Macromolecular crowding creates heterogeneous environments of gene expression in picolitre droplets

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Understanding the dynamics of complex enzymatic reactions in highly crowded small volumes is crucial for the development of synthetic minimal cells. Compartmentalized biochemical reactions in cell-sized containers exhibit a degree of randomness due to the small number of molecules involved. However, it is unknown how the physical environment contributes to the stochastic nature of multistep enzymatic processes. Here, we present a robust method to quantify gene expression noise in vitro using droplet microfluidics. We study the changes in stochasticity in the cell-free gene expression of two genes compartmentalized within droplets as a function of DNA copy number and macromolecular crowding. We find that decreased diffusion caused by a crowded environment leads to the spontaneous formation of heterogeneous microenvironments of mRNA as local production rates exceed the diffusion rates of macromolecules. This heterogeneity leads to a higher probability of the molecular machinery staying in the same microenvironment, directly increasing the system’s stochasticity.

Noise is present in all living cells. It has been studied in prokaryotes and eukaryotes\(^1\), as well as stem\(^2,3\) and cancer cells\(^4\), and cells expressing viruses\(^5\). Gene expression is a key example of a complex stochastic enzymatic process. Careful analysis of variations in mRNA and protein levels has revealed the importance of both the amplitude and typical decay time of noise and the ability of cells to exploit or suppress noise in gene expression\(^6,9\). Unlike deterministic models of gene expression, which are used to predict dynamics over large populations, stochastic models can correctly predict the dynamics of gene expression at the single cell level\(^10\). Recently, it has become apparent that the stochastic nature of many biochemical processes cannot be ignored in vivo and in vitro. The two-reporter system developed by Elowitz and colleagues\(^12\) as a reliable method to estimate \(^12\)the magnitude of variation in gene expression has been used to study noise both in vivo and in vitro\(^12–14\). Because reaction networks inside the cell involve numerous components, each at a very low concentration, an important degree of randomness is expected\(^15–18\). It is generally accepted that this stochasticity is due to both the low concentrations of reacting molecules and the random nature of molecular collisions due to diffusion. However, this inherent stochastic nature of chemical reactions is typically ignored when studying chemical reactions in dilute, well-stirred reactors. In contrast, the cell’s interior is an inhomogeneous, crowded environment. In bacteria, for example, ~30% of the cell volume is occupied by macromolecules, resulting in highly reduced diffusion\(^19,20\). Such a crowded environment can lead to spontaneous spatial organization due to the severely limited diffusion of mRNA molecules\(^21\). Moreover, macromolecular crowding affects the dynamics and thus reaction rates of cellular processes\(^22\). Further studies have shown that prokaryotic cells are less fluid-like than originally anticipated and can exhibit dynamical heterogeneity and glassy features\(^23,24\). Most studies thus far have dealt with either the quantification of noise\(^25\), or how cells exploit or suppress noise\(^1,26\). It has previously been shown in silico\(^27\) that diffusivity plays a role in gene expression noise, but no experimental work has estimated the magnitude of the effect of cellular composition or crowded environment within a cell-sized compartment on the stochasticity of biochemical reactions.

To quantify the various contributions to noise in in vitro gene expression, we have studied the transcription and translation of cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in picolitre droplets\(^14,28,29\). Picolitre droplets are ideally suited for the study of biochemical reactions involving very small numbers of reactants\(^30,31\). The microfluidic approach allows for precise control over droplet volume, producing a large number of monodisperse water-in-oil droplets at rates up to 500 droplets per second (Supplementary Fig. 1). The large number of identical droplets provides high reproducibility. We can therefore measure stochasticity in gene expression as a function of DNA copy number and macromolecular crowding. Our results emphasize the complex interplay between the cellular environment and the dynamics of cellular processes.

