Single cell sequencing in hematology

Charlotte K. Brierley[1,2,3], Adam J. Mead[1,2,3]

[1] Medical Research Council (MRC) Weatherall Institute of Molecular Medicine (WIMM); [2] MRC Molecular Haematology Unit; [3] NIHR Biomedical Research Centre, University of Oxford, Oxford, OX3 9DS, United Kingdom.

Corresponding author:

Prof Adam J. Mead

Medical Research Council (MRC) Weatherall Institute of Molecular Medicine

Headley Way

Oxford OX3 9DS

adam.mead@imm.ox.ac.uk;

Tel number: +44(0)1865 222340

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Abstract: (200 words max currently 192)

Bulk molecular profiling of hematological malignancies by next generation sequencing techniques has provided major insights into the molecular pathogenesis of blood cancers. This technology is now routinely implemented in advanced clinical diagnostics, leading to the development of novel targeted therapies. However, bulk genetic analysis can obscure key aspects of intratumoral heterogeneity which underlies critical disease events, such as treatment resistance and clonal evolution. The past few years have seen an explosion of novel techniques to analyse RNA, DNA, and protein expression at the single cell level, providing unprecedented insight into cellular heterogeneity. Given the ease of accessibility of liquid tumor biopsies, studies in hematological malignancies have been at the forefront of the application of this technology. In this review, we highlight key recent insights into hematopoiesis and hematological malignancies through the application of novel single cell approaches. We particularly focus on biological insights made through the study of stem/progenitors cells in myeloid malignancy at single cell resolution. Hematology is well positioned to move novel single cell techniques towards routine application in the clinic, and we discuss current and potential future applications for this technology in the management of patients with hematological cancers.

Keywords: Single cell analysis, intratumoral heterogeneity, cancer stem cells

Introduction

Intratumoral heterogeneity (ITH) is a key contributing factor to treatment failure and disease evolution in cancer.(1) Much of our understanding of ITH is based on genetic analysis of tumors at the bulk level, which have now comprehensively described the genetic diversity in single tumors, how this can evolve over time and under the selective pressure of therapy.(2) Clones so small as to be undetectable by bulk genetic analysis approaches may subsequently emerge as dominant clones which mediate relapse and disease progression.(3) It is important to recognise that ITH occurs at multiple levels, not only genetic but also at the cellular, epigenetic, spatial and transcriptional levels.(3) Furthermore, cellular heterogeneity in tumors encompasses not only genetically distinct subclones, but also cellular hierarchies, including presence of cancer stem cells (CSCs) as well as non-clonal cell populations such as residual normal tissue, stromal cells and immune cells.(4)

Although bulk sequencing has undoubtedly led to huge advances in the understanding of genetic ITH, it is important to recognise that this approach is subject to a number of limitations.(5) For example, inferring clonal structures from bulk variant allele fractions is inherently confounded by the presence of loss of heterozygosity or biallelic mutations and convergent evolution, where the same mutation might be acquired independently within different subclones.(5) Perhaps most importantly, characterising genetic heterogeneity in bulk analyses does little to unveil the biological differences between cells contained within specific genetic subclones.(6)

The first single cell technologies, such as flow cytometry or morphology, are already embedded in routine laboratory diagnostics in hematology. However, such techniques are restricted to a few dozen predefined markers or cell types. By contrast, single cell RNA-sequencing (scRNA-seq) can measure the expression of up to 10^4 genes simultaneously within an individual cell, providing unprecedented insights into cell state and diversity.(7) It is now feasible to profile DNA, RNA, protein, DNA methylation status, and chromatin accessibility at single cell resolution.(7) Additional developments in the field have provided advances in throughput and cost-effectiveness, the preservation of spatial information, and the ability to simultaneously detect multiple molecules in a single cell (‘multimodal profiling’) to provide a comprehensive overview of a single cell.(7) Novel computational tools to process the resultant datasets have enabled the discovery of new cell types, revised our understanding of the unperturbed hematopoietic hierarchy, and provide a powerful tool to analyse intra-and inter-tumoral heterogeneity.(6, 8-10) The challenge now lies in shifting the

application of these methodologies from description of cellular heterogeneity to a deeper understanding of disease mechanism and identification of tractable disease targets. Given the ease of accessibility of liquid tumor biopsies, studies in hematological malignancies have been at the forefront of the application of this technology. In this review, we highlight key recent insights into hematological malignancies through the application of novel single cell approaches.

