Single cell protein analysis for systems biology

Ezra Levy[1] & Nikolai Slavov[1,2]

1. Department of Biology, Northeastern University, Boston, MA 02115, USA
2. Department of Bioengineering, Northeastern University, Boston, MA 02115, USA

Abstract

The cellular abundance of proteins can vary even between isogenic single cells. This variability between single-cell protein levels can have functional roles, such as controlling cell fate during apoptosis induction or the proliferation/quiescence decision. Here, we review such examples of connecting protein levels and their dynamics in single cells to cellular functions. Such findings were made possible by the introduction of antibodies, and subsequently fluorescent proteins, for tracking protein levels in single cells. However, in heterogeneous cell populations, such as tumors or differentiating stem cells, cellular decisions are controlled by hundreds, even thousands of proteins acting in concert. Characterizing such complex systems demands measurements of thousands of proteins across thousands of single cells. This demand has inspired the development of new methods for single cell protein analysis, and we discuss their trade-offs, with emphasis on their specificity and coverage. We finish by highlighting the potential of emerging mass-spec methods to enable systems-level measurement of single-cell proteomes with unprecedented coverage and specificity. Combining such methods with methods for quantifying the trasncriptomes and metabolomes of single cells will provide essential data for advancing quantitative systems biology.

Introduction

Early experimental investigations of cellular heterogeneity focused on isogenic bacterial populations. Despite being isogenic and growing in the same culture, individual bacteria varied in persistence, lambda phage burst size, beta-galactosidase production, and chemotactic behaviour (1–4). These pioneering studies used elegant approaches to investigate heterogeneity and its functional consequences but were limited by the technology at the time, having no means of detecting gene expression in single cells. Then in 1994 Chalfie et. al (5) introduced a new technology, green fluorescent protein (GFP), that allowed researchers to measure and dynamically track protein levels in single cells. This technological innovation enabled Elowitz et. al (6) to accurately measure protein levels and their variability across thousands of isogenic cells. The measurements revealed unexpected variability in the levels of proteins expressed from the same promoter, which the authors interpreted as biochemical noise comprised of two components: intrinsic, inherent to the biochemical process of transcription and translation, and extrinsic, dominated by external environmental fluctuations.

Determinants and functions of protein

While these first studies focused on clonal cells and attributed the variability of a protein to noise in gene expression, in many cases the differences in the abundance of a protein across single cells reflects different cellular states that may lead to different functional outcomes (7). For instance, Spencer et. al (8) demonstrated that in a cell culture of mitotically cycling MCF10A cells, the level of p21, a CDK2 inhibitor, determines whether a cell enters a quiescent or proliferative state. If p21 is present above a threshold at the end of mitosis, it inhibits CDK2 and the cell enters quiescence. Conversely, if the level of p21 is below the threshold, CDK2 remains active and the cell continues to proliferate. By making measurements of single cells, the authors also found that modulating p21 levels altered the proportion of quiescent or proliferative cells, and that different cell lines exhibited different inherent proportions of each. Thus, the level of a single protein affects the proportion of cells in a quiescent or proliferative state.

Changes in genetic parameters can tune the variability in gene expression, and cells can exploit this variability to respond dynamically to environmental changes. To study the effect of genetic parameters on gene expression noise, Ozbudak et. al (9) quantified the relative contributions of transcription and translation to phenotypic noise in *B. subtilis* at various rates of transcription and translation of a single gene. They demonstrated that the efficiency of either process, and the resulting noise profile, could be altered by mutating the promoter, which affected transcription (10), or ribosomes-binding sites, which affected translation (11). Subsequently, Raser and O'Shea (12) introduced both *cis*- and *trans*- acting mutations that changed the expression noise profile of a given gene, providing further evidence how gene expression noise can be biochemically encoded and evolved. These studies indicated that gene expression variability is a selectable trait evolved to suit the gene and its particular function.

Spencer et. al (13) provided an example of how this evolved, inherent variability in protein levels between cells could lead to graded cellular responses across the population, and confer an overall survival advantage. They monitored HeLa and MCF10 cells on their path towards TRAIL-induced apoptosis and observed highly variable outcomes between single cells: most cells died, doing so at an exponentially decaying rate, but a small subpopulation always survived and continued growing. The authors measured the distribution in protein levels of five apoptotic regulators, and found that the measured inherent variability in the levels of these proteins was enough to account for the variability in cellular response time between induction and apoptosis itself. Thus, inherent distributed protein levels can lead to graded responses to stress at the population level, and can improve the chances that a small population of cells survives a particular stress. Similarly, variable response to stress as a bet-hedging strategy was theoretically predicted (14) and later experimentally demonstrated in yeast by Stewart-Ornstein et. al (15), who showed that more stochastic expression of MSN2/4 target genes increased the population survival rate under stress by 20%. The examples above demonstrate that protein expression noise plays a role in population-level cooperation, coordination, and survival. Discovering and understanding such regulatory mechanisms requires single cell measurements.

New technology can enable new biology

Just as the seminal work of Elowitz et al. (6) depended on a new technology, so do current efforts to understand and control cell function and fate. Studying regulation across heterogeneous cellular systems, such as human tissues, cancers, or differentiating cells,

demands technologies that can measure gene expression at the systems level. While single cell RNA-seq methods have made much progress at measuring single cell transcriptomes, mRNA levels alone are insufficient for characterizing gene expression and cannot detect post-translational modifications. Indeed, Franks et. al (16) showed that while mRNA levels can explain mean-level variability in protein levels, they cannot account for differences between tissue proteomes. These differences implicate post-transcriptional regulation as an important regulatory mechanism that shapes tissue-specific proteomes, and highlight the need for quantifying proteins and their all of their modified forms, termed proteoforms, in single cells in order to characterize the molecular and signaling mechanisms controlling cellular functions. A proteoform is defined as the set of all molecular forms of a protein produced from one gene (17), and comprehensive quantification of proteoforms across thousands of single-cells can enable modeling signaling networks with fewer assumptions, and even causal inference (18).

