Available online at www.sciencedirect.com
ScienceDirect
Current Opinion in Biotechnology

Studying transcriptional interactions in single cells at sufficient resolution

Mats Wallden and Johan Elf

Our ability to dissect and understand the principles of gene regulatory circuits is partly limited by the resolution of our experimental assays. In this brief review, we discuss aspects of gene expression in microbial organisms apparent only when increasing the experimental resolution from populations to single cells and sub-cellular structures, from snap-shots to high-speed time-lapse movies and from molecular ensembles to single molecules.

Address
Department of Cell and Molecular Biology, Science for Life Laboratory, Uppsala University, Husargatan 3, SE-751 24, Uppsala, Sweden

Corresponding author: Elf, Johan (johan.elf@icm.uu.se)

Current Opinion in Biotechnology 2011, **22**:81–86

This review comes from a themed issue on
Analytical biotechnology
Edited by Matthias Heinemann and Uwe Sauer

Available online 10th November 2010

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2010.10.004

Introduction

Gene regulation depends on a broad spectrum of intracellular signals encoded and processed at a variety of spatial and temporal scales. In order to unveil the regulatory principles of a specific system, the experimental assay needs to resolve the regulatory cues of the process under investigation. Obviously, to investigate stochastic differences in gene expression between cells, means of studying cells individually have to be adopted, and to understand dynamics of gene regulation, the process needs to be monitored at sufficient time resolution. Biomolecules may also change conformation, location, states of binding and modification, etc. and resolving these states may be necessary to determine their role for regulation. In this review, we present a few examples of regulatory interactions revealed only at fine experimental resolution. Further, we present the use of microfluidics as a means of meeting the greater demands of conducting high-resolution studies.

Single-cell resolution and gene expression

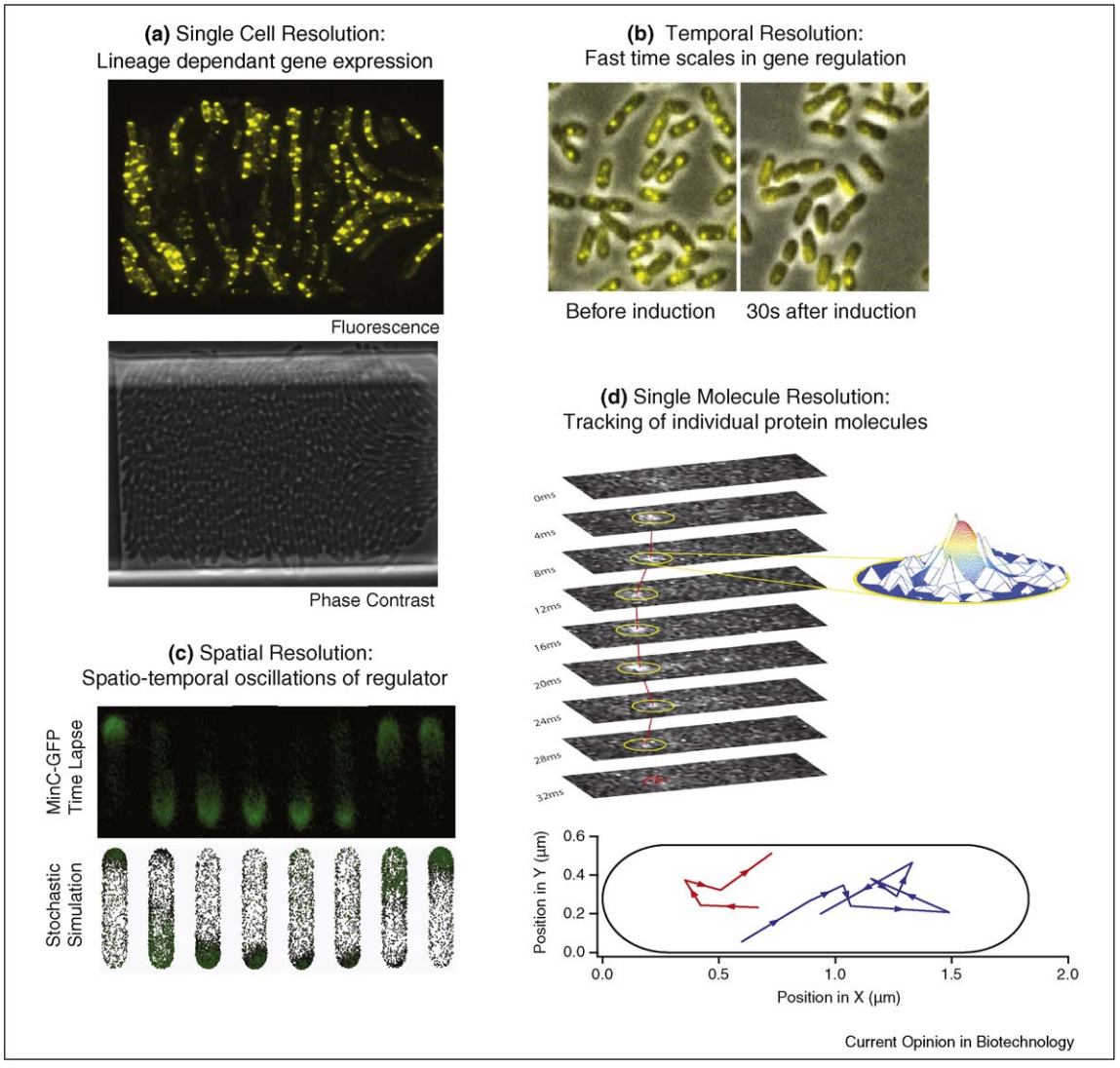
Isogenic cells living under seemingly identical conditions may display considerable heterogeneity in gene expression (Figure 1a). Population-averaged studies mask the cell-to-cell difference and assays with single-cell resolution are necessary to explore for example the reasons and consequences of heterogeneity. There are several different factors that can contribute to the variations between isogenic cells. Firstly, the chemical reactions involved in gene expression are inherently stochastic and will generate diversity [1–3]. Secondly, cells can be in different phases of the cell cycle or some other oscillating regulatory cycle [4]. Even if cells are initially synchronous, stochastic fluctuations will cause them to drift out of synchrony [5,6•]. Thirdly, there are several epigenetic phenomena that will keep lineages with different histories different for generations even when growing under identical conditions [7•,8]. Finally, unicellular organisms may also differ according to their age, as measured in the number of cell divisions they have experienced [9,10].