Effect of copy number on uncorrelated noise

One typically distinguishes two types of noise: extrinsic and intrinsic\(^11\). When comparing the expression of two identical yet independent genes in vivo, fluctuations in the amounts or states of cellular material lead to correlated fluctuations in the expression of both proteins, and this is considered intrinsic noise. On the other hand, the stochasticity of a biochemical process or other factors leading to uncorrelated fluctuations in the numbers of either protein is considered intrinsic noise. In line with these explanations, but to avoid confusion with other in vivo experiments and taking into account our experimental set-up, we make the distinction between uncorrelated and correlated noise (Fig. 1a,b)\(^8,32,33\). Correlated noise arises from the inhomogeneous Poisson

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Thus following the expression of both proteins per droplet, we can see the extent to which the output of two reactions in the same confined space differs, and is calculated as the normalized root-mean-square distance from the line CFP = YFP. Various physical and biochemical factors contribute to the uncorrelated noise in our experiments (Fig. 1c). First, as a result of our experimental design using plasmids, our uncorrelated noise is influenced by differences in the relative Poisson distributions of the CFP and YFP plasmids. This contribution is not present in the original approach taken by Elowitz and co-workers, and the uncorrelated noise we discuss here should therefore not be confused with their intrinsic noise. Second, uncorrelated noise arises from the randomness inherent to biochemical reactions and increases with decreasing numbers of reacting molecules. Third, there is a possible contribution from differences in the maturation times of CFP and YFP, and the effect of a crowded environment with limited diffusion.

By measuring the fluorescence intensity of YFP (pRSET-YFP) and CFP (pRSET-CFP) (Supplementary Fig. 2) for a population of 300 droplets every 10 min (Fig. 2a, Supplementary Fig. 3) and thus following the expression of both proteins per droplet, we can calculate the time evolution of uncorrelated, correlated and total noise in our system (Fig. 2b). There is a constant increase in protein concentrations because there is no observable protein degradation (Supplementary Fig. 4). Figure 2b shows that the noise levels decrease over time, which is in agreement with the observed increase in protein concentration over time (see Supplementary Fig. 5 for all expression and noise curves). We first varied the DNA concentration from 16,000 copies to 100 copies per droplet for both the CFP and YFP plasmids, and the concentrations of all other components were kept constant throughout the experiments. Plotting the normalized fluorescence intensities of CFP and YFP in each droplet with either high (16,000) or low (190) plasmid copy numbers 100 min after the start of fluorescence increase (Fig. 2c), we can clearly observe a significant increase in uncorrelated noise (the spread of data points orthogonal to the axis CFP = YFP) as the copy number decreases. These experiments were repeated for a wide range of initial plasmid concentrations. The correlated noise, that is, the distribution of components over the droplets decreases over time, which is in agreement with the observed increase in protein concentration over time (Supplementary Fig. 4). Figure 2b shows that the noise levels decrease over time, which is in agreement with the observed increase in protein concentration over time (see Supplementary Fig. 5 for all expression and noise curves). We first varied the DNA concentration from 16,000 copies to 100 copies per droplet for both the CFP and YFP plasmids, and the concentrations of all other components were kept constant throughout the experiments. Plotting the normalized fluorescence intensities of CFP and YFP in each droplet with either high (16,000) or low (190) plasmid copy numbers 100 min after the start of fluorescence increase (Fig. 2c), we can clearly observe a significant increase in uncorrelated noise (the spread of data points orthogonal to the axis CFP = YFP) as the copy number decreases. These experiments were repeated for a wide range of initial plasmid concentrations. The correlated noise, that is, the distribution of components over the droplets decreases over time, which is in agreement with the observed increase in protein concentration over time (Fig. 2c, red filled squares), which can be due to the gene expression becoming increasingly stochastic with a lower number of molecules involved. This is in line with the theory of stochasticity, which states that the relative importance of the fluctuations involved increases as the reactant number decreases. This trend is also visible 10, 30 and 50 min after the start of expression (Fig. 2e and Supplementary Fig. 7), indicating that it is independent of the moment the image was taken. We chose to calculate the noise...
Macromolecular crowding enhances uncorrelated noise

After exploring the range of copy numbers that can be studied reliably, we added Ficoll 70, a common macromolecular crowding agent, to mimic the crowded conditions inside cells.34,35 Remarkably, the addition of 90 mg ml\(^{-1}\) Ficoll led to strikingly different production levels for CFP and YFP over the population of droplets (that is, the uncorrelated noise increased significantly) (Fig. 3a,b and Supplementary Fig. 9).

