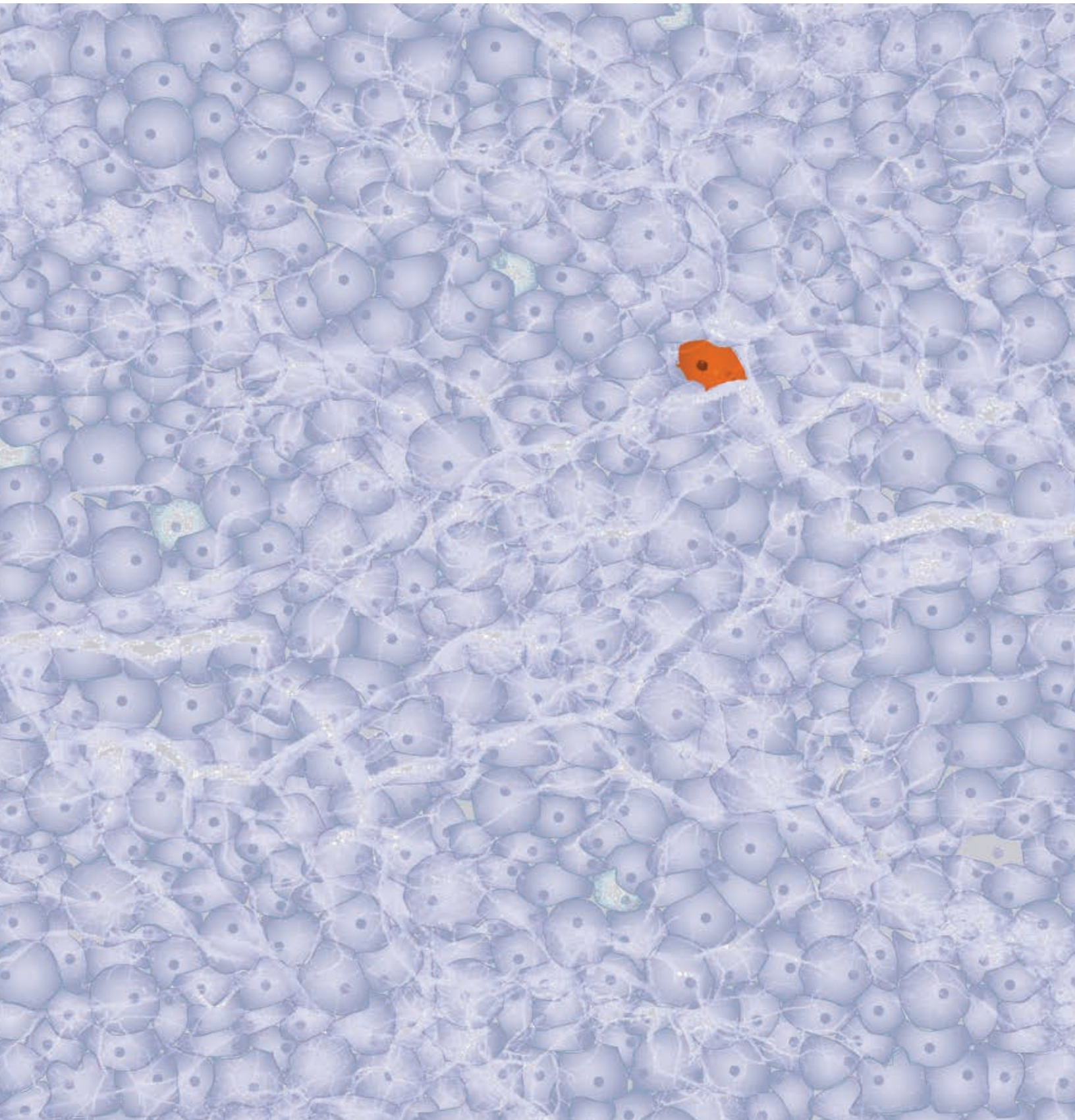
Single Cell Research

An Overview of Recent Single Cell Research Publications Featuring Illumina® Technology



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INTRODUCTION

In living tissues, such as the kidney, there are a large number of cell types. Each cell type has a distinct lineage and function, which contributes to the functioning of the tissue, the organ, and ultimately the organism. The lineage and stage of development of each cell determine how they respond to each other and to their environment. While the ultimate goal of an exhaustive understanding of tissues at their cellular level remains elusive, recent progress in single-cell analysis is offering a glimpse at the future.

Most of the impetus for single-cell tissue sequencing has come from cancer research, where cell lineage and the detection of residual disease are of paramount concern. The same approaches are being used to improve our understanding of massively complex biological systems, such as neural development and immunology.

Complex microbial populations, where many members of the communities are unculturable, are obvious candidates for single-cell analysis. A multitude of microbes and viruses have been isolated and characterized using DNA and RNA sequencing. This information has led to a new appreciation of the extent and importance of viruses in the environment and their role in shaping bacterial populations.

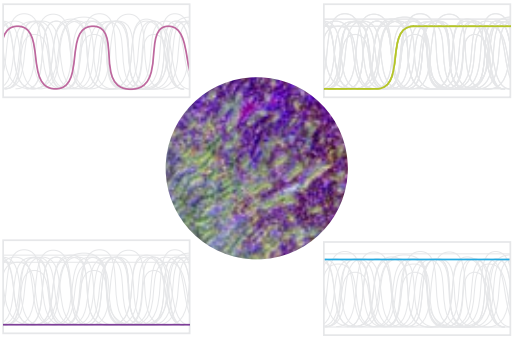
Surprisingly, colonies of bacterial cells—the paragon of homogeneity in microbiology—can also display complex collective dynamics to adapt to the local environment.[1]

The high accuracy and specificity of next-generation sequencing lends itself well to single-cell and low-level DNA/RNA sequencing. The growing armamentarium of published techniques includes the detection of DNA mutations, copy-number variants (CNVs), DNA-protein binding, RNA splicing, and the measurement of RNA expression values.

This document highlights recent publications that demonstrate the use of Illumina technology for single-cell sequencing and very low input applications and techniques. To learn more about Illumina sequencing and microarray technologies, visit www.illumina.com.

> "Single-cell approaches stand poised to revolutionize our capacity to understand the scale of genomic, epigenomic, and transcriptomic diversity that occurs during the lifetime of an individual organism." Macaulay and Voet 2014

1. Cho H., Jonsson H., Campbell K., Melke P., Williams J. W., et al. (2007) Self-organization in high-density bacterial colonies: efficient crowd control. PLoS Biol 5: e302



The same gene can be expressed at different levels, and influenced by different control mechanisms, in different cell types in the same tissue.

Reviews

Chi K. R. (2014) Singled out for sequencing. Nat Methods 11: 13-17

Eberwine J., Sul J. Y., Bartfai T. and Kim J. (2014) The promise of single-cell sequencing. Nat Methods 11: 25-27, Macaulay I. C. and Voet T. (2014) Single cell genomics: advances and future perspectives. PLoS Genet 10: e1004126

Macaulay I. C. and Voet T. (2014) Single cell genomics: advances and future perspectives. PLoS Genet 10: e1004126

Saliba A. E., Westermann A. J., Gorski S. A. and Vogel J. (2014) Single-cell RNA-seq: advances and future challenges. Nucleic Acids Res 42: 8845-8860

Wang L. and Wheeler D. A. (2014) Genomic sequencing for cancer diagnosis and therapy. Annu Rev Med 65: 33-48

Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

Lovett M. (2013) The applications of single-cell genomics. Hum Mol Genet 22: R22-26

Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

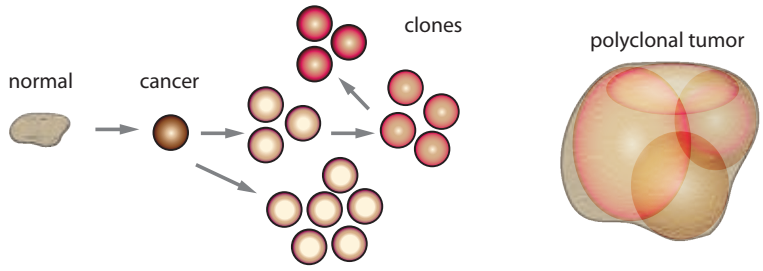
Wu J. and Tzanakakis E. S. (2013) Deconstructing stem cell population heterogeneity: single-cell analysis and modeling approaches. Biotechnol Adv 31: 1047-1062

APPLICATIONS

Cancer

Tumor progression occurs through driver mutations that undergo Darwinian selection for successive clonal expansion of tumor subclones.[2,3,4,5,6] As a result, advanced tumors may contain a number of clones where each clone has a unique set of mutations, unique pathology, and unique drug response.[7,8,9] An accurate census of all clones at diagnosis is important, because a subclone that makes up only 5.1% of the primary tumor can become the predominant clone after relapse.[10] Deep sequencing can detect subclones as low as 1% of the cellular population, but to characterize the cells fully to therapeutic efficacy of such rare populations will require single-cell analysis.[11,12]

Circulating single cells can also be used to detect cancer.[13,14] The sensitivity of this approach is limited by the ability to sample very rare cells in a typical blood specimen.[15] Cell-free detection of cancer nucleic acid markers may prove to be more sensitive and reproducible.[16,17]



Intratumor heterogeneity. The progressive accumulation of somatic mutations results in a heterogeneous polyclonal tumor, in which different clones may respond differently to treatment.

"Despite Virchow's discovery more than 150 years ago that the single cell represents the basic unit of disease, research and diagnostics are usually performed on thousands of cells without considering the different cell lineages in a body." Speicher MR et al. 2013

2. Alexandrov L. B. and Stratton M. R. (2014) Mutational signatures: the patterns of somatic mutations hidden in cancer genomes. Curr Opin Genet Dev 24: 52-60
3. Van Loo P. and Voet T. (2014) Single cell analysis of cancer genomes. Curr Opin Genet Dev 24: 82-91
4. Navin N., Kendall J., Troge J., Andrews P., Rodgers L., et al. (2011) Tumour evolution inferred by single-cell sequencing. Nature 472: 90-94
5. Stephens P. J., Greenman C. D., Fu B., Yang F., Bignell G. R., et al. (2011) Massive genomic rearrangement acquired in a single catastrophic event during cancer development. Cell 144: 27-40
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8. Landau D. A., Carter S. L., Stojanov P., McKenna A., Stevenson K., et al. (2013) Evolution and impact of subclonal mutations in chronic lymphocytic leukemia. Cell 152: 714-726
9. Navin N. E. and Hicks J. (2010) Tracing the tumor lineage. Mol Oncol 4: 267-283
10. Ding L., Ley T. J., Larson D. E., Miller C. A., Koboldt D. C., et al. (2012) Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. Nature 481: 506-510
11. Navin N. and Hicks J. (2011) Future medical applications of single-cell sequencing in cancer. Genome Med 3: 31
12. Hou Y., Song L., Zhu P., Zhang B., Tao Y., et al. (2012) Single-cell exome sequencing and monoclonal evolution of a JAK2-negative myeloproliferative neoplasm. Cell 148: 873-885
13. Cann G. M., Gulzar Z. G., Cooper S., Li R., Luo S., et al. (2012) mRNA-Seq of single prostate cancer circulating tumor cells reveals recapitulation of gene expression and pathways found in prostate cancer. PLoS One 7: e49144
14. Ramskold D., Luo S., Wang Y. C., Li R., Deng Q., et al. (2012) Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. Nat Biotechnol 30: 777-782
15. Allard W. J., Matera J., Miller M. C., Repollet M., Connelly M. C., et al. (2004) Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 10: 6897-6904
16. Swanton C. (2013) Plasma-derived tumor DNA analysis at whole-genome resolution. Clin Chem 59: 6-8
17. Newman A. M., Bratman S. V., To J., Wynne J. F., Eclov N. C., et al. (2014) An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 20: 548-554

Reviews

Adalsteinsson V. A. and Love J. C. (2014) Towards Engineered Processes for Sequencing-Based Analysis of Single Circulating Tumor Cells. Curr Opin Chem Eng 4: 97-104

Alexandrov L. B. and Stratton M. R. (2014) Mutational signatures: the patterns of somatic mutations hidden in cancer genomes. Curr Opin Genet Dev 24: 52-60

Easwaran H., Tsai H. C. and Baylin S. B. (2014) Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. Mol Cell 54: 716-727

Raphael B. J., Dobson J. R., Oesper L. and Vandin F. (2014) Identifying driver mutations in sequenced cancer genomes: computational approaches to enable precision medicine. Genome Med 6: 5

Van Loo P. and Voet T. (2014) Single cell analysis of cancer genomes. Curr Opin Genet Dev 24: 82-91

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Ding L., Raphael B. J., Chen F. and Wendl M. C. (2013) Advances for studying clonal evolution in cancer. Cancer Lett 340: 212-219

Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

Speicher M. R. (2013) Single-cell analysis: toward the clinic. Genome Med 5: 74

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Corces-Zimmerman M. R., Hong W. J., Weissman I. L., Medeiros B. C. and Majeti R. (2014) Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A 111: 2548-2553

Cancer is widely characterized by the sequential acquisition of genetic lesions in a single lineage of cells. This study examined the mutation acquisition in hematopoietic cells (HSCs) toward acute myeloid leukemia (AML). The authors used whole-exome sequencing on Illumina HiSeq to characterize the mutational profile of the cells. They discovered that mutations in "landscaping" genes (involved in global chromatin changes, such as DNA methylation, histone modification, and chromatin looping) occur early in the evolution of AML, whereas mutations in "proliferative" genes occur late.

Illumina Technology: HiSeq

Green M. R., Vicente-Duenas C., Romero-Camarero I., Long Liu C., Dai B., et al. (2014) Transient expression of Bcl6 is sufficient for oncogenic function and induction of mature B-cell lymphoma. Nat Commun 5: 3904

It is well established that cancer arises by an acquisition of somatic genomic alterations that lead to a malignant state. However, it is difficult to determine the initiation event for the cancerous development. This study examined the specific gain of 3q27.2 and its association with adverse outcome in diffuse large B-cell lymphoma (DLBCL). Using an Illumina Genome Analyzer, the researchers sequenced the whole genome and characterized the DNA methylation of murine hematopoietic progenitor cells (HSPCs). The authors identified BCL6 oncogene expression in HSPCs and demonstrated, by knock-in expression, that transient expression of Bcl6 within murine HSPCs can initiate the development into mature B-cell lymphomas.

Illumina Technology: Genome Analyzer$_{IIx}$

Melchor L., Brioli A., Wardell C. P., Murison A., Potter N. E., et al. (2014) Single-cell genetic analysis reveals the composition of initiating clones and phylogenetic patterns of branching and parallel evolution in myeloma. Leukemia 28: 1705-1715

The transformation from a normal cell to a cancer cell, and the subsequent progress of the cancer cell as a tumor, includes a series of genetic changes. Following Darwin's theory of natural selection, this diversification of the cancer cell line will result in a branching pattern of tumor evolution. The authors examine this theory via an analysis of patients with multiple myeloma (MM), using both whole-exome and single-cell sequencing with Illumina technology. The authors show that myeloma subclones exhibit different survival properties during treatment or mouse engraftment and conclude that clonal diversity, combined with varying selective pressures, is the essential foundation for tumor progression and treatment resistance.

Illumina Technology: Genome Analyzer$_{IIx}$

Lohr J. G., Adalsteinsson V. A., Cibulskis K., Choudhury A. D., Rosenberg M., et al. (2014) Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol 32: 479-484

The analysis of circulating tumor cells (CTCs) is a promising new avenue for monitoring and diagnosis of metastatic cancer. This study presents an integrated process to isolate, qualify, and sequence whole exomes of CTCs with high fidelity. The authors used the Illumina MagSweeper to enrich epithelial cell adhesion molecule (epCAM)-expressing CTCs. Individual cells were recovered and sequenced on Illumina HiSeq. The authors developed a methodology for assessing the quality and uniformity of genome-wide coverage of CTC-derived libraries to show the performance of their process. The process was validated by sequencing the metastatic CTCs of two patients with prostate cancer, and showed that 70% of CTC mutations were present in matched tissue.

Illumina Technology: HiSeq, MiSeq

Lovatt D., Ruble B. K., Lee J., Dueck H., Kim T. K., et al. (2014) Transcriptome *in vivo* analysis (TIVA) of spatially defined single cells in live tissue. Nat Methods 11: 190-196

RNA sequencing methods that rely on RNA extracted from cell mixtures do not convey the individual variability in expression among cells of the same tissue. In this paper the authors present a transcriptome *in vivo* analysis (TIVA), which is applicable to single-cell studies. In combination with Illumina sequencing technology, the authors capture and analyze the transcriptome variance across single neurons both in culture and *in vivo*. This method is furthermore non-invasive and may be applied to intact tissue. It will enable detailed studies of cell heterogeneity in complex tissues that have been intractable previously, and it opens up the possibility of use in conjunction with *in vivo* live functional imaging.

Illumina Technology: HiSeq

Papaemmanuil E., Rapado I., Li Y., Potter N. E., Wedge D. C., et al. (2014) RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. Nat Genet 46: 116-125

At least a quarter of acute lymphoblastic leukemia (ALL) cases harbor the ETV6-RUNX1 fusion gene. Although the gene fusion is characteristic for the disease, additional mutations are required for development of overt leukemia. This study used exome and low-coverage whole-genome sequencing to characterize secondary events associated with leukemic transformation. The authors found that ATF7IP and MGA are two new tumor-suppressor genes in ALL. They describe the parsimonious mutational process that transforms ETV6-RUNX1-positive lymphoblasts into leukemia.

Illumina Technology: Genome Analyzer$_{IIx}$

Picelli S., Faridani O. R., Bjorklund A. K., Winberg G., Sagasser S., et al. (2014) Full-length RNA-seq from single cells using Smart-seq2. Nat Protoc 9: 171-181

Gene expression analyses of tissues or large cell populations reflect the cell population average, but do not reveal the biological variability from cell to cell. This paper presents a new protocol for single-cell transcriptome analysis, Smart-seq2, an improved workflow based on the previously published Smart-seq protocol. Smart-seq2 includes optimized reverse transcription (RT), template switching, and pre-amplification to increase cDNA yield and lower technical variability. The protocol relies entirely on off-the-shelf reagents and can be used with all compatible Illumina sequencers, including MiSeq, HiSeq 2000, and HiSeq 2500.

Illumina Technology: MiSeq, HiSeq

Wu A. R., Neff N. F., Kalisky T., Dalerba P., Treutlein B., et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11: 41-46

In this comprehensive analysis the authors used microfluidic automation to quantitatively compare the accuracy and precision of single-cell RNA-Seq to qPCR. Commercially available single-cell RNA amplification methods, with both microliter and nanoliter volumes, were used to prepare and sequence libraries on Illumina HiSeq 2000. The study shows that single-cell RNA-Seq can generate results that are quantitatively comparable to qPCR, particularly when sample preparation is done on nanoliter-scale reaction volumes, such as in a microfluidic device.

Illumina Technology: HiSeq, Nextera DNA Sample Prep

Yu C., Yu J., Yao X., Wu W. K., Lu Y., et al. (2014) Discovery of biclonal origin and a novel oncogene SLC12A5 in colon cancer by single-cell sequencing. Cell Res 24: 701-712

The genetic heterogeneity of cancers is a major obstacle for efficient targeted therapy. This study performed single-cell sequencing analysis of a colon cancer sample to find several independent clones in the tumor cell population. The samples were subjected to whole-genome amplification (WGA) before exome sequencing using Illumina technology. The authors discovered the gene SLC12A5 had a high frequency of mutation at the single-cell level but exhibited low prevalence at the population level, suggesting that low-prevalence mutations may play a pro-tumorigenic role in the individual clones.

Illumina Technology: Sequencer unspecified

Battich N., Stoeger T. and Pelkmans L. (2013) Image-based transcriptomics in thousands of single human cells at single-molecule resolution. Nat Methods 10: 1127-1133

This paper presents a new method for high-throughput image-based transcriptomics by applying automated single-molecule fluorescent in situ hybridization (sm-FISH) to human tissue-culture cells. The authors demonstrate that the method is highly specific for low transcript counts and achieves comparable results to RNA-Seq at the mean expression level. The method has limited detection of nuclear transcripts and has a smaller dynamic range than RNA-Seq, but opens up new opportunities for scaling up the number of single cells that can be measured within the same sample. It allows for both multiplexing and the quantification of multivariate features across thousands of cells.

Illumina Technology: HiSeq

Hiatt J. B., Pritchard C. C., Salipante S. J., O'Roak B. J. and Shendure J. (2013) Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 23: 843-854

The detection and quantification of genetic heterogeneity in populations of cells is fundamentally important to diverse fields, ranging from microbial evolution to human cancer genetics. This paper presents smMIP—an assay that combines single-molecule tagging and molecular inversion probes to provide highly multiplexed and ultrasensitive detection and quantification of subclonal genetic variation. The assay is based on the Illumina MiSeq platform for rapid sequencing. The authors show that the assay is highly quantitative for frequencies as low as ~0.2%, and it is sensitive and specific for variant detection down to at least 1% frequency. This is the first paper to describe the smMIP assay together with its practicality, ability for multiplexing and scaling, and compatibility with desktop sequencing for rapid data collection.

Illumina Technology: MiSeq, HiSeq

Ni X., Zhuo M., Su Z., Duan J., Gao Y., et al. (2013) Reproducible copy number variation patterns among single circulating tumor cells of lung cancer patients. Proc Natl Acad Sci wwwU S A 110: 21083-21088

There is a great deal of interest in identifying and studying CTCs. Cells from primary tumors enter the bloodstream and can seed metastases. A major barrier to such analysis is the low DNA input amounts obtained from single cells, leading to lower coverage. This study uses multiple annealing and looping-based amplification cycles (MALBAC) for WGA sequencing of single CTCs from patients with lung cancer. The researchers identify CNVs that were consistent in patients with the same cancer subtype. Such information about cancers can help identify drug resistance and cancer subtypes, and offers potential for diagnostics leading to individualized treatment.