Gene expression can be studied in single cells using a variety of methods. For example by using flow cytometry the expression levels of fluorescent reporter proteins can be measured in many thousands of individual cells in a short time. Real-time PCR can also be used to quantify endogenous RNA molecules in single cells [11]. If the expression levels approach single copies per cell, methods with higher sensitivity are needed. For example, digital PCR makes absolute quantification of native mRNAs possible [12] and simultaneous quantification and localization of single intracellular mRNAs can be achieved by Fluorescence *in Situ* Hybridization, FISH [13]. Furthermore, expression levels of fluorescent reporter proteins can be quantified down to the level of single molecules in individual living bacterial cells by using sensitive microscopy [14]. This technique was recently used to quantify the genome-wide expression patterns of *Escherichia coli* at single-molecule sensitivity [15•].

Temporal resolution and dynamics

Static information on cell-to-cell variability can be used to infer dynamic properties of transcription based on the stationary distribution of a stochastic model [16,17]. However, important parameters are masked in time averaging and can only be obtained by monitoring cells over time [18,19]. Here, time-lapse fluorescence microscopy of fluorescent protein reporter constructs has proven an exceptionally powerful tool [20,21]. This approach has been used to study dynamic aspects of many regulatory circuits in single cells which may otherwise have been overlooked. For example, Dunlop *et al.* [22•] recently reported on how time delayed correlations in gene expression can be used to