New horizons in single cell methodologies

The past 2 years have seen a huge expansion in single-cell methodologies and associated bioinformatic toolkits designed to maximise the biological inferences that can be drawn from the data.(7, 11, 12) **Figure 1** outlines standard scRNA-seq experimental and bioinformatic workflows. The precise parameters of the sequencing approach are determined by the scientific question. Experimental variables include the number of cells to analyse (dictating ability to capture rare populations), the need for high-throughput (balanced against detection sensitivity for individual transcripts), whether to opt for full-length scRNA-seq to capture detail on splice variants versus 3' or 5'end scRNA-seq, which typically only provides absolute transcript count information. Conversion of multi-modal data into a common format enables multi-omic analysis – such as in CITE-seq, where the presence of cell surface proteins is detected by an oligonucleotide-tagged antibody.(13)

Current computational approaches to summating the cell-to-cell variability observed usually take one of two approaches: (1) Clustering of single cells into discrete groups based on differences between groups, with the aim of identifying biological distinctions such as cell type or state, or (2) Pseudotime analysis, whereby transcriptomic data is used to identify cell state transitions resulting in the ordering of cells along a temporal ('lineage differentiation') trajectory.(14-19) Learning how to best process these large and complex datasets is ongoing. Recent exciting insights include that mitochondrial (mt)DNA reads, generated as a by-product and previously discarded as experimental noise can in fact be used as natural genetic barcodes to trace somatic mtDNA mutations, track clones and infer cellullar hierarchies.(20) Another novel discovery was the fact that scRNA-seq data, rather than purely representing a single 'snapshot' of a cell in time, can be used to infer expression dynamics by exploiting the increase in unspliced (immature) to spliced (mature) mRNAs when a selected gene is upregulated, enabling prediction of the future state of individual cells.(21) To date,

single cell bioinformatics have been complex and expensive, but work packages are continuously undergoing improvement to render analysis more streamlined and user-friendly, all of which make transition to the clinical sphere more realistic.

Single cell analysis reveals how myeloid malignancies subvert the normal haematopoietic hierarchy

Haematological tumours are complex ecosystems characterised by multilevel heterogeneity.(22-24) A series of studies have applied single cell genetics to diagnostic acute myeloid leukemia (AML) patient samples to provide unprecedented resolution to the subclonal diversity observed in AML. These have demonstrated complex aberrant branching clonal hierarchies and enabled detailed analysis of clonal sequence and mutation order.(25-28) The largest study to date employed high-throughput scRNA-seq to profile the transcriptomes of over 30,000 AML bone marrow cells, correlating a proportion of these with genotyping information.(29) Machine learning algorithms were able to distinguish malignant from normal cells by correlation of genetic alteration with cell type. This study re-confirmed that AML partially recapitulates the myeloid hematopoietic hierarchy and demonstrated the variability in malignant cell type abundance across different patients. The interesting observation that AML impacted on non-mutant cell types, altering T cell number and activation, highlights how unbiased single-cell approaches can shed light on tumour cell-extrinsic effects.(29) The use of expression profiles to classify cells as AML cells or otherwise was recently challenged by a recent study integrating whole genome sequencing data with expressed mutation detection from 10x Chromium 5' high-throughput scRNA-seq data. While drop-outs were frequent, AML cells could be reliably identified by their mutant status and their expression profiles frequently displayed lineage infidelity.(30) ScRNA-seq has also generated novel insights into ontogeny-associated leukemia.(31) Here, a transgenic mouse model was used to induce the ETO2-GLIS2 fusion oncogene in either the fetal liver HSC or adult BM long-term HSC. The striking ontogeny-dependent differential gene expression observed likely accounts for the known age-dependent phenotype.(31)

ScRNA-seq methods have also significantly advanced our understanding of the cellular architecture of non-hematopoietic components of the BM niche, and how the interactions between BM HSPCs and stromal cells are perturbed during the development of leukemia.(32, 33) Additionally,

transcriptional data on >500,000 healthy human BM and cord blood cells have recently been released and provide the largest dataset to date of the hematopoietic compartment.(34) This is freely available via an interactive web portal and forms part of the “Human Cell Atlas”, a major international collaborative effort to generate a comprehensive scRNA-seq reference map of all tissues of the human body.(35) High-throughput scRNA-seq techniques has therefore enabled unbiased surveillance of large numbers of cells, leading to the characterisation of blood and bone marrow cell populations at unprecedented resolution in both health and disease.