Illumina Technology: MiSeq, HiSeq

Wang Y., Waters J., Leung M. L., Unruh A., Roh W., et al. (2014) Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature 512:155-60

Heitzer E., Ulz P., Belic J., Gutschi S., Quehenberger F., et al. (2013) Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 5: 30

Pan X., Durrett R. E., Zhu H., Tanaka Y., Li Y., et al. (2013) Two methods for full-length RNA sequencing for low quantities of cells and single cells. Proc Natl Acad Sci U S A 110: 594-599

Picelli S., Bjorklund A. K., Faridani O. R., Sagasser S., Winberg G., et al. (2013) Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 10: 1096-1098

Potter N. E., Ermini L., Papaemmanuil E., Cazzaniga G., Vijayaraghavan G., et al. (2013) Single-cell mutational profiling and clonal phylogeny in cancer. Genome Res 23: 2115-2125

Strino F., Parisi F., Micsinai M. and Kluger Y. (2013) TrAp: a tree approach for fingerprinting subclonal tumor composition. Nucleic Acids Res 41: e165

Metagenomics

The study of single cells provides the ultimate resolution of a microbial population by allowing the identification of low-abundance species that might be missed in population sequencing.[18] Single-cell sequencing is also an effective approach to characterize organisms that are difficult to culture *in vitro.* Advances in single-cell sequencing have improved detection and analysis of outbreaks, antibiotic-resistant strains, food-borne pathogens, and microbial diversities in the environment or the gut.[19,20,21]

In addition, analysis of the RNA from individual cells should provide insights into the activities as well as task division of specific microbes within a community.[22] While population sequencing would provide a mixed representation of these mechanisms without a clear image of the transcriptional kinetics underway,[24] single-cell sequencing can identify individual contributions of cis and trans mechanisms and their relationship to synchronized periodic gene activation.[24] Single-cell sequencing can not only identify unculturable microbes,[23] but it can also monitor transcriptional gene activation that acts in an oscillatory fashion via cis- or trans-acting mechanisms.[20,21,24]

18. Yilmaz S. and Singh A. K. (2012) Single cell genome sequencing. Curr Opin Biotechnol 23: 437-443
19. Bergholz T. M., Moreno Switt A. I. and Wiedmann M. (2014) Omics approaches in food safety: fulfilling the promise? Trends Microbiol 22: 275-281
20. Stepanauskas R. (2012) Single cell genomics: an individual look at microbes. Curr Opin Microbiol 15: 613-620
21. Yilmaz S. and Singh A. K. (2012) Single cell genome sequencing. Curr Opin Biotechnol 23: 437-443
22. Islam S., Zeisel A., Joost S., La Manno G., Zajac P., et al. (2014) Quantitative single-cell RNA-seq with unique molecular identifiers. Nat Methods 11: 163-166
23. Lasken R. S. (2012) Genomic sequencing of uncultured microorganisms from single cells. Nat Rev Microbiol 10: 631-640
24. Coulon A., Chow C. C., Singer R. H. and Larson D. R. (2013) Eukaryotic transcriptional dynamics: from single molecules to cell populations. Nat Rev Genet 14: 572-584

Single-cell sequencing and meta-transcriptomics can help determine the contribution of each microorganism to its surrounding environment.

> "For most of the early history of microbiology, our understanding of bacteria was largely limited to those few species that could be grown in culture." Lasken RS et al. 2012

Reviews

Bergholz T. M., Moreno Switt A. I. and Wiedmann M. (2014) Omics approaches in food safety: fulfilling the promise? Trends Microbiol 22: 275-281

Smallwood S. A., Lee H. J., Angermueller C., Krueger F., Saadeh H., et al. (2014) Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. Nat Methods 11: 817-820

Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

Blainey P. C. (2013) The future is now: single-cell genomics of bacteria and archaea. FEMS Microbiol Rev 37: 407-427

Bryant J. M. (2013) Culture-free club. Nat Rev Microbiol 11: 434

Coulon A., Chow C. C., Singer R. H. and Larson D. R. (2013) Eukaryotic transcriptional dynamics: from single molecules to cell populations. Nat Rev Genet 14: 572-584

References

Embree M., Nagarajan H., Movahedi N., Chitsaz H. and Zengler K. (2014) Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community. ISME J 8: 757-767

Microbial communities amass a wealth of biochemical processes and metagenomics approaches are often unable to decipher the key functions of individual microorganisms. This study analyzed a microbial community by first determining the genome sequence of a dominant bacterial member belonging to the genus Smithella using a single-cell sequencing approach on an Illumina Genome Analyzer. After establishing a working draft genome of Smithella, the authors used low-input metatranscriptomics to determine which genes were active during alkane degradation. The authors then designed a genome-scale metabolic model to integrate the genomic and transcriptomic data.

Illumina Technology: Genome Analyzer, MiSeq

Kaster A. K., Mayer-Blackwell K., Pasarelli B. and Spormann A. M. (2014) Single cell genomic study of Dehalococcoidetes species from deep-sea sediments of the Peruvian Margin. ISME J 8:1831-42

Dehalogenating chloroflexi (Dehalococcoidetes) were originally discovered as the key microorganisms mediating reductive dehalogenation via their key enzymes reductive dehalogenases (Rdh), as the sole mode of energy conservation in terrestrial environments. In this study, total DNA was extracted from several deep-sea sediments from the Peruvian Margin and other Pacific sites and analyzed for the presence of Dehalococcoidetes 16S rRNA and rdh genes by PCR and nanoliter-qPCR. A sample from the Peruvian Margin trench was selected for cell sorting and single-cell genome sequencing on an Illumina HiSeq system. Two of the single cells were considered to be part of a local Dehalococcoidetes population and assembled together into a 1.38-Mb genome, to about 85% completeness.

Illumina Technology: HiSeq

Mason O. U., Scott N. M., Gonzalez A., Robbins-Pianka A., Baelum J., et al. (2014) Metagenomics reveals sediment microbial community response to Deepwater Horizon oil spill. ISME J 8: 1464-1475

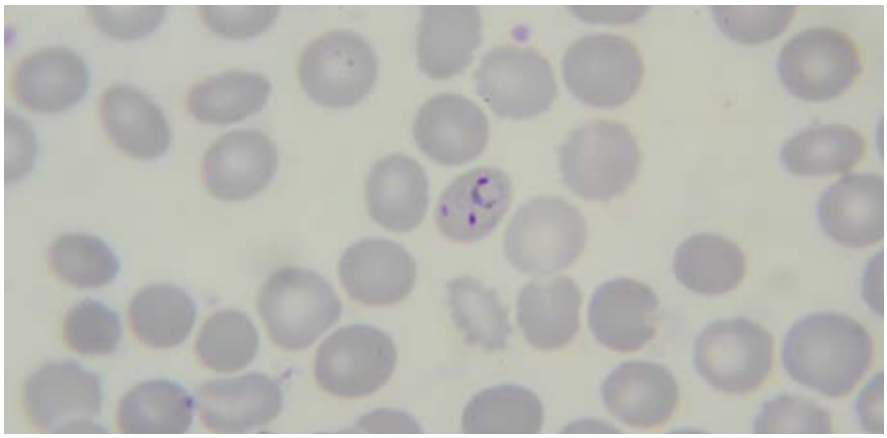
The Deepwater Horizon (DWH) oil spill in early2010 resulted in an input of ~4.1 million barrels of oil to the Gulf of Mexico; >22% of this oil is unaccounted for. This study examined the impact on marine microbial communities in surface sediments around the spill area, before and after the spill. The authors used Illumina HiSeq for metagenomic shotgun sequencing of 14 samples and targeted sequencing of 16S rRNA genes, for a total of 64 samples. Their results showed that the most heavily oil-impacted sediments were enriched in an uncultured Gammaproteobacterium and a Colwellia species. In addition, the analysis revealed an increase in abundance of genes involved in denitrification pathways in samples that exceeded the Environmental Protection Agency (EPA) benchmarks for polycyclic aromatic hydrocarbons (PAHs), compared with those that did not.

Illumina Technology: HiSeq

Nair S., Nkhoma S. C., Serre D., Zimmerman P. A., Gorena K., et al. (2014) Single-cell genomics for dissection of complex malaria infections. Genome Res 24: 1028-1038

Anti-malarial chemotherapy is central to treatment and control strategies, but drug resistance can drastically undermine these efforts. In this study, the authors used single-cell sequencing and genotyping of samples to discover the within-host diversity of a malarial infection. Using this approach, they observed four distinct drug-resistance haplotypes within the P. falciparum infection MKK2664. The authors demonstrated that the single-cell genomics approach can be used to generate parasite genome sequences directly from patient blood in order to unravel the complexity of P. vivax and P. falciparum infections.

Illumina Technology: HiSeq 2000



Malaria parasite under microscope (1,000x).

Wang F. P., Zhang Y., Chen Y., He Y., Qi J., et al. (2014) Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. ISME J 8: 1069-1078

The microbes responsible for anaerobic oxidation of methane (AOM) are the subject of intense study but information about mechanistic details have been lacking. In this study the genome of a highly active AOM enrichment dominated by the methane-oxidizing archaea ANME-2a was studied by single-aggregate sequencing on Illumina Genome Analyzer. All genes required for performing the seven steps of methanogenesis from CO2 were found present and actively expressed.

Illumina Technology: Genome Analyzer$_{IIx}$

Dodsworth J. A., Blainey P. C., Murugapiran S. K., Swingley W. D., Ross C. A., et al. (2013) Single-cell and metagenomic analyses indicate a fermentative and saccharolytic lifestyle for members of the OP9 lineage. Nat Commun 4: 1854

This study is an excellent example of the combination of single-cell and metagenomic sequencing to characterize a novel bacterial species. The OP9 bacterial lineage is found in geothermal systems, petroleum reservoirs, anaerobic digesters, and wastewater treatment facilities. This phylogenetic analysis suggests OP9 is a new phylum: Atribacteria. The metabolic reconstruction of the lineage suggests an anaerobic metabolism based on sugar fermentation, which may enable the catabolism of cellulose in thermal environments.

Illumina Technology: Genome Analyzer$_{IIx}$

Kamke J., Sczyrba A., Ivanova N., Schwientek P., Rinke C., et al. (2013) Single-cell genomics reveals complex carbohydrate degradation patterns in poribacterial symbionts of marine sponges. ISME J 7: 2287-2300

Many marine sponges are hosts to dense and phylogenetically diverse microbial communities that are located in the extracellular matrix of the animal. Single-cell sequencing was used to investigate the metabolic potential of five individual poribacterial cells, representing three phylogenetic groups almost exclusively found in sponges.

Illumina Technology: HiSeq, Genome Analyzer$_{IIx}$

McLean J. S., Lombardo M. J., Ziegler M. G., Novotny M., Yee-Greenbaum J., et al. (2013) Genome of the pathogen Porphyromonas gingivalis recovered from a biofilm in a hospital sink using a high-throughput single-cell genomics platform. Genome Res 23: 867-877

The elusive candidate phylum TM6 has evaded cultivation and characterization. This study presents the sequencing of a TM6 population from a hospital biofilm. The authors selected small random pools of single-flow-sorted cells to create a mini-metagenome by sequencing. A recently developed single-cell assembler, SPAdes, in combination with contig binning methods, allowed the reconstruction of genomes from these mini-metagenomes. Further application of this approach in other environments may greatly increase the likelihood of capturing and assembling the genomes of elusive, low-abundance microorganisms.

Illumina Technology: Genome Analyzer$_{IIx}$

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Nurk S., Bankevich A., Antipov D., Gurevich A. A., Korobeynikov A., et al. (2013) Assembling single-cell genomes and mini-metagenomes from chimeric MDA products. J Comput Biol 20: 714-737

Reyes A., Wu M., McNulty N. P., Rohwer F. L. and Gordon J. I. (2013) Gnotobiotic mouse model of phage-bacterial host dynamics in the human gut. Proc Natl Acad Sci U S A 110: 20236-20241

Rusch D. B., Lombardo M. J., Yee-Greenbaum J., Novotny M., Brinkac L. M., et al. (2013) Draft Genome Sequence of a Single Cell of SAR86 Clade Subgroup IIIa. Genome Announc 1:

Taghavi Z., Movahedi N. S., Draghici S. and Chitsaz H. (2013) Distilled single-cell genome sequencing and de novo assembly for sparse microbial communities. Bioinformatics 29: 2395-2401

Stem Cells

Life begins from a single cell, which—through subsequent mitotic cellular divisions—generates a population of cells that make up the human body.[25] Each stem cell chooses a specific lineage determined by its genetic code and response to its environment, giving rise to an array of unique heterogeneous population of cells.[26] Sequencing these single stem cells at the beginning of life and during differentiation has helped elucidate how a stem cell responds to its environment and how it chooses a specific lineage.[28,29,30,31]

As single cells differentiate to generate populations, they acquire somatic mutations that also lead to a specific phenotype.[27] In addition, these cells differ substantially at the epigenome level,[28] which dictates RNA expression and, ultimately, the cellular phenotype.[29] Advances in single-cell sequencing have shed light on the genetic, epigenetic, and expression variations unique to specific cells that elicit a unique phenotype.[30,31]

Understanding the RNA expression profiles of specific single-cell types at certain stages of development has made it possible to reverse-engineer tissues.[27,31] RNA-Seq profiling of single cells during development provides insight into the changes in protein expression that dictate lineage specification.[27,31] Sequencing uncovers transcription factors, RNA species, receptors, and ligands that direct lineage specification during development in a sequential, tissue-specific order, identifying and tracing back cell lineages.[31,32] The utilization of single-cell DNA and RNA sequencing to study stem cells is providing new breakthroughs into understanding development, tissue differentiation, diseases, and drug discovery.[32]

> "Single cells are the fundamental units of life. Therefore, single-cell analysis is not just one more step towards more-sensitive measurements, but is a decisive jump to a more-fundamental understanding of biology." Shapiro E. et al. 2013

25. Speicher M. R. (2013) Single-cell analysis: toward the clinic. Genome Med 5: 74
26. Voet T., Kumar P., Van Loo P., Cooke S. L., Marshall J., et al. (2013) Single-cell paired-end genome sequencing reveals structural variation per cell cycle. Nucleic Acids Res 41: 6119-6138
27. Streets A. M., Zhang X., Cao C., Pang Y., Wu X., et al. (2014) Microfluidic single-cell whole-transcriptome sequencing. Proc Natl Acad Sci U S A 111: 7048-7053
28. Guo H., Zhu P., Wu X., Li X., Wen L., et al. (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. Genome Res 23: 2126-2135
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32. Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

Reviews

Macaulay I. C. and Voet T. (2014) Single cell genomics: advances and future perspectives. PLoS Genet 10: e1004126

Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

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Behjati S., Huch M., van Boxtel R., Karthaus W., Wedge D. C., et al. (2014) Genome sequencing of normal cells reveals developmental lineages and mutational processes. Nature 513: 422-425

During the development from embryo to adult animal, originating cells divide, and their gene expression specializes into different tissues. As the animal ages, individual cells acquire mutations. This study investigated whether the analysis of the acquired somatic mutations across different tissues in healthy mice can bring insights into the developmental lineage tree. The authors used Illumina MiSeq to sequence the whole genomes of 12 different adult cells from two different mice. The observed differences in the numbers and types of mutations accumulated by each cell likely reflects differences in the number of cell divisions and varying contributions of different mutational processes.

Illumina Technology: MiSeq

Boroviak T., Loos R., Bertone P., Smith A. and Nichols J. (2014) The ability of inner-cell-mass cells to self-renew as embryonic stem cells is acquired following epiblast specification. Nat Cell Biol 16: 516-528

Murine embryonic stem cells (ESCs) are derived from murine inner cell mass (ICM). ESCs retain full developmental potential when cultured. This study aimed to identify the exact origin of ESCs and their relationship to ICM cells *in vivo*. The authors used Illumina HiSeq sequencing to profile the expression of early embryonic cells at a single-cell level. The findings suggest that formation of the epiblast coincides with competence for ERK-independent self-renewal *in vitro* and consequent propagation as ESC lines.

Illumina Technology: HiSeq 2000

Carter A. C., Davis-Dusenbery B. N., Koszka K., Ichida J. K. and Eggan K. (2014) Nanog-Independent Reprogramming to iPSCs with Canonical Factors. Stem Cell Reports 2: 119-126

It has been suggested that the transcription factor Nanog is essential for the establishment of pluripotency in ESCs and induced pluripotent stem cells (iPSCs). However, there may be many distinct routes to a pluripotent state. This study used Illumina HiSeq RNA sequencing to characterize iPSCs during differentiation, to test whether Nanog is necessary for reprogramming murine fibroblasts The authors showed that it is possible to reproducibly generate iPSCs from Nanog (-/-) fibroblasts that effectively contributed to the germline of chimeric mice. They conclude that although Nanog may be an important mediator of reprogramming, it is not required for establishing pluripotency in mice.

Illumina Technology: HiSeq 2500

Corces-Zimmerman M. R., Hong W. J., Weissman I. L., Medeiros B. C. and Majeti R. (2014) Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A 111: 2548-2553

Cancer is widely characterized by the sequential acquisition of genetic lesions in a single lineage of cells. This study examined the mutation acquisition in HSCs toward AML. The authors used whole-exome sequencing on Illumina HiSeq to characterize the mutational profile of the cells. They discovered that mutations in "landscaping" genes (involved in global chromatin changes, such as DNA methylation, histone modification, and chromatin looping) occur early in the evolution of AML, whereas mutations in "proliferative" genes occur late.

Illumina Technology: HiSeq 2000

Hasemann M. S., Lauridsen F. K., Waage J., Jakobsen J. S., Frank A. K., et al. (2014) C/EBPalpha is required for long-term self-renewal and lineage priming of hematopoietic stem cells and for the maintenance of epigenetic configurations in multipotent progenitors. PLoS Genet 10: e1004079

C/EBP-alpha is a well-known inducer of myeloid differentiation. It is expressed at low levels in HSCs, and its potential function in these cells has been extensively debated. In this study the authors examined the impact on C/EBP-alpha on cell renewal, differentiation, quiescence, and survival. The authors used Illumina ChIP-Seq to correlate expression with epigenetic configurations. They showed that C/EBP-alpha acts to modulate the epigenetic states of genes belonging to molecular pathways important for HSC function.

Illumina Technology: HiSeq 2000 and Genome Analyzer$_{IIx}$

Lovatt D., Ruble B. K., Lee J., Dueck H., Kim T. K., et al. (2014) Transcriptome *in vivo* analysis (TIVA) of spatially defined single cells in live tissue. Nat Methods 11: 190-196

RNA sequencing methods that rely on RNA extracted from cell mixtures do not convey the individual variability in expression among cells of the same tissue. In this paper, the authors present a transcriptome *in vivo* analysis (TIVA), which is applicable to single-cell studies. In combination with Illumina sequencing technology, the authors capture and analyze the transcriptome variance across single neurons both in culture and *in vivo*. This method is furthermore non-invasive and may be applied to intact tissue. It will enable detailed studies of cell heterogeneity in complex tissues that have been intractable previously, and it opens up the possibility of use in conjunction with *in vivo* live functional imaging.

Illumina Technology: HiSeq

Shalek A. K., Satija R., Shuga J., Trombetta J. J., Gennert D., et al. (2014) Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature 510: 363-369

Individual cells can exhibit substantial differences in gene expression, and only recently have genome profiling methods been developed to monitor the expression of single cells. This study applied the Smart-Seq single-cell RNA sequencing on Illumina HiSeq to investigate heterogeneity in the response of mouse bone-marrow-derived dendritic cells (BMDCs) to lipopolysaccharide. The authors found extensive bimodal variation in messenger RNA abundance and splicing patterns, which was subsequently validated using RNA-FISH for select transcripts.

Illumina Technology: HiSeq 2000

Streets A. M., Zhang X., Cao C., Pang Y., Wu X., et al. (2014) Microfluidic single-cell whole-transcriptome sequencing. Proc Natl Acad Sci U S A 111: 7048-7053

Single-cell whole-transcriptome analysis is a powerful tool for quantifying gene expression heterogeneity in populations of cells. This study presents a strategy for single-cell RNA-Seq implemented in a microfluidic platform for single-cell whole-transcriptome analysis. With this approach double-stranded cDNA is collected and sequenced on Illumina HiSeq 2500. The authors demonstrated the technique on single mouse embryonic cells and were able to reconstruct a majority of the bulk transcriptome from as little as 10 single cells. Using this approach the authors quantified the variation among and within different types of mouse embryonic cells.

Illumina Technology: HiSeq 2500

Trapnell C., Cacchiarelli D., Grimsby J., Pokharel P., Li S., et al. (2014) The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. Nat Biotechnol 32: 381-386

Individual cells, even when in the same tissue, exhibit high variability in expression. This study presents a new computational tool, Monocle, for clustering and determining the temporal ordering of the expression profiles of individual cells assayed by RNA-Seq. The authors applied their method to the differentiation of primary human myoblasts by single-cell transcriptome sequencing on Illumina HiSeq 2500. Their analysis revealed switch-like changes in the expression of key regulatory factors. This tool, in combination with high-throughput single-cell sequencing, has the potential to provide unique insights into transcriptional regulation and gene regulatory networks.

Illumina Technology: HiSeq 2500 100 bp paired-end reads for RNA-Seq

Treutlein B., Brownfield D. G., Wu A. R., Neff N. F., Mantalas G. L., et al. (2014) Reconstructing lineage hierarchies of the distal lung epithelium using single-cell RNA-seq. Nature 509: 371-375

Mammalian lung cell progenitor cells develop along distinct lineages. To study the development of progenitor cells into mature alveolar cell types, this study examined gene expression from single cells in development. The researchers used microfluidic single-cell transcriptome analysis by Illumina sequencing on 198 individual cells at four different stages of differentiation. They classified cells into distinct groups based on expression, and elucidated the full life cycle of the alveolar type 2 cell lineages.

Illumina Technology: Nextera XT, HiSeq

Xia H., Bodempudi V., Benyumov A., Hergert P., Tank D., et al. (2014) Identification of a cell-of-origin for fibroblasts comprising the fibrotic reticulum in idiopathic pulmonary fibrosis. Am J Pathol 184: 1369-1383

The authors showed that the lungs of patients with idiopathic pulmonary fibrosis (IPF) contain pathological mesenchymal progenitor cells that are cells of origin for fibrosis-mediating fibroblasts. These fibrogenic mesenchymal progenitors and their progeny represent unexplored drug targets.

Illumina Technology: HiSeq 2000 for RNA-Seq

Guo H., Zhu P., Wu X., Li X., Wen L., et al. (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. Genome Res 23: 2126-2135

DNA methylation is an important part of gene expression regulation and is variable across cells, even within the same tissue. This study presents a methylome analysis technique for single cells at single-base resolution. The authors created reduced-representation bisulfite sequencing libraries (scRRBS) for sequencing on Illumina HiSeq 2000. In an analysis of mouse ESCs, the authors showed for the first time that the methylome of the first polar body is comparable with that of the metaphase II oocyte within the same gamete.