We performed cell-free gene expression in the presence of 40, 70 and 90 mg ml\(^{-1}\) Ficoll for a range of DNA concentrations and compared the uncorrelated noise values to results from experiments using 0 mg ml\(^{-1}\) Ficoll (Fig. 3c and Supplementary Fig. 10). Cell-free gene expression in the presence of 40 mg ml\(^{-1}\) Ficoll shows similar levels of uncorrelated noise as in the absence of Ficoll (Fig. 3d), yet the results for both 70 mg ml\(^{-1}\) (Fig. 3e) and 90 mg ml\(^{-1}\) (Fig. 3f) Ficoll show enhancement of uncorrelated noise. At ~600 copies of each plasmid, uncorrelated noise values are 0.11, 0.10, 0.19 and 0.21 for 0, 40, 70 and 90 mg ml\(^{-1}\) Ficoll, respectively.

The average protein expression rates (Supplementary Methods) in droplets following the addition of Ficoll are comparable to droplets without Ficoll (Fig. 4a and Supplementary Fig. 11), confirming that the increase in uncorrelated noise with increasing Ficoll concentrations is not due to lower protein production. Furthermore, the uncorrelated noise at the same protein concentration shows the same trend for all Ficoll concentrations (Supplementary Fig. 12). To understand how crowding leads to enhanced levels of uncorrelated noise in cell-free protein expression we probed the influence of the physical environment on molecular processes. We used fluorescent recovery after photo-bleaching (FRAP) to determine the diffusion coefficient of Alexa 647-labelled 70S ribosomes (Supplementary Fig. 13 and Supplementary Methods). As expected, the diffusion coefficients decrease as the Ficoll concentration is increased (Fig. 4b). We find a diffusion coefficient of \(4.7 \pm 0.215 \mu m^2 s^{-1}\) in the absence of Ficoll and \(0.4 \pm 0.001 \mu m^2 s^{-1}\) in the presence of 90 mg ml\(^{-1}\) Ficoll.

Noting these significantly lower diffusion constants for ribosomes in crowded solutions, we decided to study the spatial distribution of mRNA. Limited diffusion induced by macromolecular crowding could potentially hinder the homogeneous distribution of \textit{in situ} synthesized mRNA molecules31,36, thereby increasing the heterogeneity and consequently the uncorrelated noise. To investigate the distribution and localization of the mRNA in crowded and dilute solutions, we studied an \textit{in vitro} transcription-only system using a DNA sequence encoding for a 32-repeat sequence and dilute solutions, we studied an \textit{in vitro} transcription-only system using a DNA sequence encoding for a 32-repeat sequence.

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they fall below our signal-to-noise threshold. The spots have a constant average fluorescence intensity over time (Fig. 5c), indicating that there is equilibrium between the production and dissipation of mRNA. However, the fluorescence intensity of the whole droplet increases for the first 50 min, which supports the argument that mRNA is constantly dissipating from its production point. As spots start disappearing after 50 min, the average intensity of the droplet decreases, and we verified that they did not result from aggregation of mRNA and a molecular beacon induced by Ficoll (Supplementary Fig. 15). These results imply that the spots are local production sites of mRNA. The spots were absent when no DNA was added, and we verified that they did not result from aggregation of mRNA and a molecular beacon induced by Ficoll (Supplementary Fig. 16). In other words, transcription in crowded droplets leads to a heterogeneous distribution of mRNA molecules over the time course of mRNA expression, similar to the limited diffusion of mRNA molecules observed in Escherichia coli cells. The formation of high local concentrations of mRNA can be explained by an imbalance in production rates and diffusion rates, the latter dropping significantly in crowded solutions (Fig. 4b). We note that the inhomogeneous production of proteins cannot be observed, as they will have distributed homogeneously over the droplet volume before their fluorophores matured.