Heterogeneity of blood cancer stem cells as a driver of disease evolution and therapy resistance

Cancer stem cells are rare, highly quiescent cells capable of self-renewal that underlie treatment resistance and cancer relapse.(4) There is extensive evidence that CSCs are the fundamental unit of evolution in many blood cancers and understanding ITH therefore requires a deeper understanding of the heterogeneity of CSCs. A recent study evaluated clonal evolution from myelodysplastic syndromes (MDS) to AML using single-cell targeted sequencing of stem cell populations in paired patient samples pre- and post-transformation.(36) This highlighted a previously unrecognised high degree of subclonal complexity in pre-MDS stem cells compared to MDS stem and blast cells, and implicated nonlinear, parallel clonal evolution from rare subclones within the MDS stem cell compartment in the progression of MDS to AML.

Single cell approaches are now being applied to understand response and resistance in CSC subpopulations to novel therapies. Using serial scRNA-seq in patient samples at baseline and after exposure to the *BCL2* inhibitor venetoclax, Pollyea *et al* identified that normal hematopoietic cells were preserved whereas cells identified as blasts based on lineage-specific transcript expression were rapidly depleted.(37) Cells expressing molecular signatures characteristic of primary leukemic stem cells were selectively ablated by disruption of oxidative phosphorylation, providing insight to the mechanism underlying the depth and durability of responses observed clinically.(37) Similarly, scRNA-seq was recently applied to paired AML diagnostic and post-allogeneic stem cell transplant relapse samples after bulk data implicated downregulation of MHC Class II. Single-cell data confirmed that expression of MHC Class II is high in AML cells at time of diagnosis and low in re-emergent AML cells driving post-transplant relapse, with no evidence of a low-expressing subclone at diagnosis,

supporting the hypothesis that the graft-versus-leukaemia effect may select for low-MHC Class II expressing cells.(38)

Giustacchini *et al* investigated chronic myeloid leukaemia (CML) patient samples with a modified standard scRNA-seq protocol to include targeted primers to amplify the *BCR-ABL* fusion-specific transcripts that are pathognomic of the disease.(39) This enabled high sensitivity detection of mutant CML stem cells and comparison of genes expressed by *BCR-ABL* mutant and non-mutated stem cells. This approach yielded several novel insights: firstly, that there are two key populations of CML stem cells identifiable at diagnosis, defined by a proliferative vs quiescent gene expression signature. During targeted therapy with a tyrosine kinase inhibitor (TKI), the less abundant, quiescent CML stem cells were selectively enriched with associated activation of distinct, potentially targetable, signalling pathways such as altered TGF-beta, NFkB, Wnt/beta-catenin and JAK STAT pathway activation. As CML is a unique disease entity driven by a single oncogene, this study also highlights the importance of non-genetic drivers of ITH for therapy resistance.(39)

Using single-cell analysis to evaluate responses to targeted drug therapy

Defining the single cell architecture of a tumor allows for the selection of a targeted therapy based on a patient's mutational profile. A number of single-cell studies have identified that targeted therapies induce heterogeneous, polyclonal responses that ultimately lead to relapse, questioning the utility of monotherapy with highly selective drugs. Enasidenib is a selective small molecule inhibitor of mutant *IDH2* which achieves response rates of ~40% in AML. Single-cell genotyping of serial BM cells from treated patients revealed that response is mediated by several mechanisms, including differentiation by both *IDH2* wild-type and *IDH2* mutant ancestral clones or progeny subclones. In turn, relapse does not occur by second-site *IDH2* mutations but rather due to the evolution or selection of multiple subclones exhibiting a number of mutational bypass mechanisms (including neomorphic *IDH1* mutations) capable of re-enforcing differentiation arrest.(40) Similarly, investigation of resistance mechanisms to gilteritinib, a second generation *FLT3*-ITD TKI, revealed the parallel emergence of multiple competing branching subclones of the original *FLT3*-mutated clone, all carrying off-target RAS MAPK pathway activating mutations. A much smaller subset developed resistance secondary to on-target mutations in *FLT3* kinase domain.(41)

By contrast, evaluation of the *FLT3* locus in patients who relapsed after treatment with quizartinib, another *FLT3* TKI, showed enormous mutational heterogeneity at the *FLT3* locus, with convergent second-site D835 mutations occurring both on wild type and ITD+ *FLT3* alleles. This is interesting mechanistically, as it either indicates that ancestral *FLT3*-ITD mutant and wild type clones may both be dependent on FLT3 signalling, enabling quizartinib to exert selective pressure on both with consequent branching tumor evolution, or that multiple *FLT3* mutation events occur in different HSPC compartments giving rise to polyclonal blast populations, with quizartinib therapy exerting selective pressure for D835+ve clones.(42)