Illumina Technology: HiSeq 2000

Sasagawa Y., Nikaido I., Hayashi T., Danno H., Uno K. D., et al. (2013) Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity. Genome Biol 14: R31

Individual cells may exhibit variable gene expression even if they share the same genome. The analysis of single-cell variability in gene expression requires robust protocols with a minimum of bias. This paper presents a novel single-cell RNA-Seq method, Quartz-Seq, based on Illumina sequencing that has a simpler protocol and higher reproducibility and sensitivity than existing methods. The authors implemented improvements in three main areas: 1) they optimized the protocol for suppression of byproduct synthesis; 2) they identified a robust PCR enzyme to allow single-tube reaction; and 3) they determined optimal conditions for RT and second-strand synthesis.

Illumina Technology: TruSeq RNA, HiSeq

Voet T., Kumar P., Van Loo P., Cooke S. L., Marshall J., et al. (2013) Single-cell paired-end genome sequencing reveals structural variation per cell cycle. Nucleic Acids Res 41: 6119-6138

High-throughput technologies for studying DNA variations and abundance have been hampered by the required amounts of input material for accurate analysis. In this paper, the authors demonstrate how paired-end sequence analysis of single cells after WGA enabled detection of DNA CNVs from tens of kilobases to multiple megabases in size, with accurate break-point delineation. This study also presents the first analysis to manage mapping of an interchromosomal rearrangement to base resolution, based on a single cell from a human embryo.

Illumina Technology: HiSeq, Genome Analyzer$_{IIx}$

Xue Z., Huang K., Cai C., Cai L., Jiang C. Y., et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500: 593-597

In an effort to characterize and dissect the gene regulatory mechanisms of mammalian pre-implantation development, this study examined early embryos from both human and mouse using whole-transcriptome sequencing of single cells. The authors report novel stage-specific monoallelic expression patterns for a significant portion of polymorphic transcripts. Cross-species comparisons revealed that the majority of human stage-specific modules (seven of nine) are preserved, but with developmental specificity and timing differing between human and mouse.

Illumina Technology: HiSeq

Eckersley-Maslin M. A., Thybert D., Bergmann J. H., Marioni J. C., Flicek P., et al. (2014) Random monoallelic gene expression increases upon embryonic stem cell differentiation. Dev Cell 28: 351-365

Random autosomal monoallelic gene expression refers to the transcription of a gene from one of two homologous alleles. This study examined the mechanisms for monoallelic expression in mouse cell lines through an allele-specific RNA-Seq screen using Illumina sequencing. The authors identified many random monoallelically expressed genes and discovered that neither DNA methylation nor nuclear positioning distinguished the active versus inactive alleles. However, they did find a correlation in enrichment for specific histone modifications.

Illumina Technology: Genome Analyzer

Shetzer Y., Kagan S., Koifman G., Sarig R., Kogan-Sakin I., et al. (2014) The onset of p53 loss of heterozygosity is differentially induced in various stem cell types and may involve the loss of either allele. Cell Death Differ 21: 1419-1431

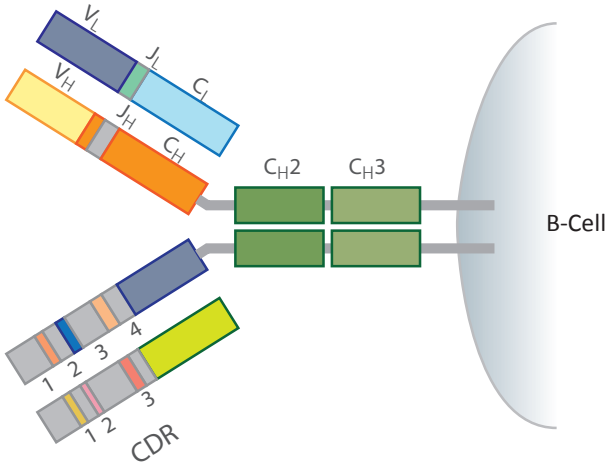
Yan L., Yang M., Guo H., Yang L., Wu J., et al. (2013) Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol 20: 1131-1139

Immunology

The immune system consists of a number of cell types, where each plays a unique role in the immune response. These dissociated single cells are usually sorted based on surface characteristics. Due to the limitations of sorting systems, sorted cells can still consist of mixed populations at various stages of development or activation. Single-cell sequencing can determine if cells have unique expression patterns and if genes within those cells have unique splice variants.[33]

The B-Cell Repertoire

Antibodies are produced by a developmentally ordered series of somatic gene rearrangement events that occur exclusively in developing B cells and continue throughout the life of an organism. Antibodies consist of heavy and light chains, which are linked by disulfide bonds. The antigen-ligand binding specificity is determined by a combination of the variable regions of both the heavy and the light chains (VH and VL). Every B cell typically contains a unique pair of heavy and light chains that are encoded by genes in different parts of the genome. To predict the antigen ligand specificity with the highest level of certainty, the VH genes should be paired with their VL genes in a single B cell.[34]



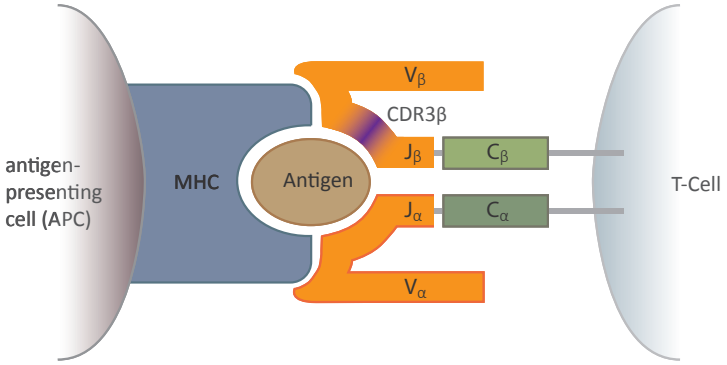
The primary antibody heavy chain repertoire is created predominantly by the somatic recombination of variable gene segments. After productive rearrangement of the heavy chain, recombination of the light chain ensues, and the heterodimeric pairing of H and L chains forms the complete antibody.[35]

> "In the genome, there are no loci that have greater complexity or extend a deeper and broader reach into human biology than those encoding the antigen receptors of T cells and B cells." Woodsworth DJ et al. 2013

33. Shalek A. K., Satija R., Adiconis X., Gertner R. S., Gaublomme J. T., et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236-240
34. Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H., et al. (2014) The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat Biotechnol 32: 158-168
35. Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H., et al. (2014) The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat Biotechnol 32: 158-168

The T-Cell Repertoire

Like B cells, functional T-cell receptors (TCRs) are heterodimeric proteins that comprise both an α and a ß chain. Every T cell contains a unique combination of α and ß chains that identify major histocompatibility complex (MHC)-presented peptide ligands.[36,37] Several single-cell sequencing methods have been developed to avoid disrupting the α and ß chain pairing through cell lysis.[38,39]



T-cell receptor-antigen-peptide-MHC interaction and TCR gene recombination. The antigen-presenting cell presents the peptide antigen bound to the MHC. The TCR (orange) binds to both the antigen and MHC. The complementarity-determining region 3 (CDR3) domain is shown in purple.[40]

For more information on repertoire sequencing, refer to the section on TCR sequencing.

Reviews

Georgiou G., Ippolito G. C., Beausang J., Busse C. E., Wardemann H., et al. (2014) The promise and challenge of high-throughput sequencing of the antibody repertoire. Nat Biotechnol 32: 158-168

Finn J. A. and Crowe J. E., Jr. (2013) Impact of new sequencing technologies on studies of the human B cell repertoire. Curr Opin Immunol 25: 613-618

Robins H. (2013) Immunosequencing: applications of immune repertoire deep sequencing. Curr Opin Immunol 25: 646-652

Warren E. H., Matsen F. A. t. and Chou J. (2013) High-throughput sequencing of B- and T-lymphocyte antigen receptors in hematology. Blood 122: 19-22

Woodsworth D. J., Castellarin M. and Holt R. A. (2013) Sequence analysis of T-cell repertoires in health and disease. Genome Med 5: 98

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Corces-Zimmerman M. R., Hong W. J., Weissman I. L., Medeiros B. C. and Majeti R. (2014) Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. Proc Natl Acad Sci U S A 111: 2548-2553

Cancer is widely characterized by the sequential acquisition of genetic lesions in a single lineage of cells. This study examined the mutation acquisition in HSCs toward AML. The authors used whole-exome sequencing on Illumina HiSeq to characterize the mutational profile of the cells. They discovered that mutations in "landscaping" genes (involved in global chromatin changes, such as DNA methylation, histone modification, and chromatin looping) occur early in the evolution of AML, whereas mutations in "proliferative" genes occur late.

Illumina Technology: HiSeq 2000

36. Birnbaum M. E., Mendoza J. L., Sethi D. K., Dong S., Glanville J., et al. (2014) Deconstructing the peptide-MHC specificity of T cell recognition. Cell 157: 1073-1087
37. Mandl J. N. and Germain R. N. (2014) Focusing in on T cell cross-reactivity. Cell 157: 1006-1008
38. Woodsworth D. J., Castellarin M. and Holt R. A. (2013) Sequence analysis of T-cell repertoires in health and disease. Genome Med 5: 98
39. Turchaninova M. A., Britanova O. V., Bolotin D. A., Shugay M., Putintseva E. V., et al. (2013) Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol 43: 2507-2515
40. Woodsworth D. J., Castellarin M. and Holt R. A. (2013) Sequence analysis of T-cell repertoires in health and disease. Genome Med 5: 98

de Almeida P. E., Meyer E. H., Kooreman N. G., Diecke S., Dey D., et al. (2014) Transplanted terminally differentiated induced pluripotent stem cells are accepted by immune mechanisms similar to self-tolerance. Nat Commun 5: 3903

iPSCs hold significant potential for sources of therapeutic cells for personalized medical applications, such as organ repair. However, little is known of the immune response elicited by iPSCs. This study examined the immune response of iPSC-derived endothelial cells in a mouse model for iPSC grafts, using Illumina MiSeq targeted sequencing to characterize the expression of factors involved in the graft immune response. The authors compared the responses of autologous iPSC-derived endothelial cells versus undifferentiated iPSCs. They concluded that the differentiation of iPSCs results in a loss of immunogenicity and leads to the induction of tolerance.

Illumina Technology: MiSeq

Han A., Glanville J., Hansmann L. and Davis M. M. (2014) Linking T-cell receptor sequence to functional phenotype at the single-cell level. Nat Biotechnol 32: 684-692

The TCRs expressed in each T cell are tremendously diverse, thanks to somatic V(D)J recombination. In this study, the authors introduce a method for characterization of the expressed TCRs for single cells to determine their antigen specificity and cell-line ancestry. The authors used targeted TCR sequencing on the Illumina MiSeq platform to reveal information about individual TCRα-TCRß pairs. The approach was applied to study the clonal ancestry and differentiation of T lymphocytes infiltrating a human colorectal carcinoma.

Illumina Technology: MiSeq

Obata Y., Furusawa Y., Endo T. A., Sharif J., Takahashi D., et al. (2014) The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. Nat Immunol 15: 571-579

Intestinal regulatory T cells (Treg cells) are necessary for the suppression of excessive immune responses to gut bacteria. This study examined the DNA methylation status and regulation in the context of exposure to gut microbiota. The authors examined mice with a T-cell specific deficiency in the DNA methylation adapter Uhrf1 using Illumina HiSeq. They found that mice with this deficiency showed defective proliferation of colonic regulatory T cells and developed severe colitis. The authors concluded that Uhrf1 is required for the epigenetic mechanism that maintains the gut immunological homeostasis.

Illumina Technology: HiSeq 1000

Shugay M., Britanova O. V., Merzlyak E. M., Turchaninova M. A., Mamedov I. Z., et al. (2014) Towards error-free profiling of immune repertoires. Nat Methods 11: 653-655

Next-generation sequencing has opened up new possibilities for analyzing genomic diversity in studies of the adaptive immune system. This paper presents MIGEC—an improved analysis method for determining TCR repertoires by Illumina sequencing. Using unique barcode labeling, combined with two-stage PCR amplification, MIGEC allows for nearly absolute error correction while fully preserving the natural diversity of complex immune repertoires.

Illumina Technology: MiSeq, HiSeq

DeKosky B. J., Ippolito G. C., Deschner R. P., Lavinder J. J., Wine Y., et al. (2013) High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire. Nat Biotechnol 31: 166-169

Previously VH:VL pairing in B-cell repertoire diversity was lost during lysis of B-cell populations. Here, the authors employed a method of single-cell mRNA capture, RT, and amplification by emulsion VH:VL linkage RT-PCR of these pairings. The linked pairings were sequenced to identify unique antibody clonotypes in healthy peripheral blood IgG+ B cells, peripheral antigen-specific plasmablasts isolated after tetanus toxoid immunization, and memory B-cell responses following influenza vaccination.

Illumina Technology: MiSeq 2 x 250 bp

Han A., Newell E. W., Glanville J., Fernandez-Becker N., Khosla C., et al. (2013) Dietary gluten triggers concomitant activation of CD4+ and CD8+ alphabeta T cells and gammadelta T cells in celiac disease. Proc Natl Acad Sci U S A 110: 13073-13078

Celiac disease is an intestinal autoimmune disease caused by dietary gluten and gluten-specific CD4+ T-cell responses. Gluten exposure also induces the appearance of activated, gut-homing CD8+ αß and γδ T cells in peripheral blood. Single-cell TCR sequence analysis indicates that both these cell populations have highly focused TCR repertoires. Such a focused repertoire usually indicates that the induction is driven by an antigen.

Illumina Technology: MiSeq paired-end sequencing

Shalek A. K., Satija R., Adiconis X., Gertner R. S., Gaublomme J. T., et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236-240
Individual cells can exhibit substantial differences in gene expression, and only recently have genome profiling methods been developed to monitor the expression of single cells. This study applied the Smart-Seq single-cell RNA sequencing on Illumina HiSeq to investigate heterogeneity in the response of mouse BMDCs to lipopolysaccharide. The authors found extensive bimodal variation in messenger RNA abundance and splicing patterns, which was subsequently validated using RNA-FISH for select transcripts.

Illumina Technology: HiSeq

Turchaninova M. A., Britanova O. V., Bolotin D. A., Shugay M., Putintseva E. V., et al. (2013) Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol 43: 2507-2515
The authors propose a single-cell-based method to identify native pairs of αß TCR CDR3 chains within emulsion droplets by reverse-transcription of α and ß chain mRNA, PCR amplification, and subsequent fusion via overlap extension. This PCR suppression technique resolves the issue of random overlap-extension of gene pairs that may create a high level of noise after the emulsion stage. The authors propose that this methodology can be applied to the identification of native pairs of variable heavy-light antibody chains.

Illumina Technology: MiSeq 2 x 150 bp

DeKosky B. J., Ippolito G. C., Deschner R. P., Lavinder J. J., Wine Y., et al. (2013) High-throughput sequencing of the paired human immunoglobulin heavy and light chain repertoire. Nat Biotechnol 31: 166-169

Nagano T., Lubling Y., Stevens T. J., Schoenfelder S., Yaffe E., et al. (2013) Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature 502: 59-64

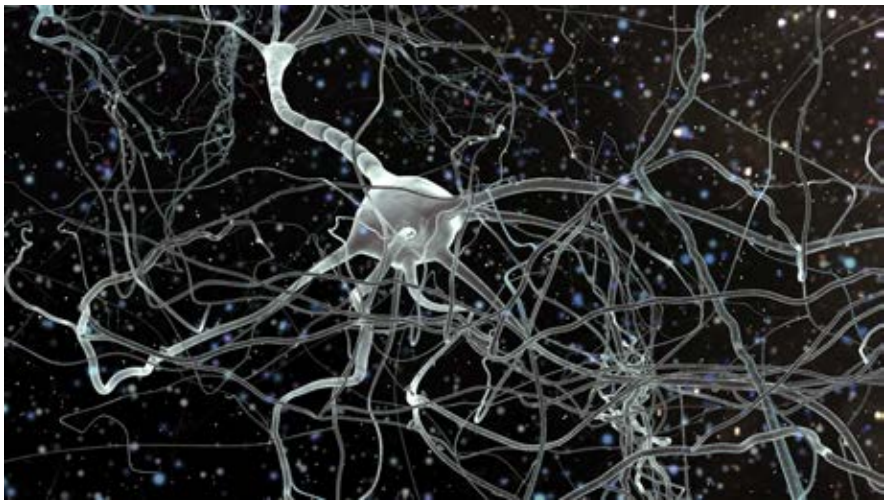
Pinto S., Michel C., Schmidt-Glenewinkel H., Harder N., Rohr K., et al. (2013) Overlapping gene coexpression patterns in human medullary thymic epithelial cells generate self-antigen diversity. Proc Natl Acad Sci U S A 110: E3497-3505

Neurology

Recent advances in research have highlighted the mosaic genomes of individual neurons, exhibiting CNVs even between cells that make up a specific region of the brain.[41] Even though genetic variations in the brain arise during fetal development,[42] the functional relevance of this mosaicism is unclear. It will be of interest not only to discover the significance of mosaicism in normal brain, but also to study its role in neurological diseases and psychological disorders.[42,43,44,45]

Research has just begun to illustrate where heterogeneity between cells is notable at the genome level. If mosaicism exists in the genetic code between single cells,[46] it is probable that there are also variations in protein expression, epigenetic changes,[47] and RNA isoforms.[48] Sequencing single cells provides a larger integrated image of the collected data, helping to ensure the effects of mosaicism on individual cellular phenotype within a given region of the brain.[43,44,45]

> "Increasingly, somatic mutations are being identified in diseases other than cancer, including neurodevelopmental diseases." Podouri A et al. 2013



Single-cell RNA-Seq has allowed the detection of mosaicism in neurons.[41]

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Chi K. R. (2014) Singled out for sequencing. Nat Methods 11: 13-17

Macaulay I. C. and Voet T. (2014) Single cell genomics: advances and future perspectives. PLoS Genet 10: e1004126

Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

Poduri A., Evrony G. D., Cai X. and Walsh C. A. (2013) Somatic mutation, genomic variation, and neurological disease. Science 341: 1237758

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Lovatt D., Ruble B. K., Lee J., Dueck H., Kim T. K., et al. (2014) Transcriptome *in vivo* analysis (TIVA) of spatially defined single cells in live tissue. Nat Methods 11: 190-196

RNA sequencing methods that rely on RNA extracted from cell mixtures do not convey the individual variability in expression among cells of the same tissue. In this paper, the authors present a transcriptome *in vivo* analysis (TIVA), which is applicable to single-cell studies. In combination with Illumina sequencing technology, the authors capture and analyze the transcriptome variance across single neurons both in culture and *in vivo*. This method is furthermore non-invasive and may be applied to intact tissue. It will enable detailed studies of cell heterogeneity in complex tissues that have been intractable previously, and it opens up the possibility of use in conjunction with *in vivo* live functional imaging

Illumina Technology: HiSeq 1000

Young G. T., Gutteridge A., Fox H. D., Wilbrey A. L., Cao L., et al. (2014) Characterizing Human Stem Cell-derived Sensory Neurons at the Single-cell Level Reveals Their Ion Channel Expression and Utility in Pain Research. Mol Ther 22: 1530-1543

Research into the biology of pain is commonly performed on animal models due to the lack of sensory neuronal cell lines. In this paper, the authors present human stem-cell derived sensory neurons and use a combination of population and single-cell techniques to perform detailed molecular, electrophysiological, and pharmacological phenotyping. The directed differentiation was monitored over 6 weeks and the gene expression characterized using Illumina BeadArrays. The authors show the derived neurons are both molecularly and functionally comparable to human sensory neurons derived from mature dorsal root ganglia.

Illumina Technology: BeadArrays

Lister R., Mukamel E. A., Nery J. R., Urich M., Puddifoot C. A., et al. (2013) Global epigenomic reconfiguration during mammalian brain development. Science 341: 1237905

DNA methylation is implicated in mammalian brain development and plasticity underlying learning and memory. This paper reports the genome-wide composition, patterning, cell specificity and dynamics of DNA methylation at single-base resolution in human and mouse frontal cortex throughout their lifespan. The extensive methylome profiling was performed with ChIP-Seq on Illumina HiSeq, revealing methylation profiles at single-base resolution.

Illumina Technology: TruSeq RNA, TruSeq DNA, HiSeq

McConnell M. J., Lindberg M. R., Brennand K. J., Piper J. C., Voet T., et al. (2013) Mosaic copy number variation in human neurons. Science 342: 632-637

The genome of a species varies not only between individuals, but also between mother and daughter cells as a result of errors and uneven distribution of DNA material after cell division. The extent of such genetic variation between somatic cells of the same individual has been hitherto unknown. With the advent of single-cell sequencing, it is now possible to examine variations within cells of the same tissue. In this study, the CNVs in individual neuron cells were studied using Illumina genome-wide sequencing. The authors discovered that CNVs are abundant even between neuronal cells from the same tissue.

Illumina Technology: Genome Analyzer$_{IIx}$, MiSeq, Nextera DNA Sample Prep Kit

Pan X., Durrett R. E., Zhu H., Tanaka Y., Li Y., et al. (2013) Two methods for full-length RNA sequencing for low quantities of cells and single cells. Proc Natl Acad Sci U S A 110: 594-599

Gene expression profiling by RNA-Seq is a powerful tool for understanding the molecular activity of specific tissues. However, the heterogeneity of gene expression within a tissue requires RNA-Seq technology that can manage single-cell amounts of input RNA. This study presents two methods: Phi29 DNA polymerase–based mRNA transcriptome amplification (PMA), and semi–random-primed PCR-based mRNA transcriptome amplification (SMA). Both techniques are coupled with Illumina sequencing for expression detection from low RNA input amounts. Both protocols produced satisfactory detection/coverage of abundant mRNAs, even from a single cell.

Illumina Technology: HiSeq

Drug Discovery

Highly targeted drugs are expected to modulate cellular processes in functional cells of interest. Unfortunately, tissues consist of a multitude of cell types, and the resultant biological noise can drown out the specific interactions of interest. Even when a sample consists of a single cell type, the cells are out of phase, which can hide subtle or transient responses. Various cell types express as many as 300-400 genes that are potential drug targets. In Parkinson's-affected neurons, it is estimated that 30-40 genes are selectively expressed at different stages of the disease.[49] Single-cell analysis, combined with next-generation sequencing, shows great promise as a tool to simplify biological systems to improve pharmaceutical screens.[50,51]

Characterization of circulating tumor cells could also include genomic analysis, pharmacological sensitivities, and assessments of metastatic potential.[52]

Bacterial populations create pharmacokinetic interference with drugs such as digoxin[53] and camptothecin.[54] The plasticity of the microbiome makes it a target of both live cell therapies and small molecules.[55] Single-cell genomics also plays an important role in this fast-growing field (see section on Metagenomics).