Although quantifying proteins in single cells is necessary for systems-level analysis, it is not sufficient. Additional measurements of other layers of biological regulation can capture important information upstream of translation that can furnish a more complete understanding of a regulatory motif or pathway. Munsky et. al (19) simulated the distributions of mRNA and protein levels for a number of gene regulatory motifs, and showed that dependent on the motif, mRNA levels were not necessarily correlated to the corresponding protein's level. The different time scales of mRNAs and proteins could account for differences between mRNA and protein levels, and thus, to characterize the interplay between various layers of regulation at a systems level (20), new single-cell methods and studies should strive towards 'multi-omics' methods (21) that enable simultaneous systems-level protein and mRNA measurements, and beyond.

Methods for quantifying protein levels in single cells

The methods for quantifying protein levels in single cells use three major modalities for identifying and quantifying proteins: (i) genetically engineered fluorescent proteins, (ii) antibodies and (iii) mass-spectrometry. The first two have so far dominated cellular protein research, and have enabled many discoveries, including those reviewed above. The third method shows the greatest promise for increasing both the specificity and the throughput of single-cell protein analysis, Figure 1. Below we summarize the distinctive advantages and weakness of the current methods.

Fluorescent proteins enable quantifying protein dynamics

Since the discovery and cloning of GFP (5), the community has engineered many fluorescent proteins with substantially enhanced functions, such as different spectral characteristics, fast folding and maturation, increased and decreased resistance to photo-bleaching, and fluorescence resonance energy transfer (22,23). Fluorescent proteins allow dynamic measurements of protein levels and location over time. Such measurements have been instrumental for discovering biological functions that depend not merely on the levels of a protein, but also on its dynamics (24–27). For example, different dynamics of p53 induce the transcription of different sets of genes and different cell fates (28). Indeed, important cellular functions are regulated by dynamic signaling mechanisms (29,30), and thus measuring protein dynamics is essential to understanding biological systems. However, the number of proteins that can be quantified per cell using fluorescent proteins remains limited by their spectral overlap (31), and fluorescent proteins have limited utility with

systems that cannot be genetically engineered, e.g., clinical samples. Furthermore, engineering new suites of fluorescent proteins for each new biological question requires much time and effort, and is often prohibitive for systems level measurements.

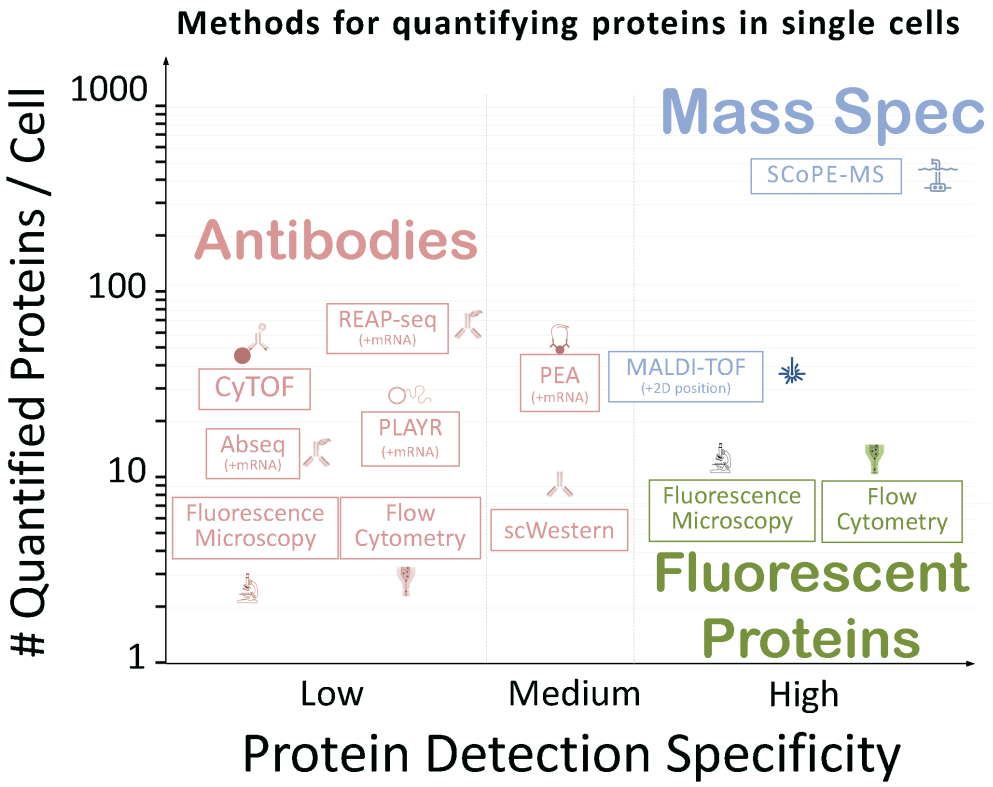

Figure 1 | Classification of single-cell protein analysis methods based on their specificity and proteome coverage. Antibody-based methods, in red, are widely utilized and generally applicable to intermediate numbers of proteins at once. Their specificity depends on the antibody and have be rather low. The specificity of antibodies can be increased by electrophoretic separation (scWestern) or using multiple antibodies per protein (PEA). Fluorescent protein-based methods, in green, are highly specific and facilitate monitoring protein levels over time, but are limited to quantifying only a few proteins per cell because of spectral overlap. Mass-spectrometry can increase both specificity and depth of coverage. MALDI-TOF has been used to study single cells and spatial questions for decades, but it offers only medium specificity and proteome coverage. SCoPE-MS enables simultaneous identification and quantitation of hundreds of proteins from single cells, and demonstrates one path towards comprehensive quantification of proteins in single cells. All values are applicable for typically sized mammalian cell, with a dimeter of about 15 μm and 500pg of total protein.