These differing mechanisms highlight how single cell approaches can be used to unravel intra-and inter-tumoral heterogeneity underlying disease resistance, providing clinically useful insights. Where treatment failure is dominated by further kinase domain mutations, novel irreversible TKIs may have therapeutic activity. Where bypass mechanisms activate new oncogenic dependencies, such as the RAS-MAPK axis, this provides a rational basis for combination therapy approaches to eliminate these rare cancer cell subclones. In combination, these studies exemplify how single-cell analyses have already yielded clinically relevant information that can be harnessed to improve patient outcomes.

Beyond scRNA-seq: Single-cell epigenetic analyses shed new light on epigenetic heterogeneity

Recent technological advances permitting evaluation of the epigenome at single-cell resolution have yielded a number of provocative observations. These include that age-associated epigenetic reprogramming occurs at huge scale in HSCs, affecting >7000 regulatory elements, and may underpin the reduced self-renewal and homing potential observed ageing HSCs.(43) There is increasing evidence that disruption of the regulatory landscape is a core factor in leukemogenesis, where AML cells coopt regulatory pathways key to normal hematopoiesis.(44) Diversity in epigenetic marks such as cytosine methylation varies during disease progression and functionally impacts on gene regulation, contributing further to tumor heterogeneity.(45) The power of integrating single-cell resolved epigenetic and transcriptomic data was recently highlighted in the disease model of chronic lymphocytic leukaemia (CLL), where analyses of patterns of DNA methylation were able to chart the lineage history and disease evolution with therapy.(46)

Single cell analysis in the hematology clinic: current and future perspectives

Leveraging recent advances in single-cell techniques to evaluate serial patient samples through diagnosis and the course of therapy provides a powerful means to evaluate disease evolution, inform prognostication and assist with treatment decisions (**Fig 2**). New techniques advancing the information that can be obtained at the single-cell level are in constant evolution, such as the recent advent of spatial transcriptomics, providing new platforms for spatial cell atlases and *in situ* visualisation of biologically processes.(47) Imaging of mRNAS for >10,000 genes in single cells is now feasible, enabling unbiased cell class identification, spatial organisation discovery, and the direct imaging of ligand-receptor pairs in neighbouring cells.(48)

However, despite the recent explosion in molecular biology techniques and computational approaches to generate and analyse multi-omic single cell techniques, challenges remain. Performing single-cell analyses in large clinical cohorts remains technically complex. Gene expression is stochastic, varying over time and secondary to sample handling, and noise in biologically irrelevant genes could mask important small variations in small numbers of key biological regulators, undetectable by current bioinformatic tools. Allelic drop-outs, low and non-uniform coverage of large genomes, and relatively high costs represent further challenges.(11) Batch variation introduced by different experiment timings and handlers, change in reagent lots, or freeze-thaw processes can induce bias and confound biological findings, and must be considered during experimental design and compensated for appropriately.(49, 50) Single cell epigenetics in particular are currently affected by poor genome coverage, and single-cell proteomics, which could help validate transcriptomic data, has lagged behind. Caveats for transfer to the wider field of oncology remain, particularly as myeloid diseases are genetically simpler than many solid organ malignancies. The isolation of viable cells from solid tumours can be challenging and carries a risk of sampling bias, and where there is more than one site of disease these may be genetically diverse.(51-53)

Nonetheless, there have been major advances. High-throughput scRNA-seq and sc-genotyping platforms are clearly capable of generating quality data at scale. Simultaneous scRNA-seq combined with high-sensitivity genotyping for selected mutations is now possible, enabling the

selective identification of cancer stem cells using a wide range of mutations, alone or in combination.(54) This throws the field wide open to evaluating how combinatorial patterns of gene mutations change transcriptomic signatures and cellular behaviours, and provides a unique opportunity to identify novel tumour-specific targets.

We believe the maturation of these technologies over the next few years will beget rich insights into previously unstudied aspects of tumor cell biology. These include the role of the tumor microenvironment in supporting or repressing tumour cell expansion. For example, high-throughput scRNA-seq data lends itself to interrogation of receptor-ligand pairs, generating insights to cellular cross-talk, which influence cell function and dictate response to treatment.(55) Whilst initial studies have highlighted the tremendous cellular heterogeneity in hematological malignancy, further disentangling of the pathways identified is required to draw biologically meaningful conclusions and identify the most promising therapeutic targets. Further analysis is required to identify the regulatory events that imbue cancer cells with the stem cell properties that underlie cancer propagation and progression, and how to target these therapeutically.