**Crowding causes microenvironment formation**

To understand under what conditions the mRNA will be distributed heterogeneously in a coupled transcription translation reaction—in other words, at what point the local synthesis rate of mRNA exceeds the local diffusion rate of mRNA (Fig. 6a)—we calculated the time it...
would take for one mRNA to be produced (Fig. 6b, black line) using Michaelis–Menten kinetics with a typical $K_m$ of 0.5 nM and $V_{max}$ of 0.1 nM min$^{-1}$ (derived experimentally, Supplementary Fig. 17). Newly produced mRNA becomes bound by ribosomes; multiple ribosomes interacting with one mRNA can associate into a polysome, and diffusion coefficients for these polysomal complexes were estimated using the Stokes–Einstein equation (Supplementary Fig. 18). We assume that the plasmids are distributed homogeneously (Supplementary Fig. 19) with an average distance of $d \sim 4 \mu$m. We thus calculated the average rate (in s$^{-1}$) for a polysome to diffuse over a distance $d/2$ (Fig. 6b, red line). We found that below 50 mg ml$^{-1}$ Ficoll, the diffusion is typically higher than mRNA production, resulting in no localization, and above 50 mg ml$^{-1}$ the opposite is the case, resulting in localization. Not much is known about the exact mechanism of localized transcription and translation in bacterial cells, although there has been much speculation that slower diffusion plays a role$^{21,38}$. Our results indicate that the decreased diffusion caused by molecular crowding could indeed play a prominent role in localized gene expression.

To estimate the relative contribution of the different factors influencing uncorrelated noise, we simulated the stochastic cell-free gene expression in 200 droplets using Gillespie’s Direct Method algorithm (Supplementary Methods and Fig. 6c). Again, we used the same $K_m$ and $V_{max}$ values for transcription previously established. For translation, a $K_m$ value from Stögbauer and co-workers was used$^{39}$, and $V_{max}$ was estimated from our experimental results. We measured protein maturation times (Supplementary Fig. 20) at different Ficoll concentrations and included these in the model. The uncorrelated noise versus plasmid copy number was plotted by taking the uncorrelated noise values after 100 min. The transcription and translation were first simulated with stochasticity as the only contributing factor, thereby excluding the Poisson distribution of plasmids, protein maturation and crowding. Later, the other factors were included in the model one by one to determine the contribution of each factor separately (Fig. 6d and Supplementary Fig. 21). The crowded droplets were simulated using a 10 times higher probability for a ribosome to rebind to the same mRNA (denoted p.n-1). An enhanced rebinding probability is supported by the observation and explanation of mRNA localization discussed above. The translationally active machinery must be co-localized with the mRNA, and this creates local micro-environments with higher concentrations of biologically active transcription and translation machinery, that is, the accumulation of ribosomes at plasmids. Owing to the lower diffusivity, this machinery has a higher probability of rebinding than anticipated from a homogeneous distribution of all components.

The results from the stochastic simulation show a decrease in uncorrelated noise with increasing copy number, which is consistent with our experimental data. Importantly, the model also shows that the trend of uncorrelated noise over plasmid copy number is not due to population differences in mean (CFP and YFP concentration) over the range of DNA copy numbers, which is also consistent with our experimental findings (Supplementary Figs 12 and 22). We find that the CFP and YFP maturation times have almost no effect on uncorrelated noise (mean of 1% over the plasmid copy numbers), while the average contributions over the plasmid copy numbers of stochasticity, Poisson distributions of plasmids and crowding are 18, 40 and 41%, respectively (Supplementary Fig. 20).