Antibody-based methods

Antibodies can target some protein portion of many cellular pathways in single cells, for instance enabling studies of emergent cancer resistance and can be applied over a broad dynamic range (32). Immunohistochemistry enables visualisation of tissue sections with single cell resolution, while immunocytochemistry does the same for monolayer cell cultures (33). Antibodies are frequently incorporated in flow cytometry, characterizing patterns of a few proteins across tens of thousands of cells (34). However, antibodies present two primary hurdles for more comprehensive protein coverage: specificity and scalability (35–38). Some new methods are attempting to increase protein multiplexing while maintaining or improving antibody specificity for protein quantitation in single cells, Figure 1.

To increase the number of simultaneously quantifiable proteins, mass cytometry (CyTOF) conjugates transition element isotopes, normally absent in biology, to antibodies. After labeling cells with these antibodies, droplets containing single cells are isolated, the cells vaporized, and the remaining transition metals analyzed by a time-of-flight (TOF) mass spectrometer. Bendall et. al (39) developed CyTOF and used it to quantify the immune response of thousands of single cells from healthy human bone marrow samples. They developed two panels of antibodies designed to interrogate different aspects of the immune response. From the 13 proteins common to both panels, the authors created a map of phenotypically and presumably functionally linked immune cell populations. The authors then overlaid the data from the 18 remaining, panel-specific proteins to refine their map, uncovering further heterogeneity within numerous subsets of the larger, annotated cell populations. Although mass cytometry begins to probe the pathway level and can detect and quantify very lowly abundant epitopes, it relies on single antibodies to do so, and is limited to 31 epitopes per cell, the number transition metals.

By substituting transition metals for DNA oligonucleotides, the Abseq, CITE-seq, and REAP-seq methods (38,40,41) fulfill the need for specific antibody labels. With Abseq, single cells are simultaneously probed and barcoded in a high-throughput microfluidic device, then subject to PCR, which ligates cell barcodes with the identifying oligonucleotides of bound antibodies, and amplifies the signal. Quantitation is accomplished by subsequent DNA sequencing, where the number of reads per cell is interpreted as a surrogate for the protein level. CITE-seq and REAP-seq also employ droplet microfluidics, and subsequently generate protein and RNA level readouts by integrating oligonucleotide-tagged antibodies into established single cell transcriptomic workflows. Although there is no practical limit to the number of unique identifiers that can be chemically conjugated to antibodies of choice, these methods are inherently limited by the number of available antibodies, their specificity, the epitope availability, and the number of antibodies that can be introduced per cell before molecular crowding becomes a limiting factor.

Approaches for increasing the specificity of antibody-based methods

The above methods depend critically on the specificity of a single antibody and will perform very poorly in the presence of non-specific binding. To alleviate such concerns, two strategies have been developed to increase the specificity of antibody-based single-cell methods as described below:

First, single cell western blotting (scWestern) increases the specificity of antibodies by physically separating proteins from single-cell lysates. This is accomplished by electrophoresis (42) or isoelectric focusing of proteins (43), and the added dimension of

separation helps single-antibody probing resolve non-specific signals. Hughes et. al (42) used in-house fabricated open-microwell arrays to separate proteins from single cells with at least 50% different masses. In the open-microwell format, the authors simultaneously applied scWestern to 5040 single-cell samples, and obtained quantitative measurements for 1608 of these samples. However, the method introduces additional protein loss of ~40% for each single cell, and introduces a limit of detection of 27,000 molecules (median murine fibroblast 50,000 molecules). Although the same blot can be stripped and reprobed for different proteins upwards of 9 times, the number of proteins simultaneously measurable by scWestern is fundamentally limited by the number of re-blotting cycles.

The second approach to increasing signal specificity for antibody-based methods is the proximity extension assay (PEA). PEA increases specificity by requiring the binding of 2 different oligonucleotide-tagged antibodies to same protein before a signal can be generated (45,46). Two antibodies bound to the same protein carry overlapping sequences that ligate upon binding, allowing subsequent extension, amplification, digestion, and quantitation of a few dozen proteins by microfluidic qPCR. This reduces background signal compared to single-antibody probing, and the less stringent specificity requirements permit the use of a wider range of antibodies. However, not all of these additional antibodies are necessarily applicable; since any given protein must have two antibody binding sites that are amenable to oligonucleotide overlap, epitope availability and molecular crowding inhibit how comprehensively PEA can be applied.

Single-cell antibody methods will continue to scale as efforts with rigorous quality control to retain or improve antibody specificity evolve.

Mass spectrometry-based methods

Proteins can be identified and quantified by mass spectrometry (MS). Indeed, MS applied to bulk samples comprised on millions of cells can already measure thousands of proteins at once with high specificity (47,48), including important post translational modifications (PTMs) that affect cell function and dynamics, such as phosphorylation (39). Although most mass spectrometry methods remain bounded to bulk samples, new methods are enabling the analysis of more complete proteomes of increasing numbers of single cells.