> "The sensitivity and specificity provided by single-cell studies have shown that as many as 300-400 druggable genes are expressed in many cell types." Eberwine et al. 2014

Single-cell genomics promise much greater efficiency in drug development and design.

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Eberwine J., Sul J. Y., Bartfai T. and Kim J. (2014) The promise of single-cell sequencing. Nat Methods 11: 25-27

Love K. R., Bagh S., Choi J. and Love J. C. (2013) Microtools for single-cell analysis in biopharmaceutical development and manufacturing. Trends Biotechnol 31: 280-286

Spaethling J. M. and Eberwine J. H. (2013) Single-cell transcriptomics for drug target discovery. Curr Opin Pharmacol 13: 786-790

Vogelstein B., Papadopoulos N., Velculescu V. E., Zhou S., Diaz L. A., Jr., et al. (2013) Cancer genome landscapes. Science 339: 1546-1558

Wilson M. C. and Piel J. (2013) Metagenomic approaches for exploiting uncultivated bacteria as a resource for novel biosynthetic enzymology. Chem Biol 20: 636-647

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Albert F. W., Treusch S., Shockley A. H., Bloom J. S. and Kruglyak L. (2014) Genetics of single-cell protein abundance variation in large yeast populations. Nature 506: 494-497

DNA variation among individuals may result in differences in protein expression. In this study, the authors examined the genetics of single-cell protein abundance by selecting single yeast cells with high and low protein expression. They subsequently detected whole-genome DNA variants with Illumina HiSeq sequencing. The method was applied to 160 genes and detected many more quantitative trait loci (QTL) per gene than previous studies. Most loci were clustered in “hotspots” that influence multiple proteins, and some hotspots were found to influence more than half of the proteins examined.

Illumina Technology: HiSeq

Lee E. C., Liang Q., Ali H., Bayliss L., Beasley A., et al. (2014) Complete humanization of the mouse immunoglobulin loci enables efficient therapeutic antibody discovery. Nat Biotechnol 32: 356-363

Antibodies are the fastest-growing class of pharmaceutical molecules produced today, thanks to their specificity and generally superior safety profiles. This study presents a novel chimeric mouse model for producing human antibodies with applications for research and therapeutics. The authors produced transgenic mice by inserting all the variable genes of the human immunoglobulin loci and verifying the sequences using Illumina MiSeq. These mice produce chimeric antibodies with human variable domains and mouse constant domains. The authors demonstrate that the transgenic mice are viable and fertile, and provide a robust system for discovery of therapeutic human monoclonal antibodies.

Illumina Technology: MiSeq for 250 bp paired-end sequencing of the V(D)J libraries

Young G. T., Gutteridge A., Fox H. D., Wilbrey A. L., Cao L., et al. (2014) Characterizing Human Stem Cell-derived Sensory Neurons at the Single-cell Level Reveals Their Ion Channel Expression and Utility in Pain Research. Mol Ther 22: 1530-1543

Research into the biology of pain is commonly performed on animal models due to the lack of sensory neuronal cell lines. In this paper, the authors present human stem-cell derived sensory neurons and use a combination of population and single-cell techniques to perform detailed molecular, electrophysiological, and pharmacological phenotyping. The directed differentiation was monitored over 6 weeks and the gene expression characterized using Illumina BeadArrays. The authors show the derived neurons are both molecularly and functionally comparable to human sensory neurons derived from mature dorsal root ganglia.

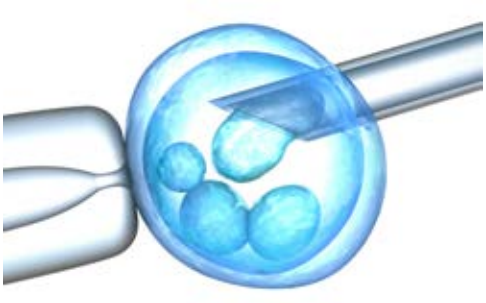
Illumina Technology: BeadArrays

Gundry M., Li W., Maqbool S. B. and Vijg J. (2012) Direct, genome-wide assessment of DNA mutations in single cells. Nucleic Acids Res 40: 2032-2040

Reproductive Health

Prenatal diagnosis refers to testing for diseases or conditions in a fetus or embryo before it is born. Birth defects can be diagnosed by detecting detrimental chromosomal or DNA aberrations. Traditionally, this has been done through invasive procedures, such as amniocentesis. Non-invasive prenatal testing (NIPT) offers greater confidence over traditional testing methods and only requires a blood draw as opposed to invasive testing.[56,57] NIPT by sequencing cell-free DNA (cfDNA) has finally made its way into the clinic, improving diagnosis.[58] Compared to traditional prenatal diagnostics, sequencing has been more accurate in detecting abnormalities.[58,59]

Single-cell sequencing can also be useful in the realm of preimplantation diagnostics, where embryos can be screened for chromosomal abnormalities prior to implantation.[60,61,62] Research shows that sequencing aids genetic testing of *in vitro* generated gametes (IVG), either for research or pregnancy.[63] Diagnostics using single-cell sequencing can accelerate research, improve early detection of embryo status, and assure transplantation of a healthy *in vitro* fertilized (IVF) embryo.[59,64]



Genetically testing an embryo can give early insights to chromosomal abnormalities that can lead to fatal diseases.

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63. Palacios-Gonzalez C., Harris J. and Testa G. (2014) Multiplex parenting: IVG and the generations to come. J Med Ethics
64. Ong F. S., Lin J. C., Das K., Grosu D. S. and Fan J. B. (2013) Translational utility of next-generation sequencing. Genomics 102: 137-139

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Bianchi D. W., Parker R. L., Wentworth J., Madankumar R., Saffer C., et al. (2014) DNA sequencing versus standard prenatal aneuploidy screening. N Engl J Med 370: 799-808

Chandrasekharan S., Minear M. A., Hung A. and Allyse M. (2014) Noninvasive prenatal testing goes global. Sci Transl Med 6: 231fs215

Macaulay I. C. and Voet T. (2014) Single cell genomics: advances and future perspectives. PLoS Genet 10: e1004126

Palacios-Gonzalez C., Harris J. and Testa G. (2014) Multiplex parenting: IVG and the generations to come. J Med Ethics

Speicher M. R. (2013) Single-cell analysis: toward the clinic. Genome Med 5: 74

Van der Aa N., Zamani Esteki M., Vermeesch J. R. and Voet T. (2013) Preimplantation genetic diagnosis guided by single-cell genomics. Genome Med 5: 71

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Kirkness E. F., Grindberg R. V., Yee-Greenbaum J., Marshall C. R., Scherer S. W., et al. (2013) Sequencing of isolated sperm cells for direct haplotyping of a human genome. Genome Res 23: 826-832

Next-generation sequencing has increased the speed and accuracy with which genotypes can be determined in a high-throughput manner. This study presents a method applying Illumina sequencing to determine the haplotypes (haploid genotypes) of single sperm cells. This approach exploits the haploid nature of sperm cells and employs a combination of genotyping and low-coverage sequencing on a short-read platform. In addition to generating chromosome-length haplotypes, the approach can directly identify recombination events (averaging 1.1 per chromosome) with a median resolution of < 100 Kb.

Illumina Technology: HumanOmni1-Quad, Genome Analyzer$_{IIx}$, HiSeq

Yan L., Yang M., Guo H., Yang L., Wu J., et al. (2013) Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol 20: 1131-1139

In a study of human embryonic development, 124 samples from human preimplantation embryos and human ESCs at different passages were profiled by transcriptome sequencing at a single-cell level. The authors characterized the maternally expressed genes and identified 2,733 novel long non-coding RNAs (lncRNAs), many of which were expressed in specific developmental stages. These results provide a comprehensive overview of the transcriptome landscape of 90 single cells of 20 morphologically normal human oocytes and early embryos, and 34 single cells of human ESCs.

Illumina Technology: HiSeq

Ong F. S., Lin J. C., Das K., Grosu D. S. and Fan J. B. (2013) Translational utility of next-generation sequencing. Genomics 102: 137-139

Next-generation sequencing techniques have opened up new avenues for genomic characterization across many areas of molecular pathology. This review discusses how the latest improvements in accuracy, throughput, and single-cell sequencing techniques have wide-ranging applications for clinical decision-making. The authors give an overview of methods and applications that are expected to become central in the development of genomic medicine.

Co-evolution

Researchers continue to discover new species and novel organisms around the planet, even today.[65] Although there are larger organisms that remain undiscovered, current technology enables researchers to understand the dynamics of evolution and host-species interactions that generates selection pressures. Antagonistic co-evolution of interacting species can help reveal the fluctuating dynamics of selection.[66] Single-cell sequencing allows tracking of selection and pressures in an environment that lead to the evolution of a species.[65,66] Sequencing single cells allows for high-resolution detection of unculturable populations.[67] Such insight is critical to understanding complex ecosystems and the many unique organisms that comprise them.[68] Sequencing enables the study of how environmental pressures lead to rapid evolution between interacting species. In addition, it enables the detection of these new species and their evolved roles in their respective environments.[69,70,71]

Single-cell analysis allows better assessment of how different organisms pressure selection, and enables study of the evolution of co-habitants as well as host-pathogen interactions.

Reviews

Martiny J. B., Riemann L., Marston M. F. and Middelboe M. (2014) Antagonistic coevolution of marine planktonic viruses and their hosts. Ann Rev Mar Sci 6: 393-414

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Cooper D. A., Jha B. K., Silverman R. H., Hesselberth J. R. and Barton D. J. (2014) Ribonuclease L and metal-ion-independent endoribonuclease cleavage sites in host and viral RNAs. Nucleic Acids Res 42: 5202-5216

Despite the biological significance of ribonuclease L (RNase L), the RNAs cleaved by this enzyme are poorly defined. In this study, the authors used Illumina sequencing to reveal the frequency and location of RNase L cleavage sites within host and viral RNAs. The method was optimized and validated using viral RNAs cleaved with RNase L and RNase A, and RNA from infected and non-infected HeLa cells. The authors identified discrete genomic regions susceptible to RNase L and other single-strand-specific endoribonucleases. Monitoring the frequency and location of these cleavage sites within host and viral RNAs may reveal how these enzymes contribute to health and disease.

Illumina Technology: Genome Analyzer$_{IIx}$, MiSeq

Kashtan N., Roggensack S. E., Rodrigue S., Thompson J. W., Biller S. J., et al. (2014) Single-cell genomics reveals hundreds of coexisting subpopulations in wild Prochlorococcus. Science 344: 416-420

The cyanobacterium Prochlorococcus is a single species by traditional measures, but it can be divided into several major clades defined by the intergenic transcribed spacer (ITS) region of rRNA genes. This study examined the cell-by-cell genomic diversity within samples of seawater collected at three separate times of the year. The authors used Illumina HiSeq 2000 single-cell sequencing to determine the individual characteristics of the clades and to measure the shift in abundance of different ITS eco-types over time.

Illumina Technology: Genome Analyzer$_{IIx}$, HiSeq

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Kaster A. K., Mayer-Blackwell K., Pasarelli B. and Spormann A. M. (2014) Single cell genomic study of Dehalococcoidetes species from deep-sea sediments of the Peruvian Margin. ISME J 8:1831-42

Dehalogenating chloroflexi (Dehalococcoidetes) were originally discovered as the key microorganisms mediating reductive dehalogenation via their key enzymes reductive dehalogenases (Rdh), as the sole mode of energy conservation in terrestrial environments. In this study, total DNA was extracted from several deep-sea sediments from the Peruvian Margin and other Pacific sites and analyzed for the presence of Dehalococcoidetes 16S rRNA and rdh genes by PCR and nanoliter-qPCR. A sample from the Peruvian Margin trench was selected for cell sorting and single-cell genome sequencing on an Illumina HiSeq system. Two of the single cells were considered to be part of a local Dehalococcoidetes population and assembled together into a 1.38-Mb genome, to about 85% completeness.

Illumina Technology: Genome Analyzer$_{IIx}$, Nextera DNA Sample Prep, HiSeq

Nair S., Nkhoma S. C., Serre D., Zimmerman P. A., Gorena K., et al. (2014) Single-cell genomics for dissection of complex malaria infections. Genome Res 24: 1028-1038

Anti-malarial chemotherapy is central to treatment and control strategies, but drug resistance can drastically undermine these efforts. In this study, the authors used single-cell sequencing and genotyping of samples to discover the within-host diversity of a malarial infection. Using this approach, they observed four distinct drug-resistance haplotypes within the P. falciparum infection MKK2664. The authors demonstrated that the single-cell genomics approach can be used to generate parasite genome sequences directly from patient blood in order to unravel the complexity of P. vivax and P. falciparum infections.

Illumina Technology: HiSeq, VeraCode Custom DASL, BeadXpress

Anopheles mosquito

Wang F. P., Zhang Y., Chen Y., He Y., Qi J., et al. (2014) Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. ISME J 8: 1069-1078

The microbes responsible for anaerobic oxidation of methane (AOM) are the subject of intense study but information about mechanistic details have been lacking. In this study, the genome of a highly active AOM enrichment dominated by the methane-oxidizing archaea ANME-2a was studied by single-aggregate sequencing on Illumina Genome Analyzer. All genes required for performing the seven steps of methanogenesis from CO2 were found present and actively expressed.

Illumina Technology: Genome Analyzer$_{IIx}$

Wasmund K., Schreiber L., Lloyd K. G., Petersen D. G., Schramm A., et al. (2014) Genome sequencing of a single cell of the widely distributed marine subsurface Dehalococcoidia, phylum Chloroflexi. ISME J 8: 383-397

Microbes in the marine subsurface are key catalysts in global biogeochemical cycles. The marine bacteria of the class Dehalococcoidia (DEH), phylum Chloroflexi, are one such widely distributed species. In this study, the authors analyzed the genomic content from a single DEH cell to determine the metabolic versatility. The genome was sequenced on an Illumina HiSeq 2000 and the authors assembled an estimated 60.8% of the full genome. Numerous genes encoding enzymes of core and auxiliary fatty acid oxidation pathways were identified. Several incomplete covered pathways suggested the organism could synthesize ATP by converting acetyl-CoA to acetate by substrate-level phosphorylation.

Illumina Technology: HiSeq

Kamke J., Sczyrba A., Ivanova N., Schwientek P., Rinke C., et al. (2013) Single-cell genomics reveals complex carbohydrate degradation patterns in poribacterial symbionts of marine sponges. ISME J 7: 2287-2300

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Reyes A., Wu M., McNulty N. P., Rohwer F. L. and Gordon J. I. (2013) Gnotobiotic mouse model of phage-bacterial host dynamics in the human gut. Proc Natl Acad Sci U S A 110: 20236-20241

SAMPLE PREPARATION

There are many options available to isolate a single cell from a heterogeneous population of cells.[72] In addition to well-established methods, such as fluorescence activated cell sorting (FACS), microscopy, and the use of antibody capture for cell separation, there is an increasingly ingenious armamentarium of modern methods to isolate single cells with ever greater accuracy and specificity.[73,74] The most critical part of single-cell analysis is sample preparation, where specific cells need to be accurately isolated from a heterogeneous population.[75] This section highlights commonly used and novel techniques for isolation of single cells from suspension or tissues.

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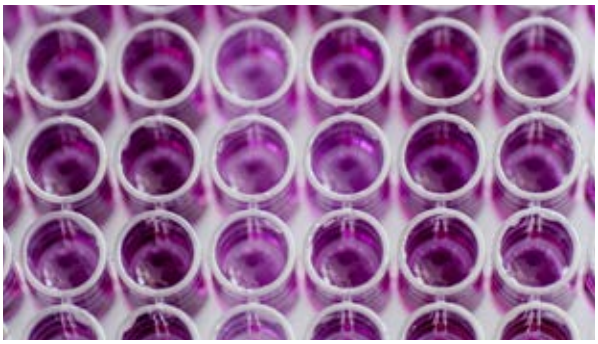

Single cells from dissociated tissues are directly sorted into 96-well plates for analysis.

Method	Sample Type	Pros[75,76,79]	Cons[75,76,79]
Fluorescence Activated Cell Sorting (FACS)[77]	Dissociated cell suspension	• Specific immuno-tagging of cell surface markers. • Multiple simultaneous tagging can assure isolation of specific cells. • Can sort cells into 96- or 384-well plates with nearly 100% purity.[75]	• Need antibodies that are specific to cell surface marker. • Cannot be scaled to large projects • Needs large starting volume. • Low throughput. • Recovered cells suffer from mechanical stress. • Optimization needed before sorting.
Microfluidics[78]	Dissociated cell suspension	• Ability to isolate non-culturable cells from small volumes. • Can isolate specific cells based on cell surface markers. • Rare circulating tumor cells have been successfully isolated.	• High cost. • Low throughput.
Optical Tweezers[79]	Dissociated cell suspension	• More focused and controlled cell isolation compared to micropipettes.[75] • Automated sorting of single cells. • High throughput. • Fluorescence tagging of cells of interest.[79]	Available to a few specialized labs due to demanding set up.[75]
Micro-manipulation[80]	Dissociated cell suspension	• Can isolate one cell from various developmental stages, such as neurons. • Can also isolate cells from a diverse population.	• Low throughput. • Cell specificity determined by microscopy and isolated using a micropipette may not be accurate. • Large starting volume needed.
TIVA[81]	Tissue sample	• Study temporal transcriptomics of single cells in live tissues.[81] • Directly capture mRNA from single cells, retaining cellular response to microenvironment.[81] • Noninvasive protocol.	• Low throughput. • Study one tissue at a time.
Laser-Capture Microdissection (LCM)[82]	Tissue sample	• Isolates cells from its natural environment from whole tissues, retaining RNA integrity within the isolated cell.[75] • Fast isolation of single cells from tissue of interest. • Can be combined with microscopy techniques to leverage single-cell temporal transcriptomics in a tissue. • No prior dissociation of tissue to cell suspension is needed.	• Tissues must be cryopreserved or fixed. • Microdissection can be challenging. • Small cells might be difficult to isolate.[83] • Proper tissue processing required to maintain integrity.[84] • Potential RNA contamination as cells are isolated and tissue cut.[81]

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Adalsteinsson V. A. and Love J. C. (2014) Towards Engineered Processes for Sequencing-Based Analysis of Single Circulating Tumor Cells. Curr Opin Chem Eng 4: 97-104

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Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

Barteneva N. S., Ketman K., Fasler-Kan E., Potashnikova D. and Vorobjev I. A. (2013) Cell sorting in cancer research--diminishing degree of cell heterogeneity. Biochim Biophys Acta 1836: 105-122

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Lohr J. G., Adalsteinsson V. A., Cibulskis K., Choudhury A. D., Rosenberg M., et al. (2014) Whole-exome sequencing of circulating tumor cells provides a window into metastatic prostate cancer. Nat Biotechnol 32: 479-484

The analysis of CTCs is a promising new avenue for monitoring and diagnosis of metastatic cancer. This study presents an integrated process to isolate, qualify, and sequence whole exomes of CTCs with high fidelity. The authors used the Illumina MagSweeper to enrich epCAM-expressing CTCs. Individual cells were recovered and sequenced on Illumina HiSeq. The authors developed a methodology for assessing the quality and uniformity of genome-wide coverage of CTC-derived libraries to show the performance of their process. The process was validated by sequencing the metastatic CTCs of two patients with prostate cancer, and showed that 70% of CTC mutations were present in matched tissue.

Illumina Technology: HiSeq, MiSeq

Lovatt D., Ruble B. K., Lee J., Dueck H., Kim T. K., et al. (2014) Transcriptome *in vivo* analysis (TIVA) of spatially defined single cells in live tissue. Nat Methods 11: 190-196

RNA sequencing methods that rely on RNA extracted from cell mixtures do not convey the individual variability in expression among cells of the same tissue. In this paper, the authors present a transcriptome *in vivo* analysis (TIVA), which is applicable to single-cell studies. In combination with Illumina sequencing technology, the authors capture and analyze the transcriptome variance across single neurons both in culture and *in vivo*. This method is furthermore non-invasive and may be applied to intact tissue. It will enable detailed studies of cell heterogeneity in complex tissues that have been intractable previously, and it opens up the possibility of use in conjunction with *in vivo* live functional imaging.

Illumina Technology: HiSeq

Marinov G. K., Williams B. A., McCue K., Schroth G. P., Gertz J., et al. (2014) From single-cell to cell-pool transcriptomes: stochasticity in gene expression and RNA splicing. Genome Res 24: 496-510

Recent studies are increasingly discovering cell-to-cell variability in gene expression levels and transcriptional regulation. This study examined the lymphoblastoid cell line GM12878 using the Smart-seq single-cell RNA-seq protocol on Illumina HiSeq 2000 to determine variation in transcription among individual cells. The authors determined, through careful quantitation, that there are significant differences in expression between individual cells, over and above technical variation. In addition, they showed that the transcriptomes from small pools of 30–100 cells approach the information content and reproducibility of contemporary pooled RNA-Seq analysis from large amounts of input material.

Illumina Technology: HiSeq 2000

Wu A. R., Neff N. F., Kalisky T., Dalerba P., Treutlein B., et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11: 41-46

In this comprehensive analysis, the authors used microfluidic automation to quantitatively compare the accuracy and precision of single-cell RNA-Seq to qPCR. Commercially available single-cell RNA amplification methods, with both microliter and nanoliter volumes, were used to prepare and sequence libraries on Illumina HiSeq 2000. The study shows that single-cell RNA-Seq can generate results that are quantitatively comparable to qPCR, particularly when sample preparation is done on nanoliter-scale reaction volumes, such as in a microfluidic device.