Conclusion

Single-cell sequencing has enormous promise to deliver translatable improvements for hematology-oncology patients. The ability to obtain, isolate and process viable tumor samples renders single-cell analysis particularly feasible in haematology, and the stage is set for these techniques to be incorporated routinely into clinical trial design. Each hematological tumor represents a complex and intricate cellular network, and studies of patient samples to date highlight that inter- and intra-clonal heterogeneity occurs more frequently than was hitherto appreciated. Pairing single-cell descriptive studies with detailed studies into mechanisms of disease initiation, therapy response and relapse are now possible and indeed underway, heralding a new era in precision oncology.

Figures (Created with BioRender.com)

Figure 1: Standard experimental (A) and computational (B) workflows for single cell analysis of hematological malignancies

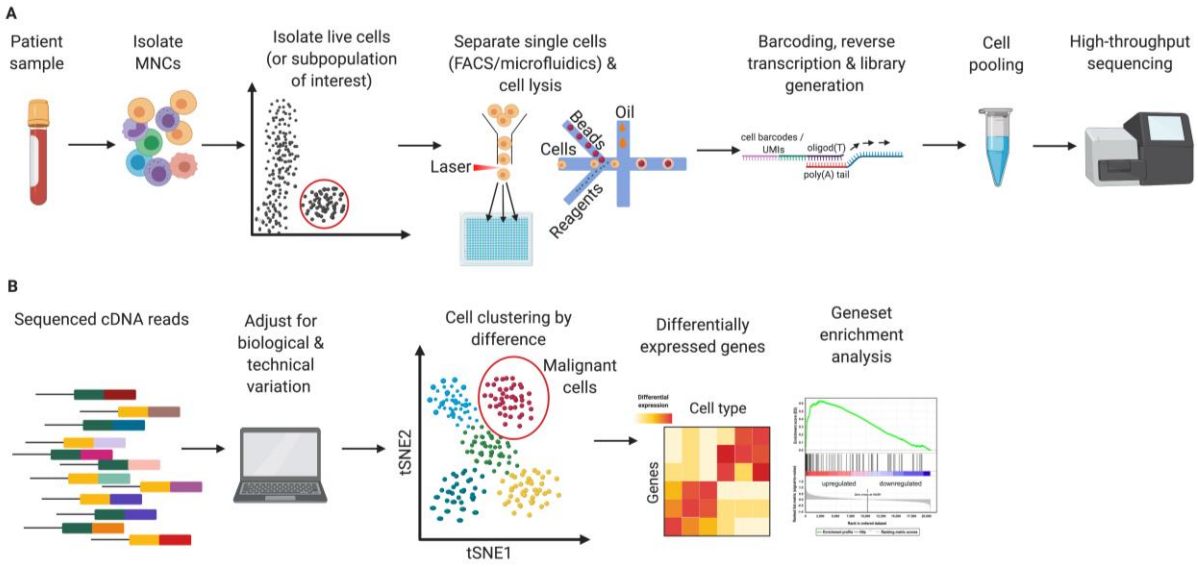
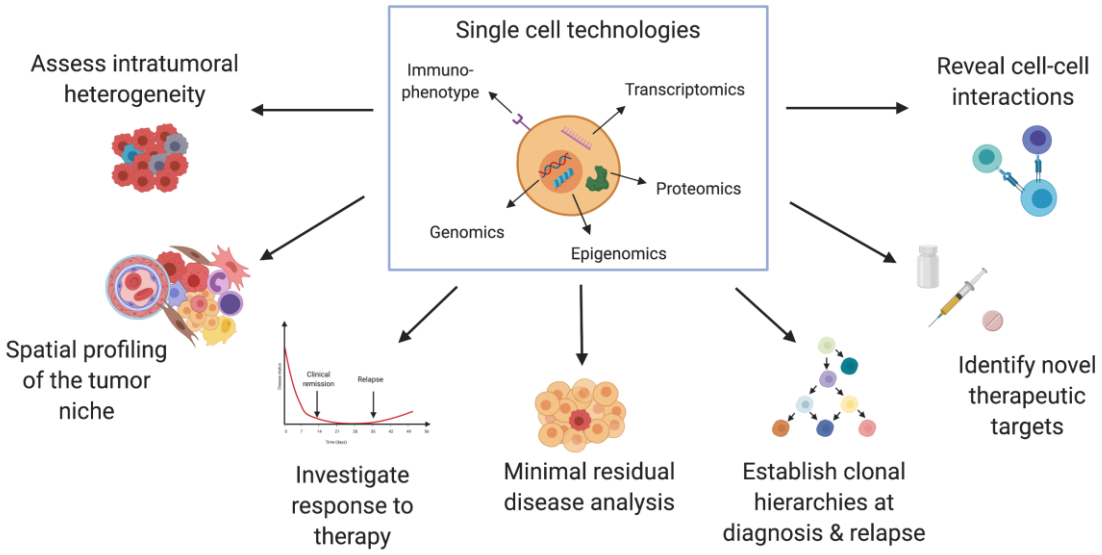