Matrix assisted laser desorption ionization coupled with time-of-flight mass spectrometry (MALDI-TOF) has been applied to single cells for about two decades (49,50), enabling identification and spatial localization of dozens of peptides, Figure 1. However, the variability in the fraction of peptides ionized across samples limits the quantitative accuracy of MALDI measurements. Furthermore, since peptides are not separated and enter the TOF instrument at the same time, the acquired spectra comprise a mixture of the spectra of many peptides. These complex spectra are hard to interpret and relatively few peptide sequences can be confidently identified. Thus, while MALDI has been employed widely in the spatial mapping of neuropeptides in single neurons (51) or proteins in tissue samples (52), it remains bounded to biological questions of localization rather than quantitation.

The MS method that has allowed for well-controlled and accurate measurements of tens of thousands of proteins is liquid chromatography (LC) combined with electrospray ionization and tandem mass-spectrometry, usually abbreviated as LC-MS/MS. Ideally, LC-MS/MS can be applied to single-cell lysates to give the deep and accurate quantification of proteins and proteoforms that it has afforded with bulk samples. However, losses during sample

preparation for LC-MS/MS and relatively low sensitivity have limited its application to very small samples.

Very sensitive workflows have begun to apply LC-MS/MS to small samples comprised of hundreds of human cells (48) and even to unusually large single cells, such as oocytes (53,54) and muscle fibers (55,56). These applications developed and applied very sensitive methods to quantify from 450~800 proteins in single human oocytes, and ~2,100 proteins in single muscle fibers. Yet the typical single mammalian cells contain orders of magnitude less protein than oocytes and muscle fibers (57). These tiny samples suffer too much loss during processing, and as a result not enough molecules are delivered to the instrument for meaningful identification and quantification. Our early attempts at using LC-MS/MS to identify and quantify proteins from typically sized single mammalian cells, dubbed Single Cell ProtEomics by Mass Spectrometry (SCoPE-MS) (58), aimed to increase the sensitivity and throughput of single-cell MS by combining two strategies:

The first strategy sought to minimize sample loss by optimizing a 'clean' sample processing pipeline that required no chemical cleanup prior to injection, but instead consisted of mechanical cell lysis and simple addition of LC- and MS-compatible reagents to reduce chemical cleanup and pipetting losses. The second strategy increased both the number of quantified cells per run and the confidence in peptide identification by using isobaric mass tags. These tags from covalent bonds with peptides and different tags can label the peptides from different samples. We used these tags to label the cell lysates of single cells as well as the lysate of 200 cells, terms carrier cells, and combined the labeled single cells and the carrier cells into a single sample. Incorporating the carrier cells into the workflow conferred two benefits: (i) it reduced the peptide loss experienced by the single cells during sample preparation and nLC separation, and (ii) it improved identification confidence by increasing the total number of ions delivered to the mass spectrometer. Combining these strategies minimized sample loss in the processing pipeline, which then delivered enough single cell sample for confident identification. This allowed reliable relative quantitation of ~600 proteins in any given single cell, and over 1000 proteins across a system of differentiating mouse embryonic stem cells.

Critically, the LC-MS/MS strategy can also measure post-translational modifications, such as phosphorylation and glycosylation (59,60). Further improvements in specificity and throughput can vastly improve single-cell mass spectrometry methods (18), positioning MS to become a new workhorse of single cell protein analysis, Figure 1. Peptides can be separated not only by LC but also by capillary electrophoresis (CE), and CE-MS/MS (54) can offer some advantages over LC-MS/MS for very small complex samples, such as the proteomes of single cells, since it allows reduced flow rates and thus improved ionization of molecules. Furthermore, CE-MS/MS is the most promising method for quantifying proteins without having to digest them in what is commonly referred to as top-down MS. Furthermore, incorporating existing automation technologies can increase assay throughput and statistical power, as well as reduce sample volumes, significantly alleviating protein adsorption. Finally, parallel technical improvements in ion accumulation and sampling will improve the sensitivity, accuracy, and depth of quantitation across sampled cells.

Simultaneous quantification of proteins and RNAs in single cells

While quantifying protein levels in single cells can be powerful alone, it is even more powerful when combined with quantifying the transcriptomes of the same single cells. Thus, an import direction in the advancement of single cell proteomics methods is making them compatible with single cell transcriptomic methods, which are generally more mature (21), and single cell metabolomics methods, many of which already employ mass spectrometry (61,62).

In initial attempts, both CyTOF and PEA, described above, have been used to quantify some transcripts and their corresponding proteins in the same single cell. To enable quantifying mRNAs by CyTOF, Frei et. al (63) developed and integrated a proximity ligation assay for RNA (PLAYR) into the CyTOF workflow. The PLAYR method is compatible with flow cytometry and mass cytometry, and the authors used both to quantify transcripts alone, then simultaneously quantify 10 transcripts and corresponding proteins in single primary human peripheral blood mononuclear cells (PBMCs). Darmanis et. al (64) extend the use of PEA to simultaneously measure ~22 mRNA and corresponding protein by performing TaqMan and PEA on the split lysate of single neural stem cells undergoing BMP4-induced differentiation. Their comparative analysis of the predictive power of mRNA and/or protein levels for assigning single cells to a treatment group demonstrated that proteins were better predictors for the functional response to BMP4-treatment than RNA, though both proteins and mRNA levels contributed unique information; the combined data predicted treatment group more effectively than mRNA or protein alone.