Figure 1



High-resolution fluorescence microscopy of gene expression. **(a)** Heterogeneous and lineage dependent gene expression from isogenic *E. coli* cells. *Top*: expression of fluorescent proteins from the lac operon in response to shift in carbon source (MW and JE, in preparation). *Bottom*: phase contrast image of the same cells. **(b)** Rapid induction of the *lac* operon as studied by dissociation of individual LacI-Venus repressors from the chromosomal binding site. The time-scale of induction would be masked in an assay based on detecting expressed reporter proteins. *Left*: cells without IPTG. *Right*: cells after addition of 100 μM IPTG. (Petter Hammar, in preparation). **(c)** MinCD oscillations in *E. coli*. *Top*: the spatial oscillations of MinC regulates the position of cell division in *E. coli* [47]. *Bottom*: stochastic reaction-diffusion simulation in 3D based on the multivariate master equation [48,49]. **(d)** Single-molecule superresolution tracking of rapidly diffusing proteins in a bacterial cell (English et al. submitted).

infer active regulatory links and separate intrinsic from extrinsic noise. Another stunning example of time-dependent gene regulation is found in a study by Cagatay *et al.*, where they showed how design of competence circuits in *Bacillus subtilis* is under selection for fluctuations in the competence time [23•].

However, the temporal resolution of assays based on expression of fluorescent proteins is limited, mainly by the reporters themselves. The instantaneous expression level of a gene is smeared over a period corresponding to the maturation time of the fluorochrome, which can amount to several hours [24]. One way to improve the time resolution is to use fast-maturing fluorescent proteins, such as Venus, a yellow fluorescent protein variant [25] with a maturation time of around 7 min [18]. Another means of increasing the time resolution is to destabilize the reporter. By including a specific protease degradation tag, such as ssrA, the fraction of early matured proteins can be increased [26]. This is at the expense of the signal strength and also introduces noise from the proteolytic pathway into the signal.

Alternatively, it is possible to monitor pre-matured fluorescent protein reporters as they bind to nascent RNA molecules. This can be achieved by utilizing the affinity that fluorescently labeled MS2 phage coat protein exhibits to a 19 nt RNA hairpin structure which can be included repeatedly in the mRNA studied [19,27]. The time resolution is in this case limited by the association rate of the fluorescent fusion proteins. Higher expression levels of the fluorescent fusion protein will obviously decrease the time for binding, but also increase the fluorescent background such that more molecules will have to bind to detect the signal. The total expression level of the reporter has to be tuned so as to allow fast detection of nascent RNA molecules.

Several transcription factors exist at sufficiently low cellular copy number to enable detection over the background of a single fluorescently tagged protein molecule bound to its chromosomal binding site. This was used by Elf *et al.* [28•] to determine the time it takes for a LacI transcription factor to find and repress its chromosomal operator in *E. coli* and its rate of dissociation after induction. In this case, the time resolution is only limited by the time it takes to distinguish specifically bound from freely diffusing proteins (<100 ms), which is much faster than the conventional indirect assays of gene regulation based on the expression of reporter proteins (Figure 1b).

Spatial resolution and gene regulation

Important information encoded in the intracellular location of transcriptional regulators may also be lost when studying cells at insufficient spatial resolution. For example, in a recent study Cai *et al.* [29•] demonstrated that the transcription factor Crz1 regulates expression of its target genes in response to the extracellular calcium concentration by oscillating between the soma and nucleus in *Saccharomyces cerevisiae* cells in a frequency modulated manner. The frequency modulation ensures that different regulated promoters can respond in proportion to the stimulus despite different binding strengths for the transcription factor. Striking examples of coordinated spatial and temporal oscillations of gene regulatory proteins can also be found in prokaryotic cells, such as in the cell cycle-dependent activities of master regulators [30] and mitotic apparatus [31] in *Caulobacter crescentus*; the regulated relocation of bacterial transcription factors between their chromosomal operators and the inner membrane [32]; as well as the irregular relocations from pole to pole or nucleoid to nucleoid of the Soj protein involved in sporulation and transcriptional regulation in *B. subtilis* [33].