Conclusions

By studying gene expression in picolitre droplets, we can reliably analyse the uncorrelated and correlated noise of protein expression of low copy numbers of DNA under different physical conditions. Surprisingly, we find that an increase in macromolecular crowding, leading to an order-of-magnitude decrease in the diffusion coefficients of RNA and proteins, leads to significantly enhanced uncorrelated noise. At the same time, we observe that mRNA becomes distributed heterogeneously over the droplet, creating local micro-environments where gene expression occurs. Owing to the formation of large polysomes, biologically active machinery will be less likely to...
diffuse away from these microenvironments. This lack of diffusion maintains the heterogeneous environment and enhances any already existing stochasticity caused by transcription and translation of low copies of DNA. Theoretical modelling strongly supports this theory, showing that the heterogeneous display of mRNA is caused by a fine balance between mRNA production rates and diffusion times. Furthermore, the results of the stochastic simulations suggest that any existing bias towards one of the two fluorescent proteins is strongly enhanced by preferential rebinding of ribosomes to the same mRNA, which we believe is the cause of our observed enhanced uncorrelated noise in crowded droplets.

These results are the first to show that the stochasticity of biochemical reactions is governed by the interplay between the rate of the reaction and its environment. Our experimental finding of heterogeneous mRNA distributions in crowded in vitro transcription systems and the concomitant increase in uncorrelated noise in similarly crowded cell-free expression systems has important implications for our understanding of living cells. It is very much conceivable that the synthesis of macromolecules (mRNA and proteins) in vivo leads to locally heterogeneous systems, as production rates will often be larger than diffusion rates\(^1\). This might explain the findings in the literature on the localization of mRNA in *E. coli*\(^2,3\), but also helps to explain the origin of experimentally determined uncorrelated noise in gene expression\(^4\). Finally, our experiments enable us not only to take into account, but also predict the magnitude of stochasticity when designing synthetic chemical pathways similar to artificial cell-like systems.

Methods

Methods and any associated references are available in the online version of the paper.

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References

1. Maamar, H., Raj, A. & Dubnau, D. Noise in gene expression determines cell fate in *Bacillus subtilis*. *Science* **317**, 526–529 (2007).
2. Chang, H. H., Hemberg, M., Barahona, M., Ingber, D. E. & Huang, S. Transcriptome-wide noise controls lineage choice in mammalian progenitor cells. *Nature* **453**, 544–547 (2008).
3. Graf, T. & Stadtfeld, M. Heterogeneity of embryonic and adult stem cells. *Cell Stem Cell* **3**, 480–483 (2008).
4. Gupta, P. B. *et al.* Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. *Cell* **146**, 633–644 (2011).
5. Weinberger, I. S., Burnett, J. C., Toeticher, J. E., Arkin, A. P. & Schaffer, D. V. Stochastic gene expression in a lentiviral positive-feedback loop: HIV-1 Tat fluctuations drive phenotypic diversity. *Cell* **122**, 169–182 (2005).
6. Yu, J., Xiao, J., Ren, X., Lao, K. & Xie, X. S. Probing gene expression in live cells, one protein molecule at a time. *Science* **311**, 1600–1603 (2006).
7. Hensel, L. *et al.* Stochastic expression dynamics of a transcription factor revealed by single-molecule noise analysis. *Nature Struct. Mol. Biol.* **19**, 797–802 (2012).
8. Munksky, B., Neuert, G. & van Oudenaarden, A. Using gene expression noise to understand gene regulation. *Science* **336**, 183–187 (2012).
9. Pedraza, J. M. & Paulsson, J. Effects of molecular memory and bursting on fluctuations in gene expression. *Science* **319**, 339–343 (2008).
10. Mettetal, J. T., Muzzey, D., Pedraza, J. M., Ozbudak, E. M. & van Oudenaarden, A. Predicting stochastic gene expression dynamics in single cells. *Proc Natl Acad. Sci. USA* **103**, 7304–7309 (2006).
11. Elowitz, M. B., Levine, A. J., Siggia, E. D. & Swain, P. S. Stochastic gene expression in a single cell. *Science* **297**, 1183–1186 (2002).
12. Hilfinger, A. & Paulsson, J. Separating intrinsic from extrinsic fluctuations in dynamic biological systems. Proc. Natl Acad. Sci. USA 108, 12167–12172 (2011).