Ultimately, we would like to comprehensively quantify the transcriptome, the proteome, and the metabolome of the same single cell. RNA-seq is steadily advancing high-throughput single-cell transcriptomics towards this end, while MS offers the most promising method for high-throughput single-cell proteomics and metabolomics. These methods have to be combined and applied to the same cell, and we expect to see much progress in this direction in the near future.

Concluding remarks

Growing evidence elaborates on how protein-level cellular heterogeneity is intricately tied to cell fate in such varied biological contexts as cancer, differentiation, and mitosis. Transcriptomic studies provide an appreciation for the heterogeneous nature of the cells making up these systems, but cannot capture most post-translational modifications, such as phosphorylation and glycosylation, which are critical layers of regulation. Ultimately, single cell proteomic measurements are required to characterize functional variability at the systems level. Mass spectrometry is poised to enable such measurements, and will continue to improve alongside parallel advances in instrumentation, automation, and computation. MS can also enable multi-layer analyses of the same single cell beyond the protein level, and begin to quantify post-translational modifications, metabolomes and transcriptomes simultaneously with proteomes. These high-powered, high-dimensional data will power systems-level measurement and characterization across many biological questions, and have an opportunity to set new standards of accuracy, applicability, and depth in quantitative systems biology.

Summary

- The levels of a protein can vary across single cells both because of stochastic influences, i.e., noise in gene-expression due to low-copy number molecules, and because of cellular regulatory mechanisms
- Protein levels in single cells influence cellular functions and determine cell fates
- Current technologies enable studying a few proteins simultaneously, but face challenges when scaled to pathway-level multiplexing
- The next generation of systems-biology needs more powerful methods for quantifying proteins in single cells
- Mass-spectrometry is poised to enable deep quantification of single-cell proteomes for the next generation of systems biology

Competing interests: The authors declare that there are no competing interests associated with the manuscript.

Abbreviations: CDK2, cyclin-dependent kinase 2; **CE**, capillary electrophoresis; **CITE-seq**, cellular indexing of transcriptomes and epitopes by sequencing; **CyTOF**, mass cytometry; **LC**, liquid chromatography; **LC-MS/MS**, liquid chromatography followed by tandem mass spectrometry; **MALDI-TOF**, matrix assisted laser desorption ionization coupled with time-of-flight mass spectrometry; **MS**, mass-spectrometry; **PEA**, proximity extension assay; **PLAYR**, proximity ligation assay for RNA; **PBMC**, peripheral blood mononuclear cell; **PTM**, post-translational modification; **REAP-seq**, RNA expression and protein sequencing; **SCoPE-MS**, single cell proteomics by mass spectrometry; **scWestern**, single cell western blot; **TRAIL**, TNF-related apoptosis-inducing ligand;

References

1. Bigger J. TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH PENICILLIN BY INTERMITTENT STERILISATION. Lancet [Internet]. 1944 Oct 14 [cited 2018 Apr 19];244(6320):497–500. Available from: https://www.sciencedirect.com/science/article/pii/S0140673600742103
2. Delbrück M. The Burst Size Distribution in the Growth of Bacterial Viruses (Bacteriophages). J Bacteriol [Internet]. 1945 Aug [cited 2018 Apr 19];50(2):131–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/16560982
3. Novick A, Weiner M. Enzyme Induction as an All-or-None Phenomenon. 1957 [cited 2018 Apr 19];43:553–66. Available from: www.pnas.org#otherarticles
4. Spudich JL, Koshland DE. Non-genetic individuality: chance in the single cell. Nature [Internet]. 1976 Aug 5 [cited 2018 Apr 19];262(5568):467–71. Available from: http://www.ncbi.nlm.nih.gov/pubmed/958399
5. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science [Internet]. 1994 Feb 11 [cited 2018 Apr 19];263(5148):802–5. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8303295
6. Elowitz MB, Levine AJ, Siggia ED, Swain PS. Stochastic gene expression in a single cell. Science [Internet]. 2002 Aug 16 [cited 2018 Apr 19];297(5584):1183–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12183631
7. Raj A, van Oudenaarden A. Nature, Nurture, or Chance: Stochastic Gene Expression and Its Consequences. Cell [Internet]. 2008 Oct 17 [cited 2018 Apr 19];135(2):216–26. Available from:

