experimental confounder: isolated leukaemia cells are removed from the native bone-marrow microenvironment; ex-vivo cytokine stimulation may not faithfully mirror in-vivo signalling crosstalk within patients [2]. By contrast, SCoPE-MS-driven mass-spectrometry based single-cell proteomics delivers large-scale unbiased protein quantification without antibody prerequisites. Comparisons between single-cell proteome and transcriptome data from differentiating embryonic stem cells have exposed widespread post-transcriptional regulation that remains invisible to scRNA-seq [3]. Still, mass-spectrometry workflows measure total protein abundance; high protein expression cannot be directly equated with activated signalling function, and phosphorylation states frequently escape detection without dedicated enrichment steps [3]. As a specialised targeted tool, scWestern combines in-gel molecular weight separation with antibody probing to reduce non-specific antibody cross reactivity, and it is well-suited for analysing rare circulating tumour cells isolated from breast-cancer patient blood [4]. Patient-derived CTCs display unique lysis-resistant biophysical properties not observed in cultured cell lines, which implies that enrichment, micromanipulation and ex-vivo handling may remodel intrinsic cellular protein profiles and complicate extrapolation back to in-vivo tumour biology [4]. Beyond commonly cited practical barriers such as limited throughput, high cost and antibody-panel dependency, these single-cell technologies face several less-emphasised analytical and translational bottlenecks that impede direct clinical deployment.

Sample pre-processing represents a substantial, often underestimated source of technical artefact shared across these platforms. Tumour-cell dissociation, fluorescence-activated cell sorting, CTC enrichment and manual micromanipulation during scWestern sample preparation can alter cellular phosphorylation status, stress-related protein expression and protein isoform patterns before any formal measurement is performed [2, 4]. Variation introduced by experimental handling can be misclassified as biologically meaningful tumour-cell heterogeneity. A second major interpretive challenge stems from causal inference constraints. Most single-cell proteomic outputs yield protein abundance values. Even with reliable quantitative data, researchers cannot automatically assign functional or prognostic significance merely based on elevated protein levels; orthogonal functional validation is required to link measured protein profiles to actual tumour-cell behaviour [3]. Third, there exists a non-trivial translational chasm between documenting high-dimensional tumour-cell heterogeneity and generating clinically actionable biomarkers. Single-cell omics studies frequently describe multiple discrete malignant sub-clones within patient specimens. Routine clinical diagnostic assays, however, demand parsimonious, compact molecular signatures. Complex multi-dimensional single-cell profiles cannot be readily condensed into simple, low cost laboratory tests suitable for patient stratification. Analogous to the limitations observed in the gastric cancer scRNA-

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seq exploratory study [1], most existing single-cell proteomic tumour investigations remain small scale proof of concept work. They uncover molecular differences among cell subsets but rarely validate candidate protein-based signatures in large, fully independent patient cohorts to test predictive performance for treatment response or survival outcomes. Consequently, encouraging observations obtained from both single-cell transcriptomic and proteomic studies have not yet been translated into standard prognostic biomarkers or therapeutic decision-making tools for oncological practice.

Given the complementary nature of transcriptomic and proteomic measurements, multi-omic integration combining scRNA-seq with single-cell proteomic or phosphoproteomic assays provides a promising path forward. scRNA-seq powered by cell barcodes and UMIs delineates cellular lineage architecture and sub-clonal transcriptional variation. Single-cell proteomic approaches add functional layers of information, including signalling pathway activity, protein isoform distribution and post-translational modifications which mRNA-level data cannot capture. To move closer towards clinical applicability, future tumour-oriented single-cell research should address several concrete priorities. Experimental protocols need optimisation to minimise artefacts introduced by cell isolation, ex-vivo perturbation and sample manipulation, and should incorporate better control conditions that mimic in-vivo microenvironmental cues. Analytical pipelines ought to implement stricter filtering criteria to separate genuine biological heterogeneity from processing driven technical noise and discourage over-interpretation of protein-abundance measurements as direct functional evidence. Most importantly, preliminary discoveries from exploratory single-cell experiments must undergo rigorous evaluation in sufficiently-powered independent clinical cohorts. Such validation will determine whether high-dimensional single-cell molecular profiles can be distilled into parsimonious biomarker panels capable of predicting metastatic potential, drug responses and patient survival.

In summary, scRNA-seq has laid a solid foundation for dissecting intratumour heterogeneity at the transcriptional level, yet it cannot fully recapitulate functional tumour phenotypes governed by proteins and their post-translational modifications. Current single-cell proteomic platforms, including antibody-based phospho-flow cytometry, mass-spectrometry driven workflows and scWestern, have demonstrated notable research value in resolving malignant subpopulations, characterising signalling network plasticity and uncovering post transcriptional regulatory events in clinical tumour specimens. Nevertheless, apart from well-known technical shortcomings, confounding factors arising from sample handling artefacts, intrinsic detection range limits, limits on causal biological inference, and difficulties in simplifying high-dimensional omics data into clinically usable signatures constitute major obstacles for routine clinical adoption. Continued technical optimisation, careful control of experimental artefacts and stringent multi-cohort clinical validation will define whether single-cell proteomics can bridge the gap between molecular tumour profiles and real-world patient therapeutic outcomes.

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