All of these studies have relied on fluorescence microscopy to provide the spatial information. Conventional light microscopy is however diffraction-limited, in the sense that it is not possible to know from where in the sample plane a photon originated with a better resolution than ~200 nm. The dynamics of cellular structures at finer resolutions have therefore remained hidden. However, several methods that allow far-field fluorescence microscopy at higher resolution have recently been devised [34]. For example Shroff *et al.* [35] used Photoactivated Localization Microscopy, PALM, to study dynamics within individual adhesion complexes in living mamalian cells at 60 nm spatial resolution and Huang *et al.* [36] used multicolor three-dimensional stochastic optical reconstruction microscopy, 3D-STORM, to visualize the topology of the mitochondrial network in fixed mammalian kidney cells at a 30 nm resolution. These methods will greatly improve our ability to study cellular structures and nanoscopic dynamics also in prokaryote cells. For example Biteen *et al.* [37] used superresolution time-lapse imaging to characterize the filamentous superstructure of the bacterial actin protein MreB in live *C. crescentus* cells at a resolution of 40 nm and Greenfield *et al.* [38] used PALM to characterize the organization and assembly of *E. coli* chemotaxis Tar receptors in fixed cells at a spatial resolution of 15 nm.

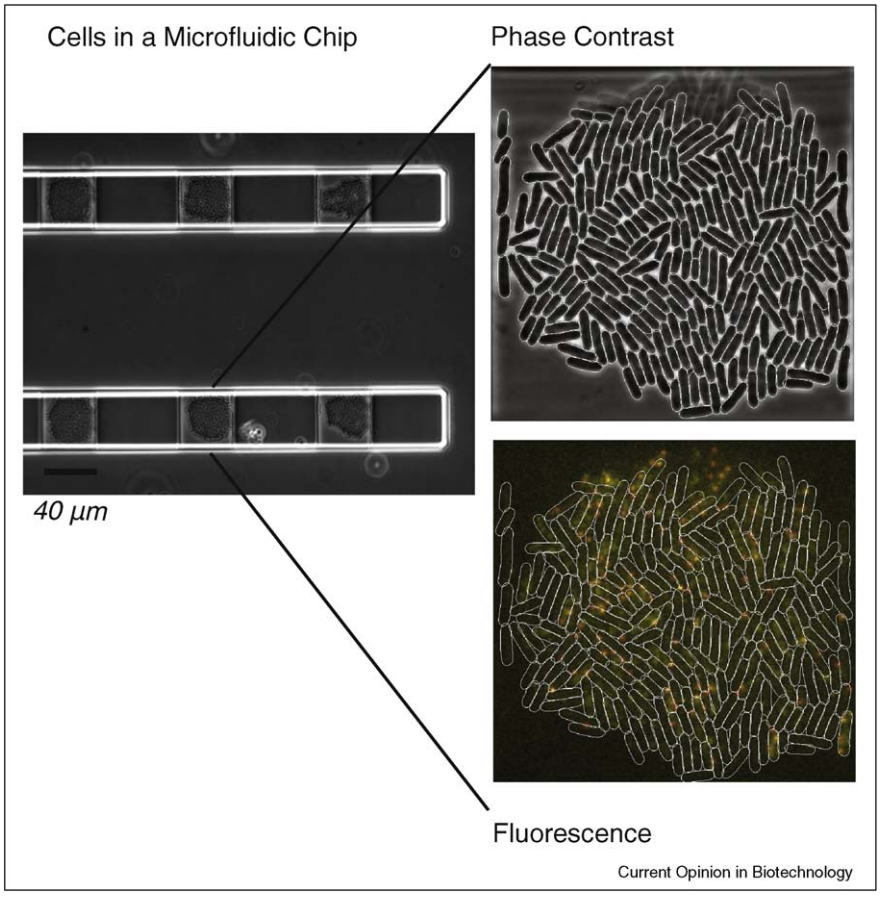
Microfluidics in single-cell microscopy

Three pressing needs arise when performing single-cell gene expression studies: firstly observations of many cells and molecules have to be accumulated in order to draw significant conclusions, secondly the cells have to be in a representative physiological state and thirdly the chemical environment must be well-defined and preferably rapidly interchangeable. Recent advances in micro-fabrication have realized microfluidic devices capable of alleviating these needs for single-cell fluorescence microscopy [39,40] (Figure 2).

In order to keep microbial cells in constant good health during experiments, several micro-chemostats have been designed. For example Danino *et al.* [41•] devised chips trapping a monolayer of bacterial cells in the focal plane. A constant occupancy of each trap was maintained as cells were released as they grew out of the trap.