https://www.sciencedirect.com/science/article/pii/S0092867408012439?via%3Dihub
8. Spencer SL, Cappell SD, Tsai F-C, Overton KW, Wang CL, Meyer T. The Proliferation-Quiescence Decision Is Controlled by a Bifurcation in CDK2 Activity at Mitotic Exit. Cell [Internet]. 2013 Oct 10 [cited 2018 Apr 19];155(2):369–83. Available from: https://www.sciencedirect.com/science/article/pii/S0092867413011434?via%3Dihub
9. Ozbudak EM, Thattai M, Kurtser I, Grossman AD, van Oudenaarden A. Regulation of noise in the expression of a single gene. Nat Genet [Internet]. 2002 May 22 [cited 2018 Apr 20];31(1):69–73. Available from: http://www.nature.com/articles/ng869
10. Ptashne M, Backman K, Humayun MZ, Jeffrey A, Maurer R, Meyer B, et al. Autoregulation and function of a repressor in bacteriophage lambda. Science [Internet]. 1976 Oct 8 [cited 2018 Apr 20];194(4261):156–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/959843
11. Coleman J, Inouye M, Nakamura K. Mutations upstream of the ribosome-binding site affect translational efficiency. J Mol Biol [Internet]. 1985 Jan 5 [cited 2018 Apr 20];181(1):139–43. Available from: https://www.sciencedirect.com/science/article/pii/0022283685903328?via%3Dihub
12. Raser JM, O'Shea EK. Control of stochasticity in eukaryotic gene expression. Science [Internet]. 2004 Jun 18 [cited 2018 Apr 19];304(5678):1811–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15166317
13. Spencer SL, Gaudet S, Albeck JG, Burke JM, Sorger PK. Non-genetic origins of cell-to-cell variability in TRAIL-induced apoptosis. Nature [Internet]. 2009 May 12 [cited 2018 Apr 19];459(7245):428–32. Available from: http://www.nature.com/articles/nature08012
14. Thattai M, van Oudenaarden A. Stochastic gene expression in fluctuating environments. Genetics [Internet]. 2004 May 1 [cited 2018 Apr 27];167(1):523–30. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15166174
15. Stewart-Ornstein J, Weissman JS, El-Samad H. Cellular Noise Regulons Underlie Fluctuations in Saccharomyces cerevisiae. Mol Cell [Internet]. 2012 Feb 24 [cited 2018 Apr 19];45(4):483–93. Available from: https://www.sciencedirect.com/science/article/pii/S109727651200038X?via%3Dihub
16. Franks A, Airoldi E, Slavov N. Post-transcriptional regulation across human tissues. Vogel C, editor. PLOS Comput Biol [Internet]. 2017 May 8 [cited 2018 Apr 19];13(5):e1005535. Available from: http://dx.plos.org/10.1371/journal.pcbi.1005535
17. Smith LM, Kelleher NL, Proteomics TC for TD, Linial M, Goodlett D, Langridge-Smith P, et al. Proteoform: a single term describing protein complexity. Nat Methods [Internet]. 2013 Mar 1 [cited 2018 May 1];10(3):186–7. Available from: http://www.nature.com/articles/nmeth.2369
18. Specht H, Slavov N. Routinely quantifying single cell proteomes: A new age in quantitative biology and medicine. 2018 Apr 2 [cited 2018 Apr 19]; Available from: https://peerj.com/preprints/26821/
19. Munsky B, Neuert G, van Oudenaarden A. Using gene expression noise to understand gene regulation. Science [Internet]. 2012 Apr 13 [cited 2018 Apr 19];336(6078):183–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22499939
20. Cheng Z, Otto GM, Powers EN, Keskin A, Mertins P, Carr SA, et al. Pervasive, Coordinated Protein-Level Changes Driven by Transcript Isoform Switching during Meiosis. Cell [Internet]. 2018 Feb 22 [cited 2018 Apr 19];172(5):910–923.e16. Available from: https://www.sciencedirect.com/science/article/pii/S0092867418301120?via%3Dihub
21. Macaulay IC, Ponting CP, Voet T. Single-Cell Multiomics: Multiple Measurements from Single Cells. Trends Genet [Internet]. 2017 Feb 1 [cited 2018 Apr 19];33(2):155–68. Available from: https://www.sciencedirect.com/science/article/pii/S016895251630169X?via%3Dihub
22. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. Nat Methods [Internet]. 2005 [cited 2018 Apr 27];2(12):905–9. Available from: http://fulltext.calis.edu.cn/nature/nmeth/2/12/nmeth819.pdf
23. Cranfill PJ, Sell BR, Baird MA, Allen JR, Lavagnino Z, de Gruiter HM, et al. Quantitative assessment of fluorescent proteins. Nat Methods [Internet]. 2016 Jul 30 [cited 2018 Apr 27];13(7):557–62. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27240257
24. Muzzey D, van Oudenaarden A. Quantitative Time-Lapse Fluorescence Microscopy in Single