Illumina Technology: Nextera DNA Sample Prep, HiSeq

Fraley S. I., Hardick J., Jo Masek B., Athamanolap P., Rothman R. E., et al. (2013) Universal digital high-resolution melt: a novel approach to broad-based profiling of heterogeneous biological samples. Nucleic Acids Res 41: e175

DATA ANALYSIS

Single-cell sequencing presents unique challenges for data analysis. Each cell contains between 50,000 and 300,000 transcripts, and there are significant differences among the expression values of individual cells.[85,86] When thousands of genes are expressed in the range of 1 to 30 mRNA copies per cell, it is important that each mRNA is copied into cDNA and ultimately represented uniformly in the library.[87,88] To distinguish between technical variability and biological changes of interest, spike-in quantification standards of known abundance[89] have shown that the bulk of the changes are due to biological variability.[90] Molecular indexing can correct for biases[91,92] and new developments in automated sample handling should reduce technical variability even more.[93]

In single-cell sequencing of DNA, the technical artifacts can also be reduced by algorithms that are specifically tuned for this application.[94]

Name	Description
Monocle	An unsupervised algorithm that increases the temporal resolution of transcriptome dynamics using single-cell RNA-Seq data collected at multiple time points.[95]
PyClone	A statistical model for inference of clonal population structures in cancers.[96]
SPAdes	Single-cell assembler for both single-cell and multi-cell assembly.[97]

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Eckersley-Maslin M. A. and Spector D. L. (2014) Random monoallelic expression: regulating gene expression one allele at a time. Trends Genet 30: 237-244

Gendrel A. V., Attia M., Chen C. J., Diabangouaya P., Servant N., et al. (2014) Developmental dynamics and disease potential of random monoallelic gene expression. Dev Cell 28: 366-380

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Saliba A. E., Westermann A. J., Gorski S. A. and Vogel J. (2014) Single-cell RNA-seq: advances and future challenges. Nucleic Acids Res 42: 8845-8860

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Albert F. W., Treusch S., Shockley A. H., Bloom J. S. and Kruglyak L. (2014) Genetics of single-cell protein abundance variation in large yeast populations. Nature 506: 494-497

DNA variation among individuals may result in differences in protein expression. In this study, the authors examined the genetics of single-cell protein abundance by selecting single yeast cells with high and low protein expression. They subsequently detected whole-genome DNA variants with Illumina HiSeq sequencing. The method was applied to 160 genes and detected many more quantitative trait loci (QTL) per gene than previous studies. Most loci were clustered in “hotspots” that influence multiple proteins, and some hotspots were found to influence more than half of the proteins examined.

Illumina Technology: HiSeq

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Eckersley-Maslin M. A., Thybert D., Bergmann J. H., Marioni J. C., Flicek P., et al. (2014) Random monoallelic gene expression increases upon embryonic stem cell differentiation. Dev Cell 28: 351-365
Random autosomal monoallelic gene expression refers to the transcription of a gene from one of two homologous alleles. This study examined the mechanisms for monoallelic expression in mouse cell lines through an allele-specific RNA-Seq screen using Illumina sequencing. The authors identified many random monoallelically expressed genes and discovered that neither DNA methylation nor nuclear positioning distinguished the active versus inactive alleles. However, they did find a correlation in enrichment for specific histone modifications.

Illumina Technology: Genome Analyzer$_{IIx}$

Marinov G. K., Williams B. A., McCue K., Schroth G. P., Gertz J., et al. (2014) From single-cell to cell-pool transcriptomes: stochasticity in gene expression and RNA splicing. Genome Res 24: 496-510
Recent studies are increasingly discovering cell-to-cell variability in gene expression levels and transcriptional regulation. This study examined the lymphoblastoid cell line GM12878 using the Smart-seq single-cell RNA-seq protocol on Illumina HiSeq 2000 to determine variation in transcription among individual cells. The authors determined, through careful quantitation, that there are significant differences in expression between individual cells, over and above technical variation. In addition, they showed that the transcriptomes from small pools of 30–100 cells approach the information content and reproducibility of contemporary pooled RNA-Seq analysis from large amounts of input material.

Illumina Technology: HiSeq, Nextera DNA Sample Prep

Streets A. M., Zhang X., Cao C., Pang Y., Wu X., et al. (2014) Microfluidic single-cell whole-transcriptome sequencing. Proc Natl Acad Sci U S A 111: 7048-7053
Single-cell whole-transcriptome analysis is a powerful tool for quantifying gene expression heterogeneity in populations of cells. This study presents a strategy for single-cell RNA-Seq implemented in a microfluidic platform for single-cell whole-transcriptome analysis. With this approach, double-stranded cDNA is collected and sequenced on Illumina HiSeq 2500. The authors demonstrated the technique on single mouse embryonic cells and were able to reconstruct a majority of the bulk transcriptome from as little as 10 single cells. Using this approach, the authors quantified the variation among and within different types of mouse embryonic cells.

Illumina Technology: HiSeq 2500

Swain Lenz D., Riles L. and Fay J. C. (2014) Heterochronic meiotic misexpression in an interspecific yeast hybrid. Mol Biol Evol 31: 1333-1342
Interspecific hybrids express genes at levels outside the range of either parental species. In this paper, the authors examined the gene expression of a sterile interspecific yeast hybrid during meiosis. They used Illumina HiSeq for RNA sequencing at multiple meiotic stages. They found the hybrid and parents exhibited similar changes in expression levels across meiosis, but the hybrid meiotic program occurred earlier than that of either parent. Coincident with the timing of misexpression, they found a transition from predominantly trans-acting to cis-acting expression divergence and an increase in the number of opposing cis-trans changes.

Illumina Technology: HiSeq

Mueller A. A., Cheung T. H. and Rando T. A. (2013) All's well that ends well: alternative polyadenylation and its implications for stem cell biology. Curr Opin Cell Biol 25: 222-232

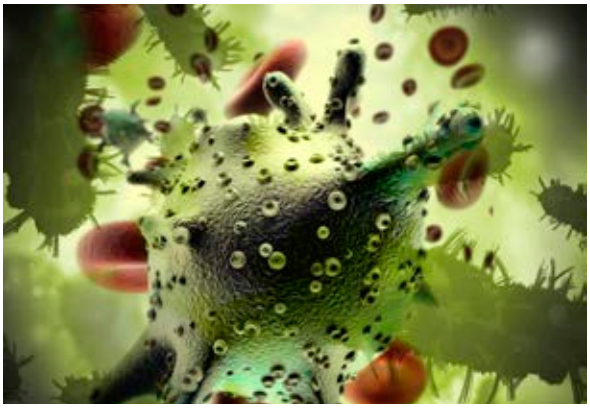
Pan X., Durrett R. E., Zhu H., Tanaka Y., Li Y., et al. (2013) Two methods for full-length RNA sequencing for low quantities of cells and single cells. Proc Natl Acad Sci U S A 110: 594-599

Xue Z., Huang K., Cai C., Cai L., Jiang C. Y., et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500: 593-597

DNA APPLICATIONS

DNA replication during cell division is not perfect; as a result, progressive generations of cells accumulate unique somatic mutations. Consequently, each cell in the body has a unique genomic signature, which allows the reconstruction of cell lineage trees with very high precision.[98] These cell lineage trees can predict the existence of small populations of stem cells. This information is important for fields as diverse as cancer development,[99,100] preimplantation, and genetic diagnosis.[101,102,103,104]

Sequencing single cells can allow researchers to trace back lineages of differentiated cells, by identifying acquired somatic mutations and/or CNVs.[101] Single-cell sequencing is also an effective approach to characterize organisms that are difficult to culture *in vitro*. Advances in single-cell sequencing have improved diagnosis of outbreaks, antibiotic-resistant strains, food-borne pathogens, and the classification of microbial diversities in the gut environment.[105,106,107]



Single-cell genomics can help characterize and identify circulating tumor cells as well as microbes.

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99. Navin N., Kendall J., Troge J., Andrews P., Rodgers L., et al. (2011) Tumour evolution inferred by single-cell sequencing. Nature 472: 90-94
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101. Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630
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Reviews

Baslan T. and Hicks J. (2014) Single cell sequencing approaches for complex biological systems. Curr Opin Genet Dev 26C: 59-65

Blainey P. C. (2013) The future is now: single-cell genomics of bacteria and archaea. FEMS Microbiol Rev 37: 407-427

Lovett M. (2013) The applications of single-cell genomics. Hum Mol Genet 22: R22-26

Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

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Voet T., Kumar P., Van Loo P., Cooke S. L., Marshall J., et al. (2013) Single-cell paired-end genome sequencing reveals structural variation per cell cycle. Nucleic Acids Res 41: 6119-6138

Spondylocostal dysostosis (SCD) is a congenital spinal defect for which autosomal dominant forms have been reported with no known genetic etiology. In this study, whole-exome sequencing was used to identify the causal mutation of SCD in two generations of a family. The finding was supported by knock-out studies in mice and demonstrates the power of detection obtainable with exome sequencing.

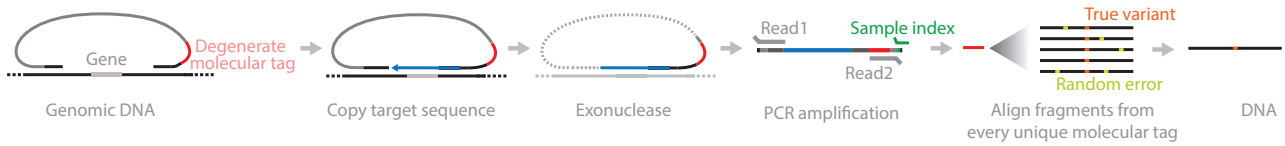
Illumina Technology: TruSeq Exome, Genome Analyzer$_{IIx}$, HiSeq

Single-Molecule Molecular Inversion Probes (smMIP)

The single-molecule molecular inversion probes (smMIP) method uses single-molecule tagging and molecular inversion probes to detect and quantify genetic variation occurring at very low frequencies.[108] In this method, probes are used to detect targets in genomic DNA. After the probed targets are copied, exonuclease digestion leaves the target with a tag, which undergoes PCR amplification and sequencing. Sequencing allows for high-resolution sequence reads of targets, while greater depth allows for better alignment for every unique molecular tag.

108. Hiatt J. B., Pritchard C. C., Salipante S. J., O'Roak B. J. and Shendure J. (2013) Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 23: 843-854

Pros	Cons
• Detection of low-frequency targets. • Can perform single-cell sequencing or sequencing for samples with very limited starting material.	• PCR amplification errors. • PCR biases can underrepresent GC-rich templates. • Targets smaller than 500 bp are preferentially amplified by polymerases during PCR.



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Hiatt J. B., Pritchard C. C., Salipante S. J., O'Roak B. J. and Shendure J. (2013) Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res 23: 843-854

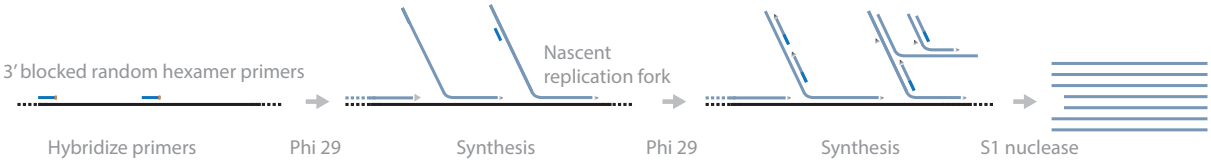
This is the first paper to describe the smMIP assay, its practicality, ability for multiplexing, scaling, and compatibility with desktop sequencing for rapid data collection. The authors demonstrated the assay by re-sequencing 33 clinically informative cancer genes in 8 cell lines and 45 clinical cancer samples, retrieving accurate data.

Illumina Technology: MiSeq, HiSeq 2000

Multiple Displacement Amplification (MDA)

Multiple displacement amplification (MDA) is a method commonly used for sequencing microbial genomes, due to its ability to amplify templates larger than 0.5 Mbp. However, it can also be used to study genomes of other sizes.[109] In this method, 3'-blocked random hexamer primers are hybridized to a template, followed by synthesis with Phi 29 polymerase. Phi 29 polymerase performs strand-displacement DNA synthesis, allowing for efficient and rapid DNA amplification. Deep sequencing of the amplified sequences permits accurate representation of reads, while sequencing depth provides better alignment and consensus for sequences.

Pros	Cons
• Templates used for this method can be circular DNA (plasmids, bacterial DNA). • Can sequence large templates. • Can perform single-cell sequencing or sequencing for samples with very limited starting material.	• Strong amplification bias. Genome coverage as low as ~6%.[110] • PCR biases can underrepresent GC-rich templates. • Contaminated reagents can impact results.[111]



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Embree M., Nagarajan H., Movahedi N., Chitsaz H. and Zengler K. (2014) Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community. ISME J 8: 757-767

Microbial communities amass a wealth of biochemical processes, and metagenomics approaches are often unable to decipher the key functions of individual microorganisms. This study analyzed a microbial community by first determining the genome sequence of a dominant bacterial member belonging to the genus Smithella using a single-cell sequencing approach on an Illumina Genome Analyzer. After establishing a working draft genome of Smithella, the authors used low-input metatranscriptomics to determine which genes were active during alkane degradation. The authors then designed a genome-scale metabolic model to integrate the genomic and transcriptomic data.

Illumina Technology: Nextera DNA Sample Prep, MiSeq, Genome Analyzer$_{IIx}$

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Kaster A. K., Mayer-Blackwell K., Pasarelli B. and Spormann A. M. (2014) Single cell genomic study of Dehalococcoidetes species from deep-sea sediments of the Peruvian Margin. ISME J 8:1831-42

Dehalogenating chloroflexi (Dehalococcoidetes) were originally discovered as the key microorganisms mediating reductive dehalogenation via their key enzymes reductive dehalogenases (Rdh), as the sole mode of energy conservation in terrestrial environments. In this study, total DNA was extracted from several deep-sea sediments from the Peruvian Margin and other Pacific sites and analyzed for the presence of Dehalococcoidetes 16S rRNA and rdh genes by PCR and nanoliter-qPCR. A sample from the Peruvian Margin trench was selected for cell sorting and single-cell genome sequencing on an Illumina HiSeq system. Two of the single cells were considered to be part of a local Dehalococcoidetes population and assembled together into a 1.38-Mb genome, to about 85% completeness.

Illumina Technology: HiSeq, Nextera DNA Sample Prep

Tsai I. J., Hunt M., Holroyd N., Huckvale T., Berriman M., et al. (2014) Summarizing specific profiles in Illumina sequencing from whole-genome amplified DNA. DNA Res 21: 243-254

Using the highly curated Caenorhabditis elegans genome as a reference, this study performed a series of sequencing experiments and identified errors and biases associated with Illumina library construction, library insert size, different WGA methods, and genome features such as GC bias and simple repeat content.

Illumina Technology: MiSeq, HiSeq

Wang F. P., Zhang Y., Chen Y., He Y., Qi J., et al. (2014) Methanotrophic archaea possessing diverging methane-oxidizing and electron-transporting pathways. ISME J 8: 1069-1078

The microbes responsible for anaerobic oxidation of methane (AOM) are the subject of intense study but information about mechanistic details have been lacking. In this study, the genome of a highly active AOM enrichment dominated by the methane-oxidizing archaea ANME-2a was studied by single-aggregate sequencing on Illumina Genome Analyzer. All genes required for performing the seven steps of methanogenesis from CO2 were found present and actively expressed.

Illumina Technology: Genome Analyzer$_{IIx}$

Wasmund K., Schreiber L., Lloyd K. G., Petersen D. G., Schramm A., et al. (2014) Genome sequencing of a single cell of the widely distributed marine subsurface Dehalococcoidia, phylum Chloroflexi. ISME J 8: 383-397

Microbes in the marine subsurface are key catalysts in global biogeochemical cycles. The marine bacteria of the class Dehalococcoidia (DEH), phylum Chloroflexi, are one such widely distributed species. In this study, the authors analyzed the genomic content from a single DEH cell to determine the metabolic versatility. The genome was sequenced on an Illumina HiSeq 2000 and the authors assembled an estimated 60.8% of the full genome. Numerous genes encoding enzymes of core and auxiliary fatty acid oxidation pathways were identified several incomplete covered pathways suggested the organism could synthesize ATP by converting acetyl-CoA to acetate by substrate-level phosphorylation.

Illumina Technology: HiSeq 2000

Depew J., Zhou B., McCorrison J. M., Wentworth D. E., Purushe J., et al. (2013) Sequencing viral genomes from a single isolated plaque. Virol J 10: 181

Sequencing of viruses and bacteriophages is usually preceded by production of viral stock or specific purification and amplification to obtain sufficient quantities of genomic material. This study presents a novel method of sequence-independent single primer amplification (SISPA) to allow sequencing from as little as 10 pg of DNA template.

Illumina Technology: HiSeq

Hou Y., Fan W., Yan L., Li R., Lian Y., et al. (2013) Genome analyses of single human oocytes. Cell 155: 1492-1506

Chromosomal crossover occurs in the oocyte, producing unique combinations of the parent chromosomes in the fertilized egg. This paper presents a protocol for single-cell genome analysis demonstrated on human oocytes. Using multiple annealing and looping-based amplification cycle (MALBAC)-based sequencing, the authors sequenced the triads of first and second polar bodies from the oocyte pronuclei from same female egg donors and phased their genomes to determine the crossover maps for the oocytes. This breakthrough assay represents important progress toward using whole-genome sequencing for meiosis research and embryo selection for *in vitro* fertilization.

Illumina Technology: HiSeq 2000

McLean J. S., Lombardo M. J., Ziegler M. G., Novotny M., Yee-Greenbaum J., et al. (2013) Genome of the pathogen Porphyromonas gingivalis recovered from a biofilm in a hospital sink using a high-throughput single-cell genomics platform. Genome Res 23: 867-877

Single-cell genomics is becoming an accepted method to capture novel genomes, primarily in the marine and soil environments. This study shows, for the first time, that it also enables comparative genomic analysis of strain variation in a pathogen captured from complex biofilm samples in a healthcare facility. The authors present a nearly complete genome representing a novel strain of the periodontal pathogen Porphyromonas gingivalis using the single-cell assembly tool SPAdes.

Illumina Technology: Nextera DNA Sample Prep, Genome Analyzer$_{IIx}$

Seth-Smith H. M., Harris S. R., Skilton R. J., Radebe F. M., Golparian D., et al. (2013) Whole-genome sequences of Chlamydia trachomatis directly from clinical samples without culture. Genome Res 23: 855-866

The use of whole-genome sequencing as a tool to study infectious bacteria is of growing clinical interest. A culture of Chlamydia trachomatis has, until now, been a prerequisite to obtain DNA for whole-genome sequencing. Unfortunately, culturing C. trachomatis is a technically demanding and time-consuming procedure. This paper presents IMS-MDA, a new approach that combines immuno-magnetic separation (IMS) and multiple-displacement amplification (MDA) for whole-genome sequencing of bacterial genomes directly from clinical samples.

Illumina Technology: Genome Analyzer$_{IIx}$, HiSeq 2000

Wang Y., Waters J., Leung M. L., Unruh A., Roh W., et al. (2014) Clonal evolution in breast cancer revealed by single nucleus genome sequencing. Nature 512:155-60

Fitzsimons M. S., Novotny M., Lo C. C., Dichosa A. E., Yee-Greenbaum J. L., et al. (2013) Nearly finished genomes produced using gel microdroplet culturing reveal substantial intraspecies genomic diversity within the human microbiome. Genome Res 23: 878-888

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Kirkness E. F., Grindberg R. V., Yee-Greenbaum J., Marshall C. R., Scherer S. W., et al. (2013) Sequencing of isolated sperm cells for direct haplotyping of a human genome. Genome Res 23: 826-832

Lloyd K. G., Schreiber L., Petersen D. G., Kjeldsen K. U., Lever M. A., et al. (2013) Predominant archaea in marine sediments degrade detrital proteins. Nature 496: 215-218

McLean J. S., Lombardo M. J., Badger J. H., Edlund A., Novotny M., et al. (2013) Candidate phylum TM6 genome recovered from a hospital sink biofilm provides genomic insights into this uncultivated phylum. Proc Natl Acad Sci U S A 110: E2390-2399

Reyes A., Wu M., McNulty N. P., Rohwer F. L. and Gordon J. I. (2013) Gnotobiotic mouse model of phage-bacterial host dynamics in the human gut. Proc Natl Acad Sci U S A 110: 20236-20241

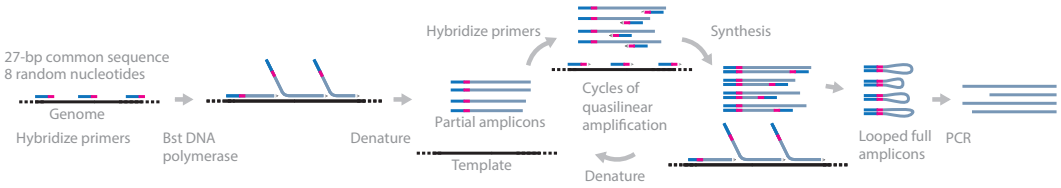
Taghavi Z., Movahedi N. S., Draghici S. and Chitsaz H. (2013) Distilled single-cell genome sequencing and de novo assembly for sparse microbial communities. Bioinformatics 29: 2395-2401

Voet T., Kumar P., Van Loo P., Cooke S. L., Marshall J., et al. (2013) Single-cell paired-end genome sequencing reveals structural variation per cell cycle. Nucleic Acids Res 41: 6119-6138

Multiple Annealing and Looping-Based Amplification Cycles (MALBAC)

Multiple annealing and looping-based amplification cycles (MALBAC) is intended to address some of the shortcomings of MDA.[112] In this method, MALBAC primers randomly anneal to a DNA template. A polymerase with displacement activity at elevated temperatures amplifies the template, generating semi-amplicons. As the amplification and annealing process is repeated, the semi-amplicons are amplified into full amplicons that have a 3' end complementary to the 5' end. As a result, full-amplicon ends hybridize to form a looped structure, inhibiting further amplification of the looped amplicon, while only the semi-amplicons and genomic DNA undergo amplification. Deep sequencing of the full-amplicon sequences allows for accurate representation of reads, while sequencing depth provides improved alignment for consensus sequences.

Pros	Cons
• Can sequence large templates. • Can perform single-cell sequencing or sequencing for samples with very limited starting material. • Full-amplicon looping inhibits over-representation of templates, reducing PCR bias. • Can amplify GC rich regions. • Uniform genome coverage. • Lower allele drop-out rate compared to MDA.	• Polymerase is relatively error-prone compared to Phi 29. • Temperature-sensitive protocol. • Genome coverage up to ~90%,[113] but some regions of the genome are consistently underrepresented.[114]



112. Zong C., Lu S., Chapman A. R. and Xie X. S. (2012) Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science 338: 1622-1626
113. Lovett M. (2013) The applications of single-cell genomics. Hum Mol Genet 22: R22-26
114. Lasken R. S. (2013) Single-cell sequencing in its prime. Nat Biotechnol 31: 211-212

References

Hou Y., Fan W., Yan L., Li R., Lian Y., et al. (2013) Genome analyses of single human oocytes. Cell 155: 1492-1506

Chromosomal crossover occurs in the oocyte, producing unique combinations of the parent chromosomes in the fertilized egg. This paper presents a protocol for single-cell genome analysis demonstrated on human oocytes. Using MALBAC-based sequencing, the authors sequenced the triads of first and second polar bodies from the oocyte pronuclei from same female egg donors and phased their genomes to determine the crossover maps for the oocytes. This breakthrough assay represents important progress toward using whole-genome sequencing for meiosis research and embryo selection for *in vitro* fertilization.