The microscopic length scale characteristic of these devices shifts the properties of the fluids into a regime in which little or no turbulent mixing occurs. This allows for predictable and automatable on-chip composition of the cells chemical environment as well as its rapid exchange, increasing both the time and inductive resolution at which gene expression can be studied [6•]. Most importantly, trapping regions are easily multiplexed on a chip, allowing a massive parallelization of a single experiment [15]. Combined with the indefinitely prolonged times that cells can be maintained and imaged [50] results in data sets, the magnitude of which can dramatically increase confidence in the conclusions drawn, even for rare events [42] and better merits the comparison with bulk experiments.

Figure 2



E. coli cells in a microfluidic device. *Right*: parallel trapping regions each containing 200–300 cells imaged with phase contrast. *Top left*: a single trap imaged with phase contrast allowing segmentation and tracking of individual cells. *Bottom left*: fluorescence image of membrane-bound Venus molecules resulting from leakage expression from the lac operon (MW and JE, in preparation).

Resolving Individual molecular states

So far we have described phenomena that may be masked in experiments lacking resolution of single cells, in time or space. However, since macromolecules with the same primary sequences, that is isoform molecules, can be in different states of activity, conformation or binding, studying only the total molecule concentration at some intracellular location may obscure important biological information. Furthermore, the average properties of a molecular ensemble can only reveal kinetics of interconversion between such states if it is first perturbed out of steady state. The situation is different if the state transitions of single molecules can be traced directly. In this case the chemical rates can be determined also at steady state without the need to synchronize the ensemble of molecules, which is often not possible *in vivo*.

In vitro, very powerful single-molecule assays based on for example Förster Resonance Energy Transfer, FRET [43] or fluorogenic product reactions have been reported [44]. Unfortunately, many such schemes remain difficult to realize *in vivo*, mainly because of high background autofluorescence, and in the case of FRET photo-instability of genetically encoded fluorescent reporters. One way to differentiate bound from free molecules in live cells would be to study their individual diffusion trajectories, as obtained by single-molecule tracking of fluorescent fusion proteins [51]. Inside living cells it has however proven a challenge to collect a sufficient number of photons from individual rapidly moving fluorescent proteins to pinpoint their positions. This has previously limited the range of possible targets to slowly diffusing molecules, such as membrane-associated proteins. For example, Niu and Yu [45] studied FtsZ labeled with the photo-convertible red fluorescent protein Dendra2b, and characterized the differences between two FtsZ subpopulations in *E. coli*.

By combining superresolution localization of individual fluorescently labeled proteins with stroboscopic laser excitation it is also possible to capture the movement of individual transcription factor molecules searching for their specific binding sites [28•]. Recently this method

was improved such that it is possible to track individual rapidly moving fluorescent proteins in *E. coli* (Figure 1d) (English *et al.*, in preparation). This capability suggests that it is also possible to track fluorescent fusion proteins diffusing through the cytoplasm in free or bound states. By monitoring the transitions between such states of different diffusivities it will be possible to acquire binding and dissociation rates for proteins in living cells.

Conclusions

Currently, gene expression and gene regulation can be studied in individual cells, and population-averaged models are now being understood from the perspective of individual contributions from heterogeneous ensembles of isogenic cells. Increased spatiotemporal resolution has enabled the correlation of expression to the diverse and dynamic states of individual regulator molecules. Furthermore, the chemical control and parallelization possible in microfluidic devices afford the exhaustive exploration of inductive responses as well as confidence in the results.

One of the challenges for the immediate future is to adapt these high-precision methods to the study of eukaryotic cells. Here the large volume and high auto fluorescent background make detection, accurate counting and fast tracking of single fluorescent proteins difficult, but probably not impossible. Also, more powerful schemes to interrogate the state of activity of individual molecules in living cells are needed. To date, single-molecule tracking can be used to measure binding kinetics and FRET to determine conformational changes *in vitro*, but the *in vivo* implementations of both methods remain limited. Brighter and more photo-stabile fluorophores, as well as improved non-perturbative and specific labeling schemes may be a prerequisite and the search for both continues [52].