Cells. Annu Rev Cell Dev Biol [Internet]. 2009 Nov 6 [cited 2018 Apr 19];25(1):301–27. Available from: http://www.annualreviews.org/doi/10.1146/annurev.cellbio.042308.113408
25. Lahav G, Rosenfeld N, Sigal A, Geva-Zatorsky N, Levine AJ, Elowitz MB, et al. Dynamics of the p53-Mdm2 feedback loop in individual cells. Nat Genet [Internet]. 2004 Feb 18 [cited 2018 Apr 19];36(2):147–50. Available from: http://www.nature.com/articles/ng1293
26. Eldar A, Elowitz MB. Functional roles for noise in genetic circuits. Nature [Internet]. 2010 Sep 9 [cited 2018 Apr 19];467(7312):167–73. Available from: http://www.nature.com/doifinder/10.1038/nature09326
27. Levine JH, Lin Y, Elowitz MB. Functional roles of pulsing in genetic circuits. Science [Internet]. 2013 Dec 6 [cited 2018 Apr 19];342(6163):1193–200. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24311681
28. Purvis JE, Karhohs KW, Mock C, Batchelor E, Loewer A, Lahav G. p53 dynamics control cell fate. Science [Internet]. 2012 Jun 15 [cited 2018 Apr 27];336(6087):1440–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22700930
29. Purvis JE, Lahav G. Encoding and Decoding Cellular Information through Signaling Dynamics. Cell [Internet]. 2013 Feb 28 [cited 2018 Apr 27];152(5):945–56. Available from: https://www.sciencedirect.com/science/article/pii/S0092867413001530?via%3Dihub
30. Slavov N, Carey J, Linse S. Calmodulin Transduces Ca $^{2+}$ Oscillations into Differential Regulation of Its Target Proteins. ACS Chem Neurosci [Internet]. 2013 Apr 17 [cited 2018 Apr 27];4(4):601–12. Available from: http://pubs.acs.org/doi/10.1021/cn300218d
31. Autissier P, Soulas C, Burdo TH, Williams KC. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. Cytom Part A [Internet]. 2010 May 1 [cited 2018 Apr 19];9999A(5):NA-NA. Available from: http://doi.wiley.com/10.1002/cyto.a.20859
32. Ullal A V, Peterson V, Agasti SS, Tuang S, Juric D, Castro CM, et al. Cancer cell profiling by barcoding allows multiplexed protein analysis in fine-needle aspirates. Sci Transl Med [Internet]. 2014 Jan 15 [cited 2018 Apr 19];6(219):219ra9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24431113, doi:10.1038/nature22794
33. Miller MA, Weissleder R. Imaging of anticancer drug action in single cells. Nat Rev Cancer [Internet]. 2017 Jun 23 [cited 2018 Apr 19];17(7):399–414. Available from: http://www.nature.com/doifinder/10.1038/nrc.2017.41
34. Adan A, Alizada G, Kiraz Y, Baran Y, Nalbant A. Flow cytometry: basic principles and applications. Crit Rev Biotechnol [Internet]. 2017 Feb 17 [cited 2018 Apr 19];37(2):163–76. Available from: https://www.tandfonline.com/doi/full/10.3109/07388551.2015.1128876
35. Michaud GA, Salcius M, Zhou F, Bangham R, Bonin J, Guo H, et al. Analyzing antibody specificity with whole proteome microarrays. Nat Biotechnol [Internet]. 2003 Dec 9 [cited 2018 Apr 19];21(12):1509–12. Available from: http://www.nature.com/articles/nbt910
36. Bumbaca D, Wong A, Drake E, Reyes II AE, Lin BC, Stephan J-P, et al. Highly specific off-target binding identified and eliminated during the humanization of an antibody against FGF receptor 4. MAbs [Internet]. 2011 Jul 27 [cited 2018 Apr 19];3(4):376–86. Available from: http://www.tandfonline.com/doi/abs/10.4161/mabs.3.4.15786
37. Persson H, Preger C, Marcon E, Lengqvist J, Gräslund S. Antibody Validation by Immunoprecipitation Followed by Mass Spectrometry Analysis. In Humana Press, New York, NY; 2017 [cited 2018 Apr 19]. p. 175–87. Available from: http://link.springer.com/10.1007/978-1-4939-6857-2_10
38. Peterson VM, Zhang KX, Kumar N, Wong J, Li L, Wilson DC, et al. Multiplexed quantification of proteins and transcripts in single cells. Nat Biotechnol [Internet]. 2017 Aug 30 [cited 2018 Apr 19];35(10):936–9. Available from: http://www.nature.com/doifinder/10.1038/nbt.3973
39. Bendall SC, Simonds EF, Qiu P, Amir ED, Krutzik PO, Finck R, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science [Internet]. 2011 May 6 [cited 2018 Apr 19];332(6030):687–96. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21551058
40. Shahi P, Kim SC, Haliburton JR, Gartner ZJ, Abate AR. Abseq: Ultrahigh-throughput single cell protein profiling with droplet microfluidic barcoding. Sci Rep [Internet]. 2017 Mar 14 [cited