Illumina Technology: HiSeq 2000

Ni X., Zhuo M., Su Z., Duan J., Gao Y., et al. (2013) Reproducible copy number variation patterns among single circulating tumor cells of lung cancer patients. Proc Natl Acad Sci U S A 110: 21083-21088

There is a great deal of interest in identifying and studying CTCs. Cells from primary tumors enter the bloodstream and can seed metastases. A major barrier to such analysis is the low DNA input amounts obtained from single cells, leading to lower coverage. This study uses MALBAC for WGA sequencing of single CTCs from patients with lung cancer. The researchers identify CNVs that were consistent in patients with the same cancer subtype. Such information about cancers can help identify drug resistance and cancer subtypes, and offers potential for diagnostics leading to individualized treatment.

Illumina Technology: MiSeq, HiSeq 2000

Zong C., Lu S., Chapman A. R. and Xie X. S. (2012) Genome-wide detection of single-nucleotide and copy-number variations of a single human cell. Science 338: 1622-1626

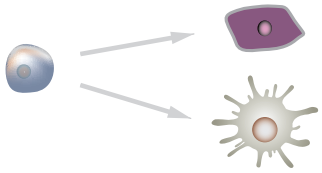
This is the first paper that describes the MALBAC method, which the authors indicate has higher detection efficiency than the traditional MDA method for single-cell studies. The authors demonstrate detection of CNVs and single-nucleotide variants (SNVs) of single cancer cells with no false positives.

Illumina Technology: HiSeq 2000

EPIGENETICS

Epigenetics plays a crucial role during embryogenesis, differentiation, lineage specification, and cancer evolution.[115,116] During embryogenesis, as cells differentiate, they do not just differ at the genome level, but also differ at the epigenome level,[115] which subsequently influences RNA expression and cellular phenotype.[115,116,117] Lifestyle, environmental factors, exposure to chemicals, and stress are some factors capable of altering epigenetic marks not only during pregnancy in stem cells, but also in differentiated somatic cells.[118] Changes in epigenetic marks can result in various diseases such as cardiovascular, respiratory and neurodegenerative diseases, and cancer.[118] Understanding the role of epigenetics in cancer evolution and subsequent tumor heterogeneity is currently of great interest.

Population sequencing cannot distinguish between which cells display a specific genotype and subsequent phenotype, given their temporal location in a tissue at a given time under specific conditions.[117] Single-cell DNA methylation and ChIP-Seq data, when integrated with RNA expression and SNV data, can precisely disclose the functions of these modifications and their roles in gene regulation. Such high-resolution sequencing data integrating the genome, epigenome, and transcriptome of single cells located within a microenvironment at a given time has not been feasible until recently.



As stem cells differentiate, changes at the epigenome level influence RNA expression and the generation of multiple cellular phenotypes.

Reviews

Baslan T. and Hicks J. (2014) Single cell sequencing approaches for complex biological systems. Curr Opin Genet Dev 26C: 59-65

Easwaran H., Tsai H. C. and Baylin S. B. (2014) Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. Mol Cell 54: 716-727

Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123

McGraw S., Shojaei Saadi H. A. and Robert C. (2013) Meeting the methodological challenges in molecular mapping of the embryonic epigenome. Mol Hum Reprod 19: 809-827

Umer M. and Herceg Z. (2013) Deciphering the epigenetic code: an overview of DNA methylation analysis methods. Antioxid Redox Signal 18: 1972-1986

115. Guo H., Zhu P., Wu X., Li X., Wen L., et al. (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. Genome Res 23: 2126-2135
116. Xue Z., Huang K., Cai C., Cai L., Jiang C. Y., et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500: 593-597
117. Weaver W. M., Tseng P., Kunze A., Masaeli M., Chung A. J., et al. (2014) Advances in high-throughput single-cell microtechnologies. Curr Opin Biotechnol 25: 114-123
118. Alegria-Torres J. A., Baccarelli A. and Bollati V. (2011) Epigenetics and lifestyle. Epigenomics 3: 267-277

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Eckersley-Maslin M. A., Thybert D., Bergmann J. H., Marioni J. C., Flicek P., et al. (2014) Random monoallelic gene expression increases upon embryonic stem cell differentiation. Dev Cell 28: 351-365

Random autosomal monoallelic gene expression refers to the transcription of a gene from one of two homologous alleles. This study examined the mechanisms for monoallelic expression in mouse cell lines through an allele-specific RNA-Seq screen using Illumina sequencing. The authors identified many random monoallelically expressed genes and discovered that neither DNA methylation nor nuclear positioning distinguished the active versus inactive alleles. However, they did find a correlation in enrichment for specific histone modifications.

Illumina Technology: Genome Analyzer$_{IIx}$

Green M. R., Vicente-Duenas C., Romero-Camarero I., Long Liu C., Dai B., et al. (2014) Transient expression of Bcl6 is sufficient for oncogenic function and induction of mature B-cell lymphoma. Nat Commun 5: 3904

It is well established that cancer arises by an acquisition of somatic genomic alterations that lead to a malignant state. However, it is difficult to determine the initiation event for the cancerous development. This study examined the specific gain of 3q27.2 and its association with adverse outcome in DLBCL. Using an Illumina Genome Analyzer, the researchers sequenced the whole genome and characterized the DNA methylation of murine HSPCs. The authors identified BCL6 oncogene expression in HSPCs and demonstrated, by knock-in expression, that transient expression of Bcl6 within murine HSPCs can initiate the development into mature B-cell lymphomas.

Illumina Technology: Genome Analyzer$_{IIx}$

Obata Y., Furusawa Y., Endo T. A., Sharif J., Takahashi D., et al. (2014) The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. Nat Immunol 15: 571-579

Treg cells are necessary for the suppression of excessive immune responses to gut bacteria. This study examined the DNA methylation status and regulation in the context of exposure to gut microbiota. The authors examined mice with a T-cell specific deficiency in the DNA methylation adapter Uhrf1 using Illumina HiSeq. They found that mice with this deficiency showed defective proliferation of colonic regulatory T cells and developed severe colitis. The authors concluded that Uhrf1 is required for the epigenetic mechanism that maintains the gut immunological homeostasis.

Illumina Technology: HiSeq 1000

Smallwood S. A., Lee H. J., Angermueller C., Krueger F., Saadeh H., et al. (2014) Single-cell genome-wide bisulfite sequencing for assessing epigenetic heterogeneity. Nat Methods 11: 817-820

DNA methylation is an important mechanism for gene regulation in the cell. This study presents a method for single-cell bisulfite sequencing (scBS-Seq) using Illumina HiSeq sequencing to characterize the genome-wide DNA methylation of individual cells. The authors demonstrate their method in mouse ESCs and show that epigenetic heterogeneity varies across different types of functional sites in the genome.

Illumina Technology: HiSeq

Guo H., Zhu P., Wu X., Li X., Wen L., et al. (2013) Single-cell methylome landscapes of mouse embryonic stem cells and early embryos analyzed using reduced representation bisulfite sequencing. Genome Res 23: 2126-2135

DNA methylation is an important part of gene expression regulation and is variable across cells, even within the same tissue. This study presents a methylome analysis technique for single cells at single-base resolution. The authors created scRRBS libraries for sequencing on Illumina HiSeq 2000. In an analysis of mouse ESCs, the authors showed for the first time that the methylome of the first polar body is comparable with that of the metaphase II oocyte within the same gamete.

Illumina Technology: HiSeq 2000

de Wit E., Bouwman B. A., Zhu Y., Klous P., Splinter E., et al. (2013) The pluripotent genome in three dimensions is shaped around pluripotency factors. Nature 501: 227-231

Kind J., Pagie L., Ortabozkoyun H., Boyle S., de Vries S. S., et al. (2013) Single-cell dynamics of genome-nuclear lamina interactions. Cell 153: 178-192

Magnusdottir E., Dietmann S., Murakami K., Gunesdogan U., Tang F., et al. (2013) A tripartite transcription factor network regulates primordial germ cell specification in mice. Nat Cell Biol 15: 905-915

Nagano T., Lubling Y., Stevens T. J., Schoenfelder S., Yaffe E., et al. (2013) Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature 502: 59-64

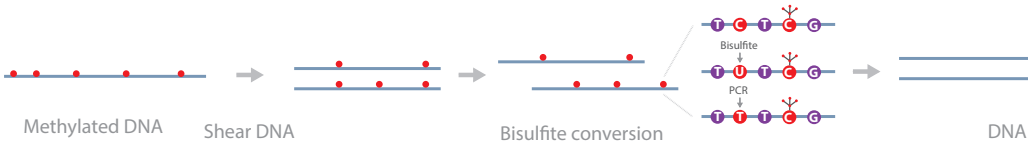
Bisulfite Sequencing (BS-Seq)

Bisulfite sequencing (BS-Seq) or whole-genome bisulfite sequencing (WGBS) is a well-established protocol to detect methylated cytosines in genomic DNA.[119] In this method, genomic DNA is treated with sodium bisulfite and then sequenced, providing single-base resolution of methylated cytosines in the genome. During bisulfite treatment, unmethylated cytosines are deaminated to uracils which, upon sequencing, are converted to thymidines. Simultaneously, methylated cytosines resist deamination and are read as cytosines. The location of the methylated cytosines can then be determined by comparing treated and untreated sequences. Bisulfite treatment of DNA converts unmethylated cytosines to thymidines, leading to reduced sequence complexity. Very accurate deep sequencing serves to mitigate this loss of complexity.

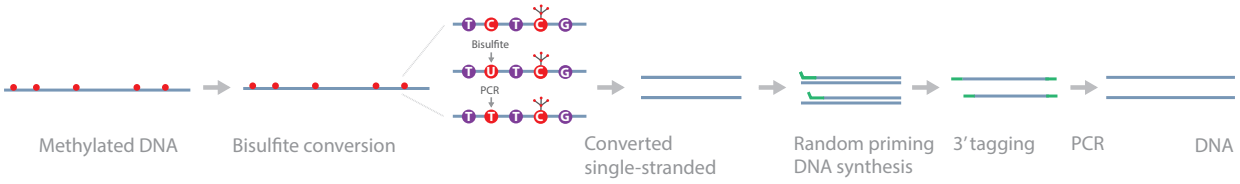
The EpiGnome Methyl-Seq kit uses a unique library construction method that incorporates bisulfite conversion as the first step. The EpiGnome method retains sample diversity while providing uniform coverage.

119. Feil R., Charlton J., Bird A. P., Walter J. and Reik W. (1994) Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res 22: 695-696

BS-Seq or WGBS



EpiGnome Methyl-Seq



Pros	Cons
BS-Seq or WGBS • Covers CpG and non-CpG methylation throughout the genome at single-base resolution. • Covers 5mC in dense, less dense, and repeat regions.	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments. • SNVs where a cytosine is converted to thymidine will be missed upon bisulfite conversion. • Bisulfite conversion does not distinguish between 5mC and 5hmC.
EpiGnome • Pre-library bisulfite conversion. • Low input genomic DNA (50 ng). • Uniform CpG, CHG, and CHH coverage. • No fragmentation and no methylated adapters. • Retention of sample diversity.	• Bisulfite converts unmethylated cytosines to thymidines, reducing sequence complexity, which can make it difficult to create alignments. • SNVs where a cytosine is converted to thymidine will be missed upon bisulfite conversion. • Bisulfite conversion does not distinguish between 5mC and 5hmC. • Higher duplicate percentage.

References:

Green M. R., Vicente-Duenas C., Romero-Camarero I., Long Liu C., Dai B., et al. (2014) Transient expression of Bcl6 is sufficient for oncogenic function and induction of mature B-cell lymphoma. Nat Commun 5: 3904

It is well established that cancer arises by an acquisition of somatic genomic alterations that lead to a malignant state. However, it is difficult to determine the initiation event for the cancerous development. This study examined the specific gain of 3q27.2 and its association with adverse outcome in DLBCL. Using an Illumina Genome Analyzer, the researchers sequenced the whole genome and characterized the DNA methylation of murine HSPCs. The authors identified BCL6 oncogene expression in HSPCs and demonstrated, by knock-in expression, that transient expression of Bcl6 within murine HSPCs can initiate the development into mature B-cell lymphomas.

Illumina Technology: Genome Analyzer$_{IIx}$

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DNA methylation is an important mechanism for gene regulation in the cell. This study presents a method for scBS-Seq using Illumina HiSeq sequencing to characterize the genome-wide DNA methylation of individual cells. The authors demonstrate their method in mouse ESCs and show that epigenetic heterogeneity varies across different types of functional sites in the genome.

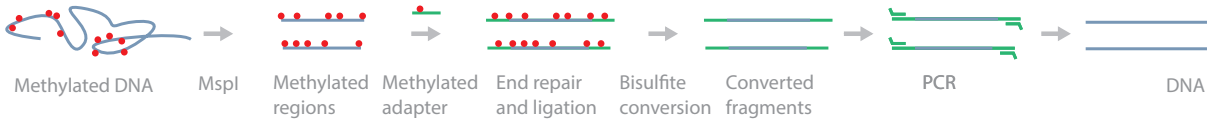
Illumina Technology: HiSeq

Reduced-Representation Bisulfite Sequencing (RRBS-Seq)

Reduced-representation bisulfite sequencing (RRBS-Seq) is a protocol that uses one or multiple restriction enzymes on the genomic DNA to produce sequence-specific fragmentation.[120] The fragmented genomic DNA is then treated with bisulfite and sequenced. This is the method of choice to study specific regions of interest. It is particularly effective where methylation is high, such as in promoters and repeat regions.

120. Meissner A., Gnirke A., Bell G. W., Ramsahoye B., Lander E. S., et al. (2005) Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Res 33: 5868-5877

Pros	Cons
• Genome-wide coverage of CpGs in islands at single-base resolution. • Covers areas dense in CpG methylation.	• Restriction enzymes cut at specific sites, providing biased sequence selection. • Method measures 10%–15% of all CpGs in the genome. • Cannot distinguish between 5mC and 5hmC. • Does not cover non-CpG areas, genome-wide CpGs, and CpGs in areas without the enzyme restriction site.



References

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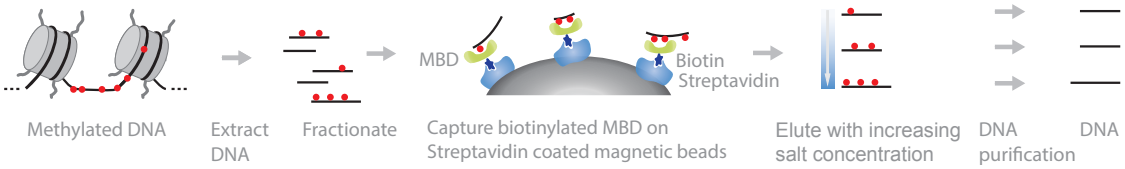
Illumina Technology: HiSeq 2000

Methylation-Capture (MethylCap) Sequencing or Methyl-Binding-Domain–Capture (MBDCap) Sequencing

MethylCap[121,122] or MBDCap[123,124] uses proteins to capture methylated DNA in the genome. Genomic DNA is first sonicated and incubated with tagged MBD proteins that can bind methylated cytosines. The protein-DNA complex is then precipitated with antibody-conjugated beads that are specific to the protein tag. Deep sequencing provides greater genome coverage, representing the majority of MBD-bound methylated DNA.

Pros	Cons
• Genome-wide coverage of 5mC in dense CpG areas and repeat regions. • MBD proteins do not interact with 5hmC.	• Genome-wide CpGs and non-CpG methylation is not covered. Areas with less dense 5mC are also missed. • Base-pair resolution is lower (~150 bp) as opposed to single-base resolution. • Protein-based selection is biased towards hypermethylated regions.



121. Bock C., Tomazou E. M., Brinkman A. B., Muller F., Simmer F., et al. (2010) Quantitative comparison of genome-wide DNA methylation mapping technologies. Nat Biotechnol 28: 1106-1114

122. Brinkman A. B., Simmer F., Ma K., Kaan A., Zhu J., et al. (2010) Whole-genome DNA methylation profiling using MethylCap-seq. Methods 52: 232-236

123. Rauch T. A., Zhong X., Wu X., Wang M., Kernstine K. H., et al. (2008) High-resolution mapping of DNA hypermethylation and hypomethylation in lung cancer. Proc Natl Acad Sci U S A 105: 252-257

124. Rauch T. A. and Pfeifer G. P. (2009) The MIRA method for DNA methylation analysis. Methods Mol Biol 507: 65-75

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Obata Y., Furusawa Y., Endo T. A., Sharif J., Takahashi D., et al. (2014) The epigenetic regulator Uhrf1 facilitates the proliferation and maturation of colonic regulatory T cells. Nat Immunol 15: 571-579

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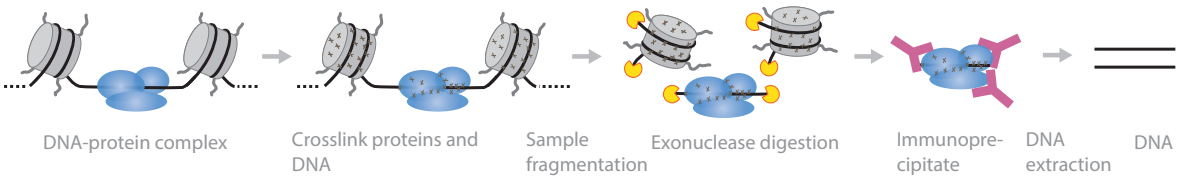
Illumina Technology: HiSeq 1000

Chromatin Immunoprecipitation Sequencing (ChIP-Seq)

Chromatin immunoprecipitation sequencing (ChIP-Seq) is a well-established method to map specific protein-binding sites.[125] In this method, DNA-protein complexes are crosslinked *in vivo*. Samples are then fragmented and treated with an exonuclease to trim unbound oligonucleotides. Protein-specific antibodies are used to immunoprecipitate the DNA-protein complex. The DNA is extracted and sequenced, giving high-resolution sequences of the protein-binding sites.

125. Solomon M. J., Larsen P. L. and Varshavsky A. (1988) Mapping protein-DNA interactions in vivo with formaldehyde: evidence that histone H4 is retained on a highly transcribed gene. Cell 53: 937-947
126. Zentner G. E. and Henikoff S. (2012) Surveying the epigenomic landscape, one base at a time. Genome Biol 13: 250

Pros	Cons
• Base-pair resolution of protein-binding site. • Specific regulatory factors or proteins can be mapped. • The use of exonuclease eliminates contamination by unbound DNA.[126]	• Nonspecific antibodies can dilute the pool of DNA-protein complexes of interest. • The target protein must be known and able to raise an antibody.



References

Magnusdottir E., Dietmann S., Murakami K., Gunesdogan U., Tang F., et al. (2013) A tripartite transcription factor network regulates primordial germ cell specification in mice. Nat Cell Biol 15: 905-915

Kind J., Pagie L., Ortabozkoyun H., Boyle S., de Vries S. S., et al. (2013) Single-cell dynamics of genome-nuclear lamina interactions. Cell 153: 178-192

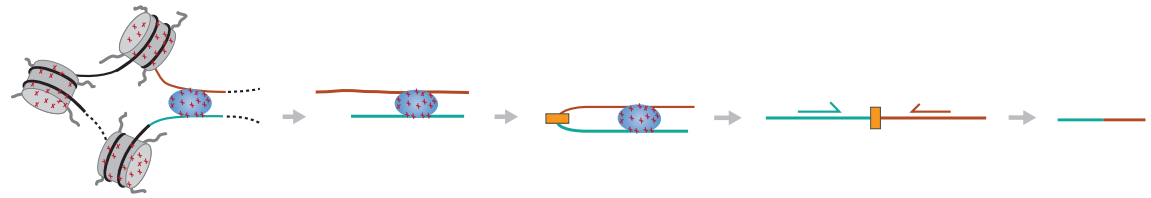
Chromatin Conformation Capture (Hi-C/3C-Seq)

Chromatin conformation capture sequencing (Hi-C)[127] or 3C-Seq[128] is used to analyze chromatin interactions. In this method, DNA-protein complexes are crosslinked using formaldehyde. The sample is fragmented, and the DNA is ligated and digested. The resulting DNA fragments are PCR-amplified and sequenced. Deep sequencing provides base-pair resolution of ligated fragments.

127. Lieberman-Aiden E., van Berkum N. L., Williams L., Imakaev M., Ragoczy T., et al. (2009) Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326: 289-293

128. Duan Z., Andronescu M., Schutz K., Lee C., Shendure J., et al. (2012) A genome-wide 3C-method for characterizing the three-dimensional architectures of genomes. Methods 58: 277-288

Pros	Cons
• Allows detection of long-range DNA interactions. • High-throughput method.	• Detection may result from random chromosomal collisions. • 3C PCR is difficult and requires careful controls and experimental design. • Needs further confirmation of interaction. • Due to multiple steps, the method requires large amounts of starting material.



References

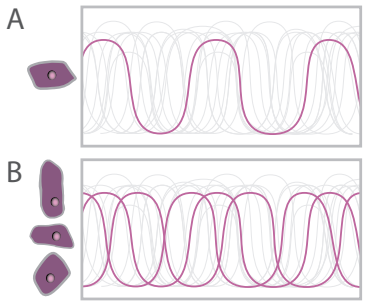
Nagano T., Lubling Y., Stevens T. J., Schoenfelder S., Yaffe E., et al. (2013) Single-cell Hi-C reveals cell-to-cell variability in chromosome structure. Nature 502: 59-64

Genomic techniques based on 3C assess contacts for millions of loci simultaneously. In this study, the authors used Illumina sequencing to perform 3C for single cells, combined with genome-wide statistical analysis and structural modeling of single-copy X chromosomes. The authors show that individual chromosomes maintain domain organization at the megabase scale, but exhibit variable cell-to-cell chromosome structures at larger scales.

Illumina Technology: Genome Analyzer$_{IIx}$, HiSeq

RNA APPLICATIONS

Low-level RNA detection refers to both detection of rare RNA molecules in a cell-free environment—such as circulating tumor RNA—or the expression patterns of single cells. Tissues consist of a multitude of different cell types, each with a distinctly different set of functions. Even within a single cell type, the transcriptomes are highly dynamic and reflect temporal, spatial, and cell-cycle–dependent changes. Cell harvesting, handling, and technical issues with sensitivity and bias during amplification add an additional level of complexity. To resolve this multi-tiered complexity would require the analysis of many thousands of cells. The use of unique barcodes has greatly increased the number of samples that can be multiplexed and pooled at little to no decrease in reads associated with each sample. Recent improvements in cell capture and sample preparation will provide more information faster, and at lower cost.[129,130] These advances promise to expand our understanding of cell function, with significant implications for research and human health.[131]



Organs, such as the kidney depicted in this cross-section, consist of a myriad of phenotypically distinct cells. Single-cell transcriptomics can characterize the function of each of these cell types.