Another important challenge is to find ways to measure the dynamics of individual metabolites in single cells as they often act as primary signals for gene regulatory systems. Beyond this, metabolite concentrations are often the entity which the cell aims to regulate and a direct readout of the investment in metabolite pools may therefore be more revealing than that of the enzymes producing them [53]. Although some fast and bright FRET probes have been developed to this end [54], a wider range of probes and targets is needed.

Finally, the large amount of hi-resolution data available from single-cell experiments may be manageable only by automated analysis tools and intelligible only within the framework of correspondingly detailed quantitative models. New challenges therefore arise in how to automate the analysis of complex data as well as in how to model and simulate intracellular processes at the right level of detail to capture their central properties [55]. We should however remember that the key details may be hidden at spatial and temporal scales that we do not yet have the tools to explore, in which case quantitative modeling should point the direction to what remains to be discovered.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

The authors would like to thank Gustaf Ullman, Gene-Wei Li and Petter Hammar for their contribution to this review and Prof. Jeff Hasty for help in constructing the μ-fluidic devises. This work was funded by the ERC (no 203083), the Swedish Research Council and the Knut and Alice Wallenberg Foundation.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest

1. Berg OG: **A model for the statistical fluctuations of protein numbers in a microbial population**. *J Theor Biol* 1978, **71**:587-603.

2. Elowitz MB, Levine AJ, Siggia ED, Swain PS: **Stochastic gene expression in a single cell**. *Science* 2002, **297**:1183-1186.

3. Mettetal JT, Muzzey D, Pedraza JM, Ozbudak EM, van Oudenaarden A: **Predicting stochastic gene expression dynamics in single cells**. *Proc Natl Acad Sci USA* 2006, **103**:7304-7309.

4. Laub MT, McAdams HH, Feldblyum T, Fraser CM, Shapiro L: **Global analysis of the genetic network controlling a bacterial cell cycle**. *Science* 2000, **290**:2144-2148.

5. Elowitz MB, Leibler S: **A synthetic oscillatory network of transcriptional regulators**. *Nature* 2000, **403**:335-338.

6. • Stricker J, Cookson S, Bennett MR, Mather WH, Tsimring LS, Hasty J: **A fast, robust and tunable synthetic gene oscillator**. *Nature* 2008, **456**:516-519.
Provides insight in how the expression from synthetic circuits can be studied in single cells and differentially induced in a microfluidic device.

7. • Robert L, Paul G, Chen Y, Taddei F, Baigl D, Lindner AB: **Predispositions and epigenetic inheritance in the Escherichia coli lactose operon bistable switch**. *Mol Syst Biol* 2010, **6**:357.
Illustrates how native genes can be monitored for expression over many generations of cells in a microfluidic device and correlated to specific regulatory events in each lineage.

8. Choi PJ, Cai L, Frieda K, Xie XS: **A stochastic single-molecule event triggers phenotype switching of a bacterial cell**. *Science* 2008, **322**:442-446.

9. Stewart EJ, Madden R, Paul G, Taddei F: **Aging and death in an organism that reproduces by morphologically symmetric division**. *PLoS Biol* 2005, **3**:e45.

10. Barker MG, Walmsley RM: **Replicative ageing in the fission yeast Schizosaccharomyces pombe**. *Yeast* 1999, **15**:1511-1518.

11. Bengtsson M, Stahlberg A, Rorsman P, Kubista M: **Gene expression profiling in single cells from the pancreatic islets of Langerhans reveals lognormal distribution of mRNA levels**. *Genome Res* 2005, **15**:1388-1392.

12. Warren L, Bryder D, Weissman IL, Quake SR: **Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR**. *Proc Natl Acad Sci USA* 2006, **103**:17807-17812.

13. Femino AM, Fay FS, Fogarty K, Singer RH: **Visualization of single RNA transcripts in situ**. *Science* 1998, **280**:585-590.

14. Xiao J, Elf J, Li G-W, Yu J, Xie X: **Imaging gene expression in living cells at the single-molecule level**. In *Single Molecule Techniques A Laboratory Manual*. Edited by Selvin P, Ha T. Cold Spring Harbor Laboratory Press; 2008.