Fig 1 (A) A standard scRNA-seq experimental workflow. Firstly, cells of interest need to be isolated in a single cell suspension. Secondly, individual cells are separated by flow cytometry or microfluidic technologies and undergo cell lysis to release RNA and DNA. Thirdly, each cell is barcoded to allow pooling while different cellular fractions can undergo parallel experimental workflows, such as reverse transcription and RNA-sequencing or genomic sequencing. Lastly, pooled cells are sequenced. The sequencing data is de-multiplexed and tracked back to an individual cell, enabling downstream bioinformatic analysis (shown in **B**).
MNCs: mononuclear cells; FACS: fluorescence assisted cell sorting; UMI: unique molecular identifier; t-SNE:t-distributed stochastic neighbor embedding.

Fig 2: Clinical applications of multi-omic single cell technologies



References

1. McGranahan N, Swanton C. Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future. Cell. 2017;168(4):613-28.
2. Turajlic S, Sottoriva A, Graham T, Swanton C. Resolving genetic heterogeneity in cancer. Nat Rev Genet. 2019;20(7):404-16.
3. O'Sullivan J, Mead AJ. Heterogeneity in myeloproliferative neoplasms: Causes and consequences. Adv Biol Regul. 2019;71:55-68.
4. Clarke MF. Clinical and Therapeutic Implications of Cancer Stem Cells. New England Journal of Medicine. 2019;380(23):2237-45.
5. Baslan T, Hicks J. Unravelling biology and shifting paradigms in cancer with single-cell sequencing. Nature reviews Cancer. 2017;17(9):557-69.
6. Giladi A, Amit I. Single-Cell Genomics: A Stepping Stone for Future Immunology Discoveries. Cell. 2018;172(1):14-21.
7. Stuart T, Satija R. Integrative single-cell analysis. Nat Rev Genet. 2019;20(5):257-72.
8. Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP, et al. Human haematopoietic stem cell lineage commitment is a continuous process. Nature cell biology. 2017;19(4):271-81.
9. Tusi BK, Wolock SL, Weinreb C, Hwang Y, Hidalgo D, Zilionis R, et al. Population snapshots predict early haematopoietic and erythroid hierarchies. Nature. 2018;555(7694):54.
10. Villani A-C, Satija R, Reynolds G, Sarkizova S, Shekhar K, Fletcher J, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science. 2017;356(6335):eaah4573.
11. Mincarelli L, Lister A, Lipscombe J, Macaulay IC. Defining Cell Identity with Single-Cell Omics. PROTEOMICS. 2018;18(18):1700312.
12. Svensson V, Vento-Tormo R, Teichmann SA. Exponential scaling of single-cell RNA-seq in the past decade. Nature Protocols. 2018;13:599.
13. Stoeckius M, Hafemeister C, Stephenson W, Houck-Loomis B, Chattopadhyay PK, Swerdlow H, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods. 2017;14(9):865-8.
14. Haghverdi L, Buettner M, Wolf FA, Buettner F, Theis FJ. Diffusion pseudotime robustly reconstructs lineage branching. Nature methods. 2016;13(10):845.
15. Weinreb C, Wolock S, Tusi BK, Socolovsky M, Klein AM. Fundamental limits on dynamic inference from single-cell snapshots. Proceedings of the National Academy of Sciences. 2018;115(10):E2467-E76.
16. Wolf FA, Hamey FK, Plass M, Solana J, Dahlin JS, Göttgens B, et al. PAGA: graph abstraction reconciles clustering with trajectory inference through a topology preserving map of single cells. Genome Biology. 2019;20(1):59.
17. Chen H, Albergante L, Hsu JY, Lareau CA, Lo Bosco G, Guan J, et al. Single-cell trajectories reconstruction, exploration and mapping of omics data with STREAM. Nature communications. 2019;10(1):1903.
18. Herman JS, Sagar, Grun D. FateID infers cell fate bias in multipotent progenitors from single-cell RNA-seq data. Nat Methods. 2018;15(5):379-86.
19. Da Rocha EL, Rowe RG, Lundin V, Malleshaiah M, Jha DK, Rambo CR, et al. Reconstruction of complex single-cell trajectories using CellRouter. Nature communications. 2018;9(1):892.
20. Ludwig LS, Lareau CA, Ulirsch JC, Christian E, Muus C, Li LH, et al. Lineage Tracing in Humans Enabled by Mitochondrial Mutations and Single-Cell Genomics. Cell. 2019;176(6):1325-39 e22.
21. La Manno G, Soldatov R, Zeisel A, Braun E, Hochgerner H, Petukhov V, et al. RNA velocity of single cells. Nature. 2018;560(7719):494-8.
22. Woll PS, Kjallquist U, Chowdhury O, Doolittle H, Wedge DC, Thongjuea S, et al. Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo. Cancer cell. 2014;25(6):794-808.