> "Single-cell transcriptomics has the power to dissect mixed populations of cells; conversely, if only limited material is available, it may permit characterization of the transcriptome of extremely rare cells, such as CTCs."
> Macauley and Voet 2014

Reviews

Baslan T. and Hicks J. (2014) Single cell sequencing approaches for complex biological systems. Curr Opin Genet Dev 26C: 59-65

Eckersley-Maslin M. A. and Spector D. L. (2014) Random monoallelic expression: regulating gene expression one allele at a time. Trends Genet 30: 237-244

Saliba A. E., Westermann A. J., Gorski S. A. and Vogel J. (2014) Single-cell RNA-seq: advances and future challenges. Nucleic Acids Res 42: 8845-8860

Blainey P. C. (2013) The future is now: single-cell genomics of bacteria and archaea. FEMS Microbiol Rev 37: 407-427

Lovett M. (2013) The applications of single-cell genomics. Hum Mol Genet 22: R22-26

Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

Spaethling J. M. and Eberwine J. H. (2013) Single-cell transcriptomics for drug target discovery. Curr Opin Pharmacol 13: 786-790

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References

Bajikar S. S., Fuchs C., Roller A., Theis F. J. and Janes K. A. (2014) Parameterizing cell-to-cell regulatory heterogeneities via stochastic transcriptional profiles. Proc Natl Acad Sci U S A 111: E626-635

Cell-to-cell variations in gene regulation occur in a number of biological contexts, such as development and cancer. It is difficult to discover regulatory heterogeneities in an unbiased manner, due to the population averaging that is required for most global molecular methods. This study shows how single-cell regulatory states can be inferred by mathematical deconvolution of global measurements, i.e., from averages over a small number of cells. The authors found that population-level inferences were much more accurate with pooled samples than with one-cell samples when the extent of sampling was limited.

Lovatt D., Ruble B. K., Lee J., Dueck H., Kim T. K., et al. (2014) Transcriptome *in vivo* analysis (TIVA) of spatially defined single cells in live tissue. Nat Methods 11: 190-196

RNA sequencing methods that rely on RNA extracted from cell mixtures do not convey the individual variability in expression among cells of the same tissue. In this paper, the authors present a transcriptome *in vivo* analysis (TIVA), which is applicable to single-cell studies. In combination with Illumina sequencing technology, the authors capture and analyze the transcriptome variance across single neurons both in culture and *in vivo*. This method is furthermore non-invasive and may be applied to intact tissue. It will enable detailed studies of cell heterogeneity in complex tissues that have been intractable previously, and it opens up the possibility of use in conjunction with *in vivo* live functional imaging.

Illumina Technology: HiSeq

Wu A. R., Neff N. F., Kalisky T., Dalerba P., Treutlein B., et al. (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11: 41-46

In this comprehensive analysis, the authors used microfluidic automation to quantitatively compare the accuracy and precision of single-cell RNA-Seq to qPCR. Commercially available single-cell RNA amplification methods, with both microliter and nanoliter volumes, were used to prepare and sequence libraries on Illumina HiSeq 2000. The study shows that single-cell RNA-Seq can generate results that are quantitatively comparable to qPCR, particularly when sample preparation is done on nanoliter-scale reaction volumes, such as in a microfluidic device.

Illumina Technology: HiSeq

Shalek A. K., Satija R., Adiconis X., Gertner R. S., Gaublomme J. T., et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236-240

Individual cells can exhibit substantial differences in gene expression, and only recently have genome profiling methods been developed to monitor the expression of single cells. This study applied the Smart-Seq single-cell RNA sequencing on Illumina HiSeq to investigate heterogeneity in the response of mouse BMDCs to lipopolysaccharide. The authors found extensive bimodal variation in messenger RNA abundance and splicing patterns, which was subsequently validated using RNA-FISH for select transcripts.

Illumina Technology: HiSeq

Xue Z., Huang K., Cai C., Cai L., Jiang C. Y., et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500: 593-597

In an effort to characterize and dissect the gene regulatory mechanisms of mammalian pre-implantation development, this study examined early embryos from both human and mouse using whole-transcriptome sequencing of single cells. The authors report novel stage-specific monoallelic expression patterns for a significant portion of polymorphic transcripts. Cross-species comparisons revealed that the majority of human stage-specific modules (seven of nine) are preserved, but with developmental specificity and timing differing between human and mouse.

Illumina Technology: HiSeq

Shalek A. K., Satija R., Shuga J., Trombetta J. J., Gennert D., et al. (2014) Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. Nature 510: 363-369

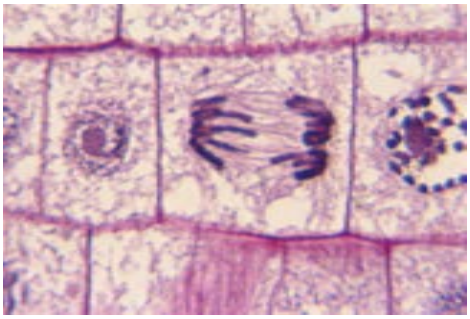
Yan L., Yang M., Guo H., Yang L., Wu J., et al. (2013) Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. Nat Struct Mol Biol 20: 1131-1139

Goetz J. J. and Trimarchi J. M. (2012) Transcriptome sequencing of single cells with Smart-Seq. Nat Biotechnol 30: 763-765

Allele-Specific Gene Expression

Diploid organisms have two sets of chromosomes, one from each parent. Genes are transcribed either from one allele (monoallelic expression) or from both alleles (biallelic expression). Population sequencing provides a global representation of gene expression, but the expression levels of rare isoforms may be lost. Single-cell sequencing can detect these rare isoforms, as well as changes between monoallelic or biallelic expression.

Compared to established methods such as RNA-FISH, RNA-Seq, PCR, and live-cell imaging, single-cell RNA-Seq provides the most accurate representation of monoallelic or biallelic expression in individual cells within a population.[132] Combining RNA expression and SNV data can provide an integrated look into which SNVs lead to preferential expression or silencing of alleles and their subsequent roles in diseases, cancer, or tissue-specific differentiation.[133,134] In a study using single-cell RNA-Seq of mouse embryos of mixed background, the monoallelic expression was consistent with models of transcriptional bursting.[135] In each cell, independent bursts of transcription occurred from both alleles over time, but often RNA from only one allele was present at any given time.[136] This level of detailed analysis can only be achieved with single cells.



As cells divide during differentiation, they accumulate SNVs, which can play a role in determining random monoallelic gene expression.[132,137]

Reviews

Eckersley-Maslin M. A. and Spector D. L. (2014) Random monoallelic expression: regulating gene expression one allele at a time. Trends Genet 30: 237-244

Gendrel A. V., Attia M., Chen C. J., Diabangouaya P., Servant N., et al. (2014) Developmental dynamics and disease potential of random monoallelic gene expression. Dev Cell 28: 366-380

Saliba A. E., Westermann A. J., Gorski S. A. and Vogel J. (2014) Single-cell RNA-seq: advances and future challenges. Nucleic Acids Res 42: 8845-8860

Barlow D. P. (2011) Genomic imprinting: a mammalian epigenetic discovery model. Annu Rev Genet 45: 379-403

132. Eckersley-Maslin M. A. and Spector D. L. (2014) Random monoallelic expression: regulating gene expression one allele at a time. Trends Genet 30: 237-244
133. Gendrel A. V., Attia M., Chen C. J., Diabangouaya P., Servant N., et al. (2014) Developmental dynamics and disease potential of random monoallelic gene expression. Dev Cell 28: 366-380
134. Saliba A. E., Westermann A. J., Gorski S. A. and Vogel J. (2014) Single-cell RNA-seq: advances and future challenges. Nucleic Acids Res 42: 8845-8860
135. Raj A. and van Oudenaarden A. (2008) Nature, nurture, or chance: stochastic gene expression and its consequences. Cell 135: 216-226
136. Deng Q., Ramskold D., Reinius B. and Sandberg R. (2014) Single-cell RNA-seq reveals dynamic, random monoallelic gene expression in mammalian cells. Science 343: 193-196
137. Eckersley-Maslin M. A., Thybert D., Bergmann J. H., Marioni J. C., Flicek P., et al. (2014) Random monoallelic gene expression increases upon embryonic stem cell differentiation. Dev Cell 28: 351-365

References:

Deng Q., Ramskold D., Reinius B. and Sandberg R. (2014) Single-cell RNA-seq reveals dynamic, random monoallelic gene expression in mammalian cells. Science 343: 193-196

The authors identified many random monoallelically expressed genes and discovered that neither DNA methylation nor nuclear positioning distinguished active versus inactive alleles. However, they did find a correlation in enrichment for specific histone modifications.

Illumina Technology: Genome Analyzer$_{IIx}$

Marinov G. K., Williams B. A., McCue K., Schroth G. P., Gertz J., et al. (2014) From single-cell to cell-pool transcriptomes: stochasticity in gene expression and RNA splicing. Genome Res 24: 496-510

Recent studies are increasingly discovering cell-to-cell variability in gene expression levels and transcriptional regulation. This study examined the lymphoblastoid cell line GM12878 using the Smart-seq single-cell RNA-seq protocol on Illumina HiSeq 2000 to determine variation in transcription among individual cells. The authors determined, through careful quantitation, that there are significant differences in expression between individual cells, over and above technical variation. In addition, they showed that the transcriptomes from small pools of 30–100 cells approach the information content and reproducibility of contemporary pooled RNA-Seq analysis from large amounts of input material.

Illumina Technology: HiSeq

Pan X., Durrett R. E., Zhu H., Tanaka Y., Li Y., et al. (2013) Two methods for full-length RNA sequencing for low quantities of cells and single cells. Proc Natl Acad Sci U S A 110: 594-599

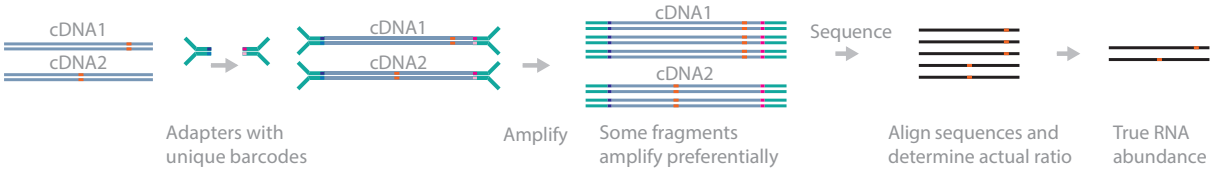
Xue Z., Huang K., Cai C., Cai L., Jiang C. Y., et al. (2013) Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature 500: 593-597

Digital RNA Sequencing

Digital RNA sequencing is an approach to RNA-Seq that removes sequence-dependent PCR amplification biases by barcoding the RNA molecules before amplification.[138] RNA is reverse-transcribed to cDNA, then an excess of adapters, each with a unique barcode, is added to the reaction. The barcoded cDNA is then amplified and sequenced. Deep sequencing reads are compared, and barcodes are used to determine the actual ratio of RNA abundance.

138. Shiroguchi K., Jia T. Z., Sims P. A. and Xie X. S. (2012) Digital RNA sequencing minimizes sequence-dependent bias and amplification noise with optimized single-molecule barcodes. Proc Natl Acad Sci U S A 109: 1347-1352

Pros	Cons
• Low amplification bias during PCR. • Information about abundance of RNA. • Detection of low-copy number RNA. • Single copy resolution.	• Some amplification bias still persists. • Barcodes may miss targets during ligation.



References

Shiroguchi K., Jia T. Z., Sims P. A. and Xie X. S. (2012) Digital RNA sequencing minimizes sequence-dependent bias and amplification noise with optimized single-molecule barcodes. Proc Natl Acad Sci U S A 109: 1347-1352

Experimental protocols that include PCR as an amplification step are subject to sequence-dependent bias. For RNA-Seq, this bias results in difficulties in quantifying expression levels, especially at very low copy numbers. In this study, digital RNA-Seq is introduced as an accurate method for quantitative measurements by appending unique barcode sequences to the pool of RNA fragments. The authors demonstrate how digital RNA-Seq allows transcriptome profiling of Escherichia coli with more accurate and reproducible quantification than conventional RNA-Seq. The efficacy of optimization was estimated by comparison to simulated data.

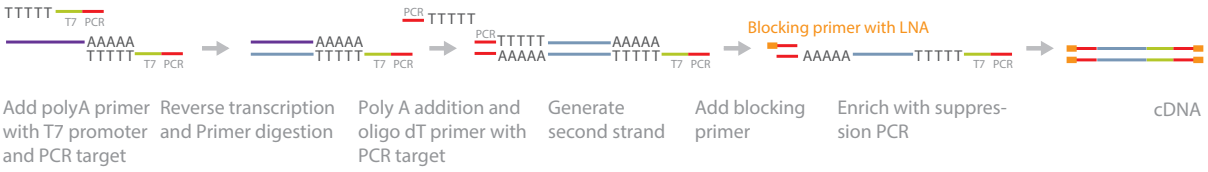
Illumina Technology: Genome Analyzer$_{IIx}$

Whole-Transcript Amplification for Single Cells (Quartz-Seq)

The Quartz-Seq method optimizes whole-transcript amplification (WTA) of single cells.[139] In this method, an RT primer with a T7 promoter and PCR target is added to extracted mRNA. Reverse transcription synthesizes first-strand cDNA, following which the RT primer is digested by exonuclease I. A poly(A) tail is then added to the 3' ends of first-strand cDNA, along with a dT primer containing a PCR target. After second-strand generation, a blocking primer is added to ensure PCR enrichment in sufficient quantity for sequencing. Deep sequencing allows for accurate, high-resolution representation of the whole transcriptome of a single cell.

139. Sasagawa Y., Nikaido I., Hayashi T., Danno H., Uno K. D., et al. (2013) Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity. Genome Biol 14: R31

Pros	Cons
• Single-tube reaction suitable for automation. • Digestion of RT primers by exonuclease I eliminates amplification of byproducts. • Short fragments and byproducts are suppressed during enrichment.	• PCR biases can underrepresent GC-rich templates. • Amplification errors caused by polymerases will be represented and sequenced incorrectly. • Targets smaller than 500 bp are preferentially amplified by polymerases during PCR.



References

Sasagawa Y., Nikaido I., Hayashi T., Danno H., Uno K. D., et al. (2013) Quartz-Seq: a highly reproducible and sensitive single-cell RNA sequencing method, reveals non-genetic gene-expression heterogeneity. Genome Biol 14: R31

Individual cells may exhibit variable gene expression even if they share the same genome. The analysis of single-cell variability in gene expression requires robust protocols with a minimum of bias. This paper presents a novel single-cell RNA-Seq method, Quartz-Seq, based on Illumina sequencing that has a simpler protocol and higher reproducibility and sensitivity than existing methods. The authors implemented improvements in three main areas: 1) they optimized the protocol for suppression of byproduct synthesis; 2) they identified a robust PCR enzyme to allow single-tube reaction; and 3) they determined optimal conditions for RT and second-strand synthesis.

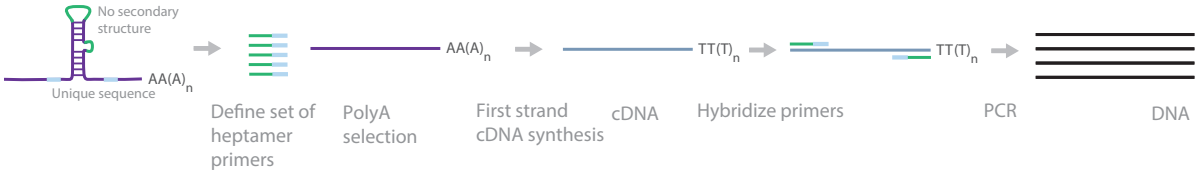
Illumina Technology: TruSeq RNA Sample Prep, HiSeq 2000

Designed Primer-Based RNA Sequencing (DP-Seq)

Designed-primer based RNA sequencing (DP-Seq) is a method that amplifies mRNA from limited amounts of starting material, as low as 50 pg.[140] In this method, a specific set of heptamer primers are first designed. Enriched poly(A)-selected mRNA undergoes first-strand cDNA synthesis. Designed primers are then hybridized to first-strand cDNA, followed by second-strand synthesis and PCR. Deep sequencing of amplified DNA allows for accurate detection of specific mRNA expression at the single-cell level.

140. Bhargava V., Ko P., Willems E., Mercola M. and Subramaniam S. (2013) Quantitative transcriptomics using designed primer-based amplification. Sci Rep 3: 1740
141. Bhargava V., Head S. R., Ordoukhanian P., Mercola M. and Subramaniam S. (2014) Technical variations in low-input RNA-seq methodologies. Sci Rep 4: 3678

Pros	Cons
• As little as 50 pg of starting material can be used. • Low transcript-length bias.	• The sequences of the target areas must be known to design the heptamers. • Exponential amplification during PCR can lead to primer-dimers and spurious PCR products.[141] • Some read-length bias.



References

Bhargava V., Head S. R., Ordoukhanian P., Mercola M. and Subramaniam S. (2014) Technical variations in low-input RNA-seq methodologies. Sci Rep 4: 3678

Recent advances in RNA-Seq methodologies from limiting amounts of mRNA have facilitated the characterization of rare cell-types in various biological systems. In this paper, the authors compare three amplification-based methods: Smart-Seq, DP-Seq, and CEL-Seq. The authors showed that these libraries exhibit significant technical variations, including inefficient amplification of the majority of low to moderately expressed transcripts. The sequencing errors are negligible, but the high technical variations introduced from amplification are still a hurdle to the use of these methods for detecting subtle biological differences.

Illumina Technology: HiSeq

Bhargava V., Ko P., Willems E., Mercola M. and Subramaniam S. (2013) Quantitative transcriptomics using designed primer-based amplification. Sci Rep 3: 1740

Standard amplification of RNA-transcripts before sequencing is prone to bias. This paper presents a protocol for selecting a unique subset of primers to target the majority of expressed transcripts in mouse for amplification while preserving their relative abundance. This protocol was developed for Illumina sequencing platforms, and the authors demonstrate that it yields high levels of amplification from as little as 50 pg of mRNA, while offering a dynamic range of over five orders of magnitude.

Illumina Technology: Genome Analyzer$_{IIx}$

Switch Mechanism at the 5’ End of RNA Templates (Smart-Seq)

Smart-Seq was developed as a single-cell sequencing protocol with improved read coverage across transcripts.[142] Complete coverage across the genome allows the detection of alternative transcript isoforms and SNVs. In this protocol, cells are lysed and the RNA hybridized to an oligo(dT)-containing primer. The first strand is then created with the addition of a few untemplated C nucleotides. This poly(C) overhang is added exclusively to full-length transcripts. An oligonucleotide primer is then hybridized to the poly(C) overhang and used to synthesize the second strand. Full-length cDNAs are PCR-amplified to obtain nanogram amounts of DNA. The PCR products are purified for sequencing.

Pros	Cons
• The sequence of the mRNA does not have to be known. • As little as 50 pg of starting material can be used. • Improved coverage across transcripts. • High level of mappable reads.	• Not strand-specific. • No early multiplexing.[143] • Transcript length bias with inefficient transcription of reads over 4 Kb.[144] • Preferential amplification of high-abundance transcripts. • The purification step may lead to loss of material. • Could be subject to strand-invasion bias.[145]

142. Ramskold D., Luo S., Wang Y. C., Li R., Deng Q., et al. (2012) Full-length mRNA-Seq from single-cell levels of RNA and individual circulating tumor cells. Nat Biotechnol 30: 777-782
143. Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630
144. Bhargava V., Head S. R., Ordoukhanian P., Mercola M. and Subramaniam S. (2014) Technical variations in low-input RNA-seq methodologies. Sci Rep 4: 3678
145. Tang D. T., Plessy C., Salimullah M., Suzuki A. M., Calligaris R., et al. (2013) Suppression of artifacts and barcode bias in high-throughput transcriptome analyses utilizing template switching. Nucleic Acids Res 41: e44

References

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Illumina Technology: HiSeq

Kadkhodaei B., Alvarsson A., Schintu N., Ramskold D., Volakakis N., et al. (2013) Transcription factor Nurr1 maintains fiber integrity and nuclear-encoded mitochondrial gene expression in dopamine neurons. Proc Natl Acad Sci U S A 110: 2360-2365

Developmental transcription factors important in early neuron differentiation are often found expressed also in the adult brain. This study set out to investigate the development of ventral midbrain dopamine (DA) neurons by studying the transcriptional expression in a mouse model system. By using the Smart-Seq method, which allows sequencing from low amounts of total RNA, the authors could sequence RNA from laser-microdissected DA neurons. Their analysis showed transcriptional activation of the essential transcription factor Nurr1 and its key role in sustaining healthy DA cells.

Illumina Technology: HiSeq 2000, Genomic DNA Sample Prep Kit (FC-102-1001; Illumina)

Marinov G. K., Williams B. A., McCue K., Schroth G. P., Gertz J., et al. (2014) From single-cell to cell-pool transcriptomes: stochasticity in gene expression and RNA splicing. Genome Res 24: 496-510

Recent studies are increasingly discovering cell-to-cell variability in gene expression levels and transcriptional regulation. This study examined the lymphoblastoid cell line GM12878 using the Smart-seq single-cell RNA-seq protocol on Illumina HiSeq 2000 to determine variation in transcription among individual cells. The authors determined, through careful quantitation, that there are significant differences in expression between individual cells, over and above technical variation. In addition, they showed that the transcriptomes from small pools of 30–100 cells approach the information content and reproducibility of contemporary pooled RNA-Seq analysis from large amounts of input material.

Illumina Technology: Nextera DNA Sample Prep, HiSeq 2000

Shalek A. K., Satija R., Adiconis X., Gertner R. S., Gaublomme J. T., et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236-240

Individual cells can exhibit substantial differences in gene expression, and only recently have genome profiling methods been developed to monitor the expression of single cells. This study applied the Smart-Seq single-cell RNA sequencing on Illumina HiSeq to investigate heterogeneity in the response of mouse BMDCs to lipopolysaccharide. The authors found extensive bimodal variation in messenger RNA abundance and splicing patterns, which was subsequently validated using RNA-FISH for select transcripts.