15. Taniguchi Y, Choi PJ, Li GW, Chen H, Babu M, Hearn J, Emili A, Xie XS: **Quantifying E. coli proteome and transcriptome with single-molecule sensitivity in single cells**. *Science* 2010, **329**:533-538.

Genome-wide gene expression levels at single-molecule sensitivity.

16. Friedman N, Cai L, Xie XS: **Linking stochastic dynamics to population distribution: an analytical framework of gene expression**. *Phys Rev Lett* 2006, **97**:168302.

17. Paulsson J: **Summing up the noise in gene networks**. *Nature* 2004, **427**:415-418.

18. Yu J, Xiao J, Ren X, Lao K, Xie XS: **Probing gene expression in live cells, one protein molecule at a time**. *Science* 2006, **311**:1600-1603.

19. Golding I, Paulsson J, Zawilski SM, Cox EC: **Real-time kinetics of gene activity in individual bacteria**. *Cell* 2005, **123**: 1025-1036.

20. Pedraza JM, van Oudenaarden A: **Noise propagation in gene networks**. *Science* 2005, **307**:1965-1969.

21. Rosenfeld N, Young JW, Alon U, Swain PS, Elowitz MB: **Gene regulation at the single-cell level**. *Science* 2005, **307**:1962-1965.

22. Dunlop MJ, Cox RS III, Levine JH, Murray RM, Elowitz MB: **Regulatory activity revealed by dynamic correlations in gene expression noise**. *Nat Genet* 2008, **40**:1493-1498.

Shows how dynamic correlations can be used to infer activity in regulatory links.

23. Cagatay T, Turcotte M, Elowitz MB, Garcia-Ojalvo J, Suel GM: **Architecture-dependent noise discriminates functionally analogous differentiation circuits**. *Cell* 2009, **139**:512-522.

Shows how selection of noise distributions may be needed to understand evolution of biological circuits.

24. Dong GQ, McMillen DR: **Effects of protein maturation on the noise in gene expression**. *Phys Rev E Stat Nonlin Soft Matter Phys* 2008, **77**:021908.

25. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A: **A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications**. *Nat Biotechnol* 2002, **20**:87-90.

26. Andersen JB, Sternberg C, Poulsen LK, Bjorn SP, Givskov M, Molin S: **New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria**. *Appl Environ Microbiol* 1998, **64**:2240-2246.

27. Fusco D, Accornero N, Lavoie B, Shenoy SM, Blanchard JM, Singer RH, Bertrand E: **Single mRNA molecules demonstrate probabilistic movement in living mammalian cells**. *Curr Biol* 2003, **13**:161-167.

28. Elf J, Li GW, Xie XS: **Probing transcription factor dynamics at the single-molecule level in a living cell**. *Science* 2007, **316**:1191-1194.

Shows how single-molecule imaging can be used to study gene regulation directly.

29. Cai L, Dalal CK, Elowitz MB: **Frequency-modulated nuclear localization bursts coordinate gene regulation**. *Nature* 2008, **455**:485-490.

Illustrates the need for spatial resolution understand biological phenomena.

30. Collier J, Shapiro L: **Spatial complexity and control of a bacterial cell cycle**. *Curr Opin Biotechnol* 2007, **18**:333-340.

31. Ptacin JL, Lee SF, Garner EC, Toro E, Eckart M, Comolli LR, Moerner WE, Shapiro L: **A spindle-like apparatus guides bacterial chromosome segregation**. *Nat Cell Biol* 2010, **12**:791-798.

32. Bohm A, Boos W: **Gene regulation in prokaryotes by subcellular relocalization of transcription factors**. *Curr Opin Microbiol* 2004, **7**:151-156.

33. Doubrovinski K, Howard M: **Stochastic model for Soj relocation dynamics in Bacillus subtilis**. *Proc Natl Acad Sci USA* 2005, **102**:9808-9813.

34. Hell SW: **Far-field optical nanoscopy**. *Science* 2007, **316**: 1153-1158.

35. Shroff H, Galbraith CG, Galbraith JA, Betzig E: **Live-cell photoactivated localization microscopy of nanoscale adhesion dynamics**. *Nat Methods* 2008, **5**:417-423.