2018 Apr 19];7:44447. Available from: http://www.ncbi.nlm.nih.gov/pubmed/28290550
41. Stoeckius M, Hafemeister C, Stephenson W, Houck-Loomis B, Chattopadhyay PK, Swerdlow H, et al. Simultaneous epitope and transcriptome measurement in single cells. Nat Methods [Internet]. 2017 Jul 31 [cited 2018 May 1];14(9):865–8. Available from: http://www.nature.com/doifinder/10.1038/nmeth.4380
42. Hughes AJ, Spelke DP, Xu Z, Kang C-C, Schaffer D V, Herr AE. Single-cell western blotting. Nat Methods [Internet]. 2014 Jul 1 [cited 2018 Apr 19];11(7):749–55. Available from: http://www.nature.com/articles/nmeth.2992
43. Tentori AM, Yamauchi KA, Herr AE. Detection of Isoforms Differing by a Single Charge Unit in Individual Cells. Angew Chemie Int Ed [Internet]. 2016 Sep 26 [cited 2018 Apr 27];55(40):12431–5. Available from: http://doi.wiley.com/10.1002/anie.201606039
44. Kang C-C, Ward TM, Bockhorn J, Schiffman C, Huang H, Pegram MD, et al. Electrophoretic cytopathology resolves ERBB2 forms with single-cell resolution. npj Precis Oncol [Internet]. 2018 Dec 22 [cited 2018 Apr 27];2(1):10. Available from: http://www.nature.com/articles/s41698-018-0052-3
45. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous antibody-based proximity extension assays provide sensitive and specific detection of low-abundant proteins in human blood. Nucleic Acids Res [Internet]. 2011 Aug 1 [cited 2018 Apr 19];39(15):e102–e102. Available from: https://academic.oup.com/nar/article-lookup/doi/10.1093/nar/gkr424
46. Assarsson E, Lundberg M, Holmquist G, Björkesten J, Bucht Thorsen S, Ekman D, et al. Homogenous 96-Plex PEA Immunoassay Exhibiting High Sensitivity, Specificity, and Excellent Scalability. Hoheisel JD, editor. PLoS One [Internet]. 2014 Apr 22 [cited 2018 Apr 19];9(4):e95192. Available from: http://dx.plos.org/10.1371/journal.pone.0095192
47. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature [Internet]. 2003 Mar 13 [cited 2018 Apr 19];422(6928):198–207. Available from: http://www.nature.com/articles/nature01511
48. Li S, Plouffe BD, Belov AM, Ray S, Wang X, Murthy SK, et al. An Integrated Platform for Isolation, Processing, and Mass Spectrometry-based Proteomic Profiling of Rare Cells in Whole Blood. Mol Cell Proteomics [Internet]. 2015 Jun 1 [cited 2018 Apr 19];14(6):1672–83. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25755294
49. Richard M. Caprioli *, Terry B. Farmer and, Gile J. Molecular Imaging of Biological Samples: Localization of Peptides and Proteins Using MALDI-TOF MS. 1997 [cited 2018 Apr 19]; Available from: https://pubs.acs.org/doi/abs/10.1021/ac970888i
50. Li L, Garden RW, Sweedler J V. Single-cell MALDI: a new tool for direct peptide profiling. Trends Biotechnol [Internet]. 2000 Apr 1 [cited 2018 Apr 19];18(4):151–60. Available from: https://www.sciencedirect.com/science/article/pii/S016777990001427X?via%3Dihub
51. Hummon AB, Amare A, Sweedler J V. Discovering new invertebrate neuropeptides using mass spectrometry. Mass Spectrom Rev [Internet]. 2006 Jan 1 [cited 2018 Apr 19];25(1):77–98. Available from: http://doi.wiley.com/10.1002/mas.20055
52. Schwartz SA, Reyzer ML, Caprioli RM. Direct tissue analysis using matrix-assisted laser desorption/ionization mass spectrometry: practical aspects of sample preparation. J Mass Spectrom [Internet]. 2003 Jul 1 [cited 2018 Apr 19];38(7):699–708. Available from: http://doi.wiley.com/10.1002/jms.505
53. Virant-Klun I, Leicht S, Hughes C, Krijgsveld J. Identification of Maturation-Specific Proteins by Single-Cell Proteomics of Human Oocytes. Mol Cell Proteomics [Internet]. 2016 Aug [cited 2018 Apr 19];15(8):2616–27. Available from: http://www.mcponline.org/lookup/doi/10.1074/mcp.M115.056887
54. Lombard-Banek C, Moody SA, Nemes P. Single-Cell Mass Spectrometry for Discovery Proteomics: Quantifying Translational Cell Heterogeneity in the 16-Cell Frog (*Xenopus*) Embryo. Angew Chemie Int Ed [Internet]. 2016 Feb 12 [cited 2018 Apr 19];55(7):2454–8. Available from: http://doi.wiley.com/10.1002/anie.201510411
55. Murgia M, Nagaraj N, Deshmukh AS, Zeiler M, Cancellara P, Moretti I, et al. Single muscle fiber proteomics reveals unexpected mitochondrial specialization. EMBO Rep [Internet]. 2015 Mar 1 [cited 2018 Apr 19];16(3):387–95. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/25643707
56. Murgia M, Toniolo L, Nagaraj N, Ciciliot S, Vindigni V, Schiaffino S, et al. Single Muscle Fiber Proteomics Reveals Fiber-Type-Specific Features of Human Muscle Aging. Cell Rep [Internet]. 2017 Jun 13 [cited 2018 Apr 19];19(11):2396–409. Available from: https://www.sciencedirect.com/science/article/pii/S2211124717307118?via%3Dihub
57. Milo R, Jorgensen P, Moran U, Weber G, Springer M. BioNumbers—the database of key numbers in molecular and cell biology. [cited 2018 Apr 20]; Available from: https://www.weizmann.ac.il/plants/Milo/sites/plants.Milo/files/publications/nucl._acids_res.-2010-milo-d750-3.pdf
58. Budnik B, Levy E, Harmange G, Slavov N. Mass-spectrometry of single mammalian cells quantifies proteome heterogeneity during cell differentiation. bioRxiv [Internet]. 2018 Jan 1 [cited 2018 Apr 19];102681. Available from: https://www.biorxiv.org/content/early/2018/01/01/102681
59. Steen H, Jebanathirajah JA, Rush J, Morrice N, Kirschner MW. Phosphorylation Analysis by Mass Spectrometry MYTHS, FACTS, AND THE CONSEQUENCES FOR QUALITATIVE AND QUANTITATIVE MEASUREMENTS*. 2005 [cited 2018 Apr 20]; Available from: http://kirschner.med.harvard.edu/files/papers/Steen-MolCellProteo2005-5.1-172.pdf
60. Morelle W, Michalski J-C. Analysis of protein glycosylation by mass spectrometry. Nat Protoc [Internet]. 2007 Jul 1 [cited 2018 Apr 20];2(7):1585–602. Available from: http://www.nature.com/articles/nprot.2007.227
61. Zenobi R. Single-cell metabolomics: analytical and biological perspectives. Science [Internet]. 2013 Dec 6 [cited 2018 Apr 27];342(6163):1243259. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24311695
62. Rubakhin SS, Lanni EJ, Sweedler J V. Progress toward single cell metabolomics. Curr Opin Biotechnol [Internet]. 2013 Feb [cited 2018 Apr 27];24(1):95–104. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23246232
63. Frei AP, Bava F-A, Zunder ER, Hsieh EWY, Chen S-Y, Nolan GP, et al. Highly multiplexed simultaneous detection of RNAs and proteins in single cells. Nat Methods [Internet]. 2016 Mar 25 [cited 2018 Apr 19];13(3):269–75. Available from: http://www.nature.com/articles/nmeth.3742
64. Darmanis S, Gallant CJ, Marinescu VD, Niklasson M, Segerman A, Flamourakis G, et al. Simultaneous Multiplexed Measurement of RNA and Proteins in Single Cells. Cell Rep [Internet]. 2016 Jan 12 [cited 2018 Apr 19];14(2):380–9. Available from: https://www.sciencedirect.com/science/article/pii/S2211124715014345?via%3Dihub