Illumina Technology: HiSeq 2000

Yamaguchi S., Hong K., Liu R., Inoue A., Shen L., et al. (2013) Dynamics of 5-methylcytosine and 5-hydroxymethylcytosine during germ cell reprogramming. Cell Res 23: 329-339

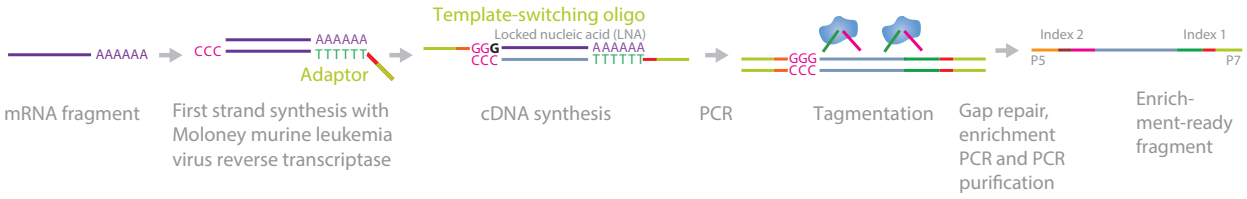
Mouse primordial germ cells (PGCs) undergo genome-wide DNA methylation reprogramming to reset the epigenome for totipotency. In this study, the dynamics between 5mC and 5hmC were characterized using immunostaining techniques and analyzed in combination with transcriptome profiles obtained with Illumina RNA-Seq. The study revealed that the dynamics of 5mC and 5hmC during PGC reprogramming supports a model in which DNA demethylation in PGCs occurs through multiple steps, with both active and passive mechanisms. In addition, the transcriptome study suggests that PGC reprogramming may have an important role in the activation of a subset of meiotic and imprinted genes.

Illumina Technology: HiSeq 2000

Switch Mechanism at the 5’ End of RNA Templates Version 2 (Smart-Seq2)

Smart-Seq2 includes several improvements over the original Smart-Seq protocol.[146,147] The new protocol includes a locked nucleic acid (LNA), an increased $MgCl_2$ concentration, betaine, and it eliminates the purification step to significantly improve the yield. In this protocol, single cells are lysed in a buffer that contains free dNTPs and tailed oligo(dT) oligonucleotides with a universal 5' anchor sequence. Reverse transcription is performed, which also adds 2–5 untemplated nucleotides to the cDNA 3' end. A template-switching oligo (TSO) is added, carrying two riboguanosines and a modified guanosine to produce a LNA as the last base at the 3' end. After the first-strand reaction, the cDNA is amplified using a limited number of cycles. Tagmentation is then used to quickly and efficiently construct sequencing libraries from the amplified cDNA.

Pros	Cons
• As little as 50 pg of starting material can be used. • The sequence of the mRNA does not have to be known. • Improved coverage across transcripts. • High level of mappable reads.	• Not strand-specific. • No early multiplexing. • Only sequences poly(A)+ RNA.



146. Picelli S., Bjorklund A. K., Faridani O. R., Sagasser S., Winberg G., et al. (2013) Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 10: 1096-1098

147. Picelli S., Faridani O. R., Björklund Å. K., Winberg G., Sagasser S., et al. (2014) Full-length RNA-seq from single cells using Smart-seq2. Nat. Protocols 9: 171-181

References

Picelli S., Bjorklund A. K., Faridani O. R., Sagasser S., Winberg G., et al. (2013) Smart-seq2 for sensitive full-length transcriptome profiling in single cells. Nat Methods 10: 1096-1098

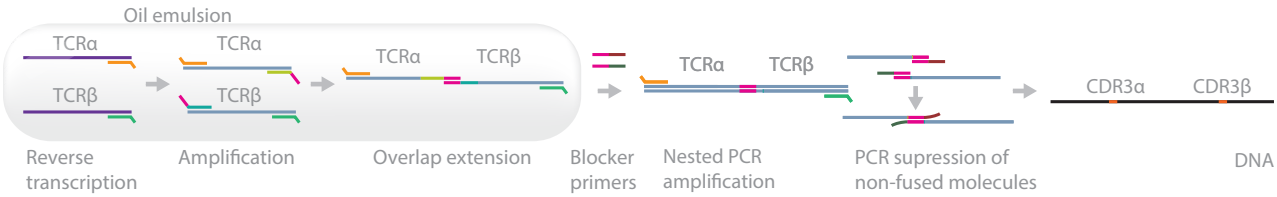
Single-cell gene expression analyses hold promise for characterizing cellular heterogeneity, but current methods compromise on the coverage, sensitivity, or throughput. This paper introduces Smart-Seq2, with improved RT, template-switching, and preamplification to increase both yield and length of cDNA libraries generated from individual cells. The authors evaluated the efficacy of the Smart-Seq2 protocol using Illumina HiSeq and concluded that the Smart-Seq2 transcriptome libraries have improved detection, coverage, bias and accuracy compared to Smart-Seq libraries. In addition, they are generated with off-the-shelf reagents at lower cost.

Illumina Technology: Nextera DNA Sample Prep, HiSeq 2000

TCR Sequencing

Functional TCRs are heterodimeric proteins that comprise both an α and a ß chain. Every T cell contains a unique combination of α and ß chains. For an accurate functional analysis, both subunits must be sequenced together to avoid disrupting the α and ß chain pairing during the cell lysis step.[148,149]

148. Woodsworth D. J., Castellarin M. and Holt R. A. (2013) Sequence analysis of T-cell repertoires in health and disease. Genome Med 5: 98
149. Turchaninova M. A., Britanova O. V., Bolotin D. A., Shugay M., Putintseva E. V., et al. (2013) Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol 43: 2507-2515
150. Turchaninova M. A., Britanova O. V., Bolotin D. A., Shugay M., Putintseva E. V., et al. (2013) Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol 43: 2507-2515



Cell-based emulsion RT-PCR technique for identifying TCR α-ß chain pairing. Released TCR α and ß mRNAs are reverse-transcribed, amplified, and overlap-extended within each droplet. Products are extracted from the emulsion, and fused molecules of interest are selectively amplified. Non-fused molecules are suppressed with blocking primers.[150]

References

Ma Y., Mattarollo S. R., Adjemian S., Yang H., Aymeric L., et al. (2014) CCL2/CCR2-dependent recruitment of functional antigen-presenting cells into tumors upon chemotherapy. Cancer Res 74: 436-445

The therapeutic efficacy of anthracyclines as cancer chemotherapy relies on the induction of dendritic cell and T-lymphocyte-dependent anticancer immune responses. This study investigated the effects of anthracycline-based chemotherapy on the chemokine CCL2 and its receptor CCR2 in a mouse cancer model. The authors used Illumina Mouse BeadArray to characterize differential gene expression. They found that anthracycline-based chemotherapy promotes the intra-tumor accumulation of myeloid cells, including cells that mediate antigen presentation. These findings add to the understanding of the anticancer immune response elicited by immunogenic cell death.

Illumina Technology: Mouse BeadArray

Papaemmanuil E., Rapado I., Li Y., Potter N. E., Wedge D. C., et al. (2014) RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia. Nat Genet 46: 116-125

At least a quarter of acute lymphoblastic leukemia (ALL) cases harbor the ETV6-RUNX1 fusion gene. Although this gene fusion is characteristic for the disease, additional mutations are required for the development of overt leukemia. This study used exome and low-coverage whole-genome sequencing to characterize secondary events associated with leukemic transformation. The authors found that ATF7IP and MGA are two new tumor-suppressor genes in ALL. They describe the parsimonious mutational process that transforms ETV6-RUNX1-positive lymphoblasts into leukemia.

Illumina Technology: Genome Analyzer$_{IIx}$

Gao C., Kozlowska A., Nechaev S., Li H., Zhang Q., et al. (2013) TLR9 signaling in the tumor microenvironment initiates cancer recurrence after radiotherapy. Cancer Res 73: 7211-7221

This study investigated the mechanism of the reported immunogenic potential of cancer radiotherapy and the response of nucleic acid receptors before and after local radiotherapy. The authors used Illumina HiSeq 2000 for RNA-Seq to characterize differential expression patterns. The study suggests that combining localized tumor irradiation with myeloid cell-specific inhibition of TLR9/STAT3 signaling may help eliminate radioresistant cancers.

Illumina Technology: HiSeq 2000 for RNA-Seq

Linnemann C., Heemskerk B., Kvistborg P., Kluin R. J., Bolotin D. A., et al. (2013) High-throughput identification of antigen-specific TCRs by TCR gene capture. Nat Med 19: 1534-1541

The transfer of TCR genes into patient T cells is a promising approach for the treatment of both viral infections and cancer. This study presents a new high-throughput assay for identifying TCR sequences by capture and sequencing of TCR genes on the Illumina HiSeq sequencing platform. The approach was validated by the assembly of a large library of cancer germline tumor antigen-reactive TCRs. The authors demonstrated the feasibility of identifying antigen-specific TCRs in oligoclonal T-cell populations from either human material or TCR-humanized mice.

Illumina Technology: TruSeq on Illumina HiSeq 2000

Mamedov I. Z., Britanova O. V., Zvyagin I. V., Turchaninova M. A., Bolotin D. A., et al. (2013) Preparing unbiased T-cell receptor and antibody cDNA libraries for the deep next generation sequencing profiling. Front Immunol 4: 456

This paper presents a detailed protocol, similar to SmartSeq, for the preparation of TCR and IgG cDNA libraries. The protocol can be performed in 1–2 days.

Illumina Technology: MiSeq, HiSeq 2000

Shalek A. K., Satija R., Adiconis X., Gertner R. S., Gaublomme J. T., et al. (2013) Single-cell transcriptomics reveals bimodality in expression and splicing in immune cells. Nature 498: 236-240

Individual cells can exhibit substantial differences in gene expression, and only recently have genome profiling methods been developed to monitor the expression of single cells. This study applied the Smart-Seq single-cell RNA sequencing on Illumina HiSeq to investigate heterogeneity in the response of mouse BMDCs to lipopolysaccharide. The authors found extensive bimodal variation in messenger RNA abundance and splicing patterns, which was subsequently validated using RNA-FISH for select transcripts.

Illumina Technology: HiSeq

Turchaninova M. A., Britanova O. V., Bolotin D. A., Shugay M., Putintseva E. V., et al. (2013) Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol 43: 2507-2515

The authors propose a single-cell-based method to identify native pairs of αß TCR CDR3 chains within emulsion droplets by reverse-transcription of α and ß chain mRNA, PCR amplification, and subsequent fusion via overlap extension. This PCR suppression technique resolves the issue of random overlap-extension of gene pairs that may create a high level of noise after the emulsion stage. The authors propose that this methodology can be applied to the identification of native pairs of variable heavy-light antibody chains.

Illumina Technology: MiSeq 2 x 150 bp

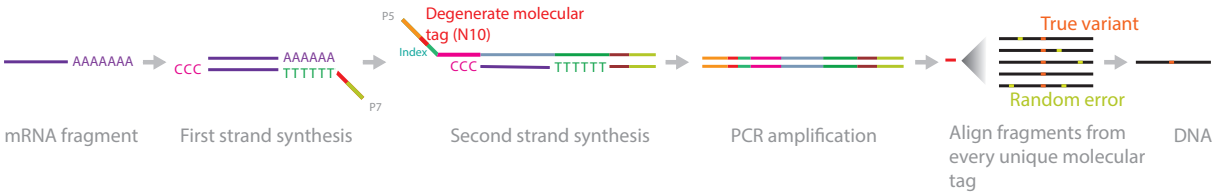
Bolotin D. A., Shugay M., Mamedov I. Z., Putintseva E. V., Turchaninova M. A., et al. (2013) MiTCR: software for T-cell receptor sequencing data analysis. Nat Methods 10: 813-814

Unique Molecular Identifiers (UMI)

Unique molecular identifiers (UMI) is a method that uses molecular tags to detect and quantify unique mRNA transcripts.[151] In this method, mRNA libraries are generated by fragmentation and then reverse-transcribed to cDNA. Oligo(dT) primers with specific sequencing linkers are added to cDNA. Another sequencing linker with a 10 bp random label and an index sequence is added to the 5' end of the template, which is amplified and sequenced. Sequencing allows for high-resolution sequence reads, enabling accurate detection of true variants.

151. Kivioja T., Vaharautio A., Karlsson K., Bonke M., Enge M., et al. (2012) Counting absolute numbers of molecules using unique molecular identifiers. Nat Methods 9: 72-74

Pros	Cons
• Can sequence unique mRNA transcripts. • Can be used to detect transcripts occurring at low frequencies. • Transcripts can be quantified based on sequencing reads specific to each barcode. • Can be applied to multiple platforms to karyotype chromosomes.	• Targets smaller than 500 bp are preferentially amplified by polymerases during PCR.



References

Cooper D. A., Jha B. K., Silverman R. H., Hesselberth J. R. and Barton D. J. (2014) Ribonuclease L and metal-ion-independent endoribonuclease cleavage sites in host and viral RNAs. Nucleic Acids Res 42: 5202-5216

Despite the biological significance of ribonuclease L (RNase L), the RNAs cleaved by this enzyme are poorly defined. In this study, the authors used Illumina sequencing to reveal the frequency and location of RNase L cleavage sites within host and viral RNAs. The method was optimized and validated using viral RNAs cleaved with RNase L and RNase A, and RNA from infected and non-infected HeLa cells. The authors identified discrete genomic regions susceptible to RNase L and other single-strand-specific endoribonucleases. Monitoring the frequency and location of these cleavage sites within host and viral RNAs may reveal how these enzymes contribute to health and disease.

Illumina Technology: Genome Analyzer$_{IIx}$, MiSeq

Islam S., Zeisel A., Joost S., La Manno G., Zajac P., et al. (2014) Quantitative single-cell RNA-seq with unique molecular identifiers. Nat Methods 11: 163-166

Gene expression varies among different tissues, in effect giving rise to different tissue types out of undifferentiated cells; however, expression also varies among different cells in the same tissue. Most assays for measuring gene expression depend on multiple cell input material, but this study presents a method for single-cell RNA-Seq, based on Illumina sequencing technology. This technology can be applied to characterize sources of transcriptional noise, or to study expression in early embryos and other sample types where the cell count is naturally limited. One attractive possibility is the application of single-cell sequencing to assess cell type diversity in complex tissues.

Illumina Technology: HiSeq 2000

Murtaza M., Dawson S. J., Tsui D. W., Gale D., Forshew T., et al. (2013) Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 497: 108-112

Recent studies have shown that genomic alterations in solid cancers can be characterized by sequencing of circulating cell-free tumor DNA released from cancer cells into plasma, representing a non-invasive liquid biopsy. This study describes how this approach was applied using Illumina HiSeq sequencing to track the genomic evolution of metastatic cancers in response to therapy. Six patients with breast, ovarian, and lung cancers were followed over 1–2 years. For two cases, synchronous biopsies were also analyzed, confirming genome-wide representation of the tumor genome in plasma and establishing the proof-of-principle of exome-wide analysis of circulating tumor DNA.

Illumina Technology: TruSeq Exome Enrichment Kit, HiSeq 2000

Kivioja T., Vaharautio A., Karlsson K., Bonke M., Enge M., et al. (2012) Counting absolute numbers of molecules using unique molecular identifiers. Nat Methods 9: 72-74

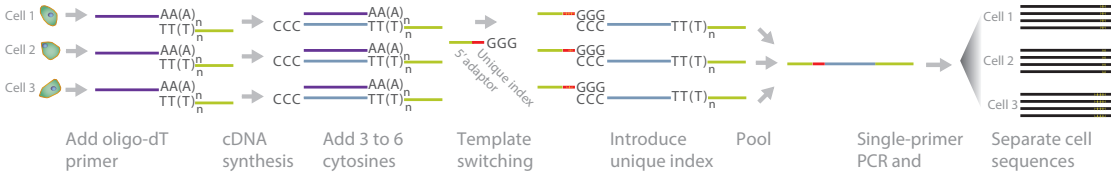
This is the first paper to describe the UMI method and its utility as a tool for sequencing. The authors use UMIs, which make each molecule in a population distinct, for genome-scale karyotyping and mRNA sequencing.

Illumina Technology: Genome Analyzer$_{IIx}$, HiSeq 2000

Cell Expression by Linear Amplification Sequencing (CEL-Seq)

Cell expression by linear amplification sequencing (CEL-Seq) utilizes barcoding and pooling of RNA to overcome challenges from low input.[152] In this method, each cell undergoes RT with a unique barcoded primer in its individual tube. After second-strand synthesis, cDNA from all reaction tubes are pooled, and PCR-amplified. Paired-end deep sequencing of the amplified DNA allows accurate detection of sequence derived from sequencing both strands.

Pros	Cons
• Barcoding and pooling allows for multiplexing and studying many different single cells at a time. • Contamination among samples is greatly reduced due to processing one tube per cell. • Fewer steps than STRT-Seq. • Very little read-length bias.[153] • Strand-specific.	• Strongly 3' biased.[154] • Abundant transcripts are preferentially amplified. • Requires at least 400 pg of total RNA.



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152. Hashimshony T., Wagner F., Sher N. and Yanai I. (2012) CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. Cell Rep 2: 666-673
153. Bhargava V., Head S. R., Ordoukhanian P., Mercola M. and Subramaniam S. (2014) Technical variations in low-input RNA-seq methodologies. Sci Rep 4: 3678
154. Shapiro E., Biezuner T. and Linnarsson S. (2013) Single-cell sequencing-based technologies will revolutionize whole-organism science. Nat Rev Genet 14: 618-630

Bhargava V., Head S. R., Ordoukhanian P., Mercola M. and Subramaniam S. (2014) Technical variations in low-input RNA-seq methodologies. Sci Rep 4: 3678

Recent advances in RNA-Seq methodologies from limiting amounts of mRNA have facilitated the characterization of rare cell-types in various biological systems. In this paper, the authors compare three amplification-based methods: Smart-Seq, DP-Seq, and CEL-Seq. The authors showed that these libraries exhibit significant technical variations, including inefficient amplification of the majority of low to moderately expressed transcripts. The sequencing errors are negligible, but the high technical variations introduced from amplification are still a hurdle to the use of these methods for detecting subtle biological differences.

Illumina Technology: HiSeq

Hashimshony T., Wagner F., Sher N. and Yanai I. (2012) CEL-Seq: single-cell RNA-Seq by multiplexed linear amplification. Cell Rep 2: 666-673

High-throughput sequencing has allowed for unprecedented detail in gene expression analyses, yet its efficient application to single cells is challenged by the small starting amounts of RNA. This paper presents a single-cell RNA-Seq protocol, CEL-Seq, that uses barcoding, pooling of samples, and linear amplification with one round of *in vitro* transcription. The assay is designed around a modified version of the Illumina directional RNA-Seq protocol, and sequencing is done on the Illumina HiSeq. The authors demonstrate their method by single-cell expression profiling of early C. elegans embryonic development.

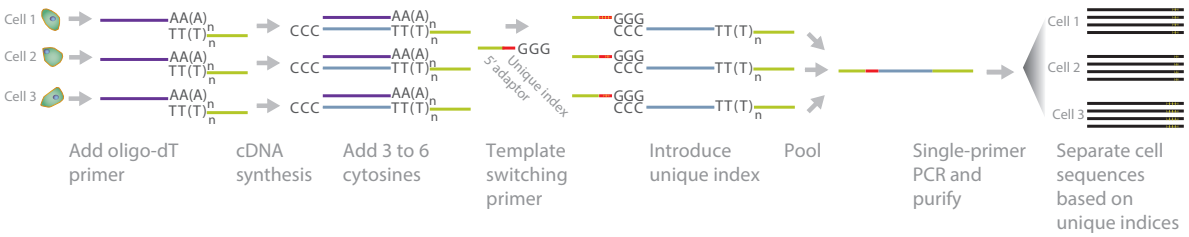
Illumina Technology: Genome Analyzer$_{IIx}$

Single-Cell Tagged Reverse Transcription (STRT-Seq)

Single-cell tagged reverse transcription sequencing (STRT-Seq) is a method similar to CEL-Seq that involves unique barcoding and sample pooling to overcome the challenges of samples with limited material.[155] In this method, single cells are first picked in individual tubes, where first-strand cDNA synthesis occurs using an oligo(dT) primer with the addition of 3–6 cytosines. A helper oligo promotes template switching, which introduces the barcode on the cDNA. Barcoded cDNA is then amplified by single-primer PCR. Deep sequencing allows for accurate transcriptome sequencing of individual cells.

155. 155 Islam S., Kjallquist U., Moliner A., Zajac P., Fan J. B., et al. (2011) Characterization of the single-cell transcriptional landscape by highly multiplex RNA-seq. Genome Res 21: 1160-1167

Pros	Cons
• Barcoding and pooling allows for multiplexing and studying many different single cells at a time. • Contamination among samples is greatly reduced due to processing one tube per cell.	• PCR biases can underrepresent GC-rich templates. • Non-linear PCR amplification can lead to biases affecting reproducibility. • Amplification errors caused by polymerases will be represented and sequenced incorrectly. • Loss of accuracy due to PCR bias. • Targets smaller than 500 bp are preferentially amplified by polymerases during PCR.



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Islam S., Zeisel A., Joost S., La Manno G., Zajac P., et al. (2014) Quantitative single-cell RNA-seq with unique molecular identifiers. Nat Methods 11: 163-166

Gene expression varies among different tissues, in effect giving rise to different tissue types out of undifferentiated cells; however, expression also varies among different cells in the same tissue. Most assays for measuring gene expression depend on multiple cell input material, but this study presents a method for single-cell RNA-Seq, based on Illumina sequencing technology. This technology can be applied to characterize sources of transcriptional noise, or to study expression in early embryos and other sample types where the cell count is naturally limited. One attractive possibility is the application of single-cell sequencing to assess cell type diversity in complex tissues.

Illumina Technology: HiSeq 2000

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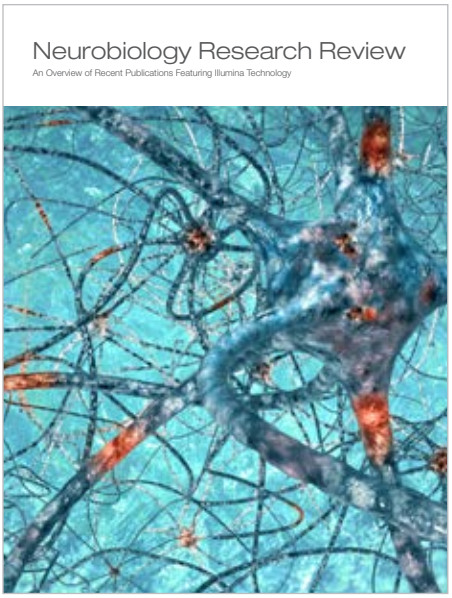

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