23. Shlush LI, Zandi S, Mitchell A, Chen WC, Brandwein JM, Gupta V, et al. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. Nature. 2014;506(7488):328-33.
24. Jan M, Snyder TM, Corces-Zimmerman MR, Vyas P, Weissman IL, Quake SR, et al. Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. Sci Transl Med. 2012;4(149):149ra18.
25. Paguirigan AL, Smith J, Meshinchi S, Carroll M, Maley C, Radich JP. Single-cell genotyping demonstrates complex clonal diversity in acute myeloid leukemia. Science Translational Medicine. 2015;7(281):281re2.
26. Klco JM, Spencer DH, Miller CA, Griffith M, Lamprecht TL, O'Laughlin M, et al. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. Cancer cell. 2014;25(3):379-92.
27. Quek L, Otto GW, Garnett C, Lhermitte L, Karamitros D, Stoilova B, et al. Genetically distinct leukemic stem cells in human CD34- acute myeloid leukemia are arrested at a hemopoietic precursor-like stage. The Journal of experimental medicine. 2016;213(8):1513-35.
28. Potter N, Miraki-Moud F, Ermini L, Titley I, Vijayaraghavan G, Papaemmanuil E, et al. Single cell analysis of clonal architecture in acute myeloid leukaemia. Leukemia. 2019;33(5):1113-23.
29. van Galen P, Hovestadt V, Wadsworth Ii MH, Hughes TK, Griffin GK, Battaglia S, et al. Single-Cell RNA-Seq Reveals AML Hierarchies Relevant to Disease Progression and Immunity. Cell. 2019;176(6):1265-81.e24.
30. Petti AA, Williams SR, Miller CA, Fiddes IT, Srivatsan SN, Chen DY, et al. A general approach for detecting expressed mutations in AML cells using single cell RNA-sequencing. Nature communications. 2019;10(1):3660.
31. Lopez CK, Noguera E, Stavropoulou V, Robert E, Aid Z, Ballerini P, et al. Ontogenic changes in hematopoietic hierarchy determine pediatric specificity and disease phenotype in fusion oncogene-driven myeloid leukemia. Cancer Discov. 2019.
32. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, et al. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. Cell. 2019;177(7):1915-32 e16.
33. Tikhonova AN, Dolgalev I, Hu H, Sivaraj KK, Hoxha E, Cuesta-Dominguez A, et al. The bone marrow microenvironment at single-cell resolution. Nature. 2019;569(7755):222-8.
34. Hay SB, Ferchen K, Chetal K, Grimes HL, Salomonis N. The Human Cell Atlas bone marrow single-cell interactive web portal. Experimental hematology. 2018;68:51-61.
35. Regev A, Teichmann SA, Lander ES, Amit I, Benoist C, Birney E, et al. The Human Cell Atlas. Elife. 2017;6.
36. Chen J, Kao YR, Sun D, Todorova TI, Reynolds D, Narayanagari SR, et al. Myelodysplastic syndrome progression to acute myeloid leukemia at the stem cell level. Nature medicine. 2018.
37. Pollyea DA, Stevens BM, Jones CL, Winters A, Pei S, Minhajuddin M, et al. Venetoclax with azacitidine disrupts energy metabolism and targets leukemia stem cells in patients with acute myeloid leukemia. Nature medicine. 2018;24(12):1859-66.
38. Christopher MJ, Petti AA, Rettig MP, Miller CA, Chendamarai E, Duncavage EJ, et al. Immune Escape of Relapsed AML Cells after Allogeneic Transplantation. The New England journal of medicine. 2018;379(24):2330-41.
39. Giustacchini A, Thongjuea S, Barkas N, Woll PS, Povinelli BJ, Booth CAG, et al. Single-cell transcriptomics uncovers distinct molecular signatures of stem cells in chronic myeloid leukemia. Nature medicine. 2017;23(6):692-702.
40. Quek L, David MD, Kennedy A, Metzner M, Amatangelo M, Shih A, et al. Clonal heterogeneity of acute myeloid leukemia treated with the IDH2 inhibitor enasidenib. Nature medicine. 2018;24(8):1167-77.
41. McMahon CM, Ferng T, Canaani J, Wang ES, Morrissette JJD, Eastburn DJ, et al. Clonal Selection with RAS Pathway Activation Mediates Secondary Clinical Resistance to Selective FLT3 Inhibition in Acute Myeloid Leukemia. Cancer Discov. 2019;9(8):1050-63.