36. Huang B, Jones SA, Brandenburg B, Zhuang X: **Whole-cell 3D STORM reveals interactions between cellular structures with nanometer-scale resolution**. *Nat Methods* 2008, **5**:1047-1052.

37. Biteen JS, Thompson MA, Tselentis NK, Bowman GR, Shapiro L, Moerner WE: **Super-resolution imaging in live Caulobacter crescentus cells using photoswitchable EYFP**. *Nat Methods* 2008, **5**:947-949.

38. Greenfield D, McEvoy AL, Shroff H, Crooks GE, Wingreen NS, Betzig E, Liphardt J: **Self-organization of the Escherichia coli chemotaxis network imaged with super-resolution light microscopy**. *PLoS Biol* 2009, **7**:e1000137.

39. Weibel DB, Diluzio WR, Whitesides GM: **Microfabrication meets microbiology**. *Nat Rev Microbiol* 2007, **5**:209-218.

40. Breslauer DN, Lee PJ, Lee LP: **Microfluidics-based systems biology**. *Mol Biosyst* 2006, **2**:97-112.

41. Danino T, Mondragon-Palomino O, Tsimring L, Hasty J: **A synchronized quorum of genetic clocks**. *Nature* 2010, **463**:326-330.

Contains interesting design principles for genetic circuitry as well as microfluidic devices for maintaining cells for prolonged times.

42. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S: **Bacterial persistence as a phenotypic switch**. *Science* 2004, **305**:1622-1625.

43. Roy R, Hohng S, Ha T: **A practical guide to single-molecule FRET**. *Nat Methods* 2008, **5**:507-516.

44. English BP, Min W, van Oijen AM, Lee KT, Luo G, Sun H, Cherayil BJ, Kou SC, Xie XS: **Ever-fluctuating single enzyme molecules: Michaelis–Menten equation revisited**. *Nat Chem Biol* 2006, **2**:87-94.

45. Niu L, Yu J: **Investigating intracellular dynamics of FtsZ cytoskeleton with photoactivation single-molecule tracking**. *Biophys J* 2008, **95**:2009-2016.

47. Meinhardt H, de Boer PA: **Pattern formation in Escherichia coli: a model for the pole-to-pole oscillations of Min proteins and the localization of the division site**. *Proc Natl Acad Sci USA* 2001, **98**:14202-14207.

48. Hattne J, Fange D, Elf J: **Stochastic reaction-diffusion simulation with MesoRD**. *Bioinformatics* 2005, **21**:2923-2924.

49. Fange D, Elf J: **Noise-induced Min phenotypes in E. coli**. *PLoS Comput Biol* 2006, **2**:e80.

50. Balagaddé FK, You L, Hansen CL, Arnold FH, Quake SR: **Long-term monitoring of bacteria undergoing programmed population control in a microchemostat**. *Science* 2005, **309**:137-140.

51. Manley S, Gillette JM, Patterson GH, Shroff H, Hess HF, Betzig E, Lippincott-Schwartz J: **High-density mapping of single-molecule trajectories with photoactivated localization microscopy**. *Nat Methods* 2008, **5**:155-157.

52. Uttamapinant C, White KA, Baruah H, Thompson S, Fernández-Suárez M, Puthenveetil S, Ting AY: **A fluorophore ligase for site-specific protein labeling inside living cells**. *Proc Natl Acad Sci USA* 2010, **107**:10914-10919.

53. Elf J, Ehrenberg M: **Near-critical behavior of aminoacyl-tRNA pools in E. coli at rate-limiting supply of amino acids**. *Biophys J* 2005, **88**:132-146.

54. Horikawa K, Yamada Y, Matsuda T, Kobayashi K, Hashimoto M, Matsu-ura T, Miyawaki A, Michikawa T, Mikoshiba K, Nagai T: **Spontaneous network activity visualized by ultrasensitive Ca(2+) indicators, yellow Cameleon-Nano**. *Nat Methods* 2010, **7**:729-732.

55. David Fange, Otto Berg, Paul Sjöberg, Johan Elf: **Stochastic reaction-diffusion kinetics in the microscopic limit**. *Proc Natl Acad Sci* 2010, doi:10.1073/pnas.1006565107.