42. Smith CC, Paguirigan A, Jeschke GR, Lin KC, Massi E, Tarver T, et al. Heterogeneous resistance to quizartinib in acute myeloid leukemia revealed by single-cell analysis. Blood. 2017;130(1):48-58.
43. Adelman ER, Huang HT, Roisman A, Olsson A, Colaprico A, Qin T, et al. Aging Human Hematopoietic Stem Cells Manifest Profound Epigenetic Reprogramming of Enhancers That May Predispose to Leukemia. Cancer Discov. 2019;9(8):1080-101.
44. Corces MR, Buenrostro JD, Wu B, Greenside PG, Chan SM, Koenig JL, et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and leukemia evolution. Nat Genet. 2016;48(10):1193-203.
45. Li S, Garrett-Bakelman FE, Chung SS, Sanders MA, Hricik T, Rapaport F, et al. Distinct evolution and dynamics of epigenetic and genetic heterogeneity in acute myeloid leukemia. Nature medicine. 2016;22(7):792-9.
46. Gaiti F, Chaligne R, Gu H, Brand RM, Kothen-Hill S, Schulman RC, et al. Epigenetic evolution and lineage histories of chronic lymphocytic leukaemia. Nature. 2019;569(7757):576-80.
47. Crosetto N, Bienko M, van Oudenaarden A. Spatially resolved transcriptomics and beyond. Nat Rev Genet. 2015;16(1):57-66.
48. Eng C-HL, Lawson M, Zhu Q, Dries R, Koulena N, Takei Y, et al. Transcriptome-scale super-resolved imaging in tissues by RNA seqFISH+. Nature. 2019;568(7751):235-9.
49. Haghverdi L, Lun ATL, Morgan MD, Marioni JC. Batch effects in single-cell RNA-sequencing data are corrected by matching mutual nearest neighbors. Nat Biotechnol. 2018;36(5):421-7.
50. Goh WWB, Wang W, Wong L. Why Batch Effects Matter in Omics Data, and How to Avoid Them. Trends Biotechnol. 2017;35(6):498-507.
51. Turajlic S, Xu H, Litchfield K, Rowan A, Chambers T, Lopez JI, et al. Tracking Cancer Evolution Reveals Constrained Routes to Metastases: TRACERx Renal. Cell. 2018;173(3):581-94 e12.
52. Jamal-Hanjani M, Wilson GA, McGranahan N, Birkbak NJ, Watkins TBK, Veeriah S, et al. Tracking the Evolution of Non-Small-Cell Lung Cancer. The New England journal of medicine. 2017;376(22):2109-21.
53. Kim C, Gao R, Sei E, Brandt R, Hartman J, Hatschek T, et al. Chemoresistance Evolution in Triple-Negative Breast Cancer Delineated by Single-Cell Sequencing. Cell. 2018;173(4):879-93 e13.
54. Rodriguez-Meira A, Buck G, Clark SA, Povinelli BJ, Alcolea V, Louka E, et al. Unravelling Intratumoral Heterogeneity through High-Sensitivity Single-Cell Mutational Analysis and Parallel RNA Sequencing. Molecular cell. 2019;73(6):1292-305.e8.
55. Efremova M, Vento-Tormo M, Teichmann SA, Vento-Tormo R. CellPhoneDB v2.0: Inferring cell-cell communication from combined expression of multi-subunit receptor-ligand complexes. bioRxiv. 2019:680926.