13. Raser, J. M. & O’Shea, E. K. Control of stochasticity in eukaryotic gene expression. Science 304, 1811–1814 (2004).

14. Nishimura, K., Tsuru, S., Suzuki, H. & Yomo, T. Stochasticity in gene expression in a cell-sized compartment. ACS Synth. Biol. 4, 566–576 (2015).

15. Shahrezaei, V. & Swain, P. S. The stochastic nature of biochemical networks. Curr. Opin. Biotechnol. 19, 369–374 (2008).

16. Weitz, M. et al. The dynamical behaviour of a compartmentalized programmable biochemical oscillator. Nature Chem. 6, 295–302 (2014).

17. Bratsun, D., Volfson, D., Tsimring, L. S. & Hasty, J. Delay-induced stochastic oscillations in gene regulation. Proc. Natl Acad. Sci. USA 102, 14593–14598 (2005).

18. Elowitz, M. B. & Leibler, S. A synthetic oscillatory network of transcriptional translation and cell growth. Science 297, 1110–1113 (2002).

19. Zimmerman, S. B. & Harrison, B. Macromolecular crowding increases binding of DNA polymerase to DNA: an adaptive effect. Proc. Natl Acad. Sci. USA 84, 1871–1875 (1987).

20. Minton, A. P. How can biochemical reactions within cells differ from those in test tubes? J. Cell Sci. 119, 2863–2869 (2006).

21. Minton, A. P. How can biochemical reactions within cells differ from those in test tubes? J. Cell Sci. 119, 2863–2869 (2006).

22. Klumpp, S., Scott, M., Pedersen, S. & Hwa, T. Molecular crowding limits translation and cell growth. Proc. Natl Acad. Sci. USA 107, 17008–17013 (2010).

23. Parry, B. R. et al. The bacterial cytoplasm has glass-like properties and is fluidized by metabolic activity. Cell 156, 183–194 (2014).

24. Swain, P. S., Elowitz, M. B. & Sigcia, E. D. Intrinsic and extrinsic contributions to stochasticity in gene expression. Proc. Natl Acad. Sci. USA 99, 12795–12800 (2002).

25. Dunlop, M. J., Cox, R. S., Levine, J. H., Murray, R. M. & Elowitz, M. B. Regulatory activity revealed by dynamic correlations in gene expression noise. Nature Genet. 40, 1493–1498 (2008).

26. Elis, R. J. Macromolecular crowding: obvious but underappreciated. Trends Biochem. Sci. 26, 597–604 (2001).

27. Ge, X., Liao, D. & Xu, J. Cell-free protein expression under macromolecular crowding conditions. PLoS ONE 6, e28707 (2011).

28. Gillespie, D. T., Petzold, L. R. & Seitaridou, E. Validity conditions for stochastic chemical kinetics in diffusion-limited systems. J. Chem. Phys. 140, 054111 (2014).

29. Vargas, D. Y., Raj, A., Marras, S. A. E., Kramer, F. R. & Tyagi, S. Mechanism of mRNA transport in the nucleus. Proc. Natl Acad. Sci. USA 102, 17008–17013 (2005).

30. Kim, S., Mlodzianoski, M., Bewersdorf, J. & Jacobs-Wagner, C. Probing spatial organization of mRNA in bacterial cells using 3D super-resolution microscopy. Biophys. J. 102, 278a (2012).

31. Stogbauer, T., Windhager, L., Zimmer, R. & Radler, J. O. Experiment and mathematical modeling of gene expression dynamics in a cell-free system. Integr. Biol. 4, 494–501 (2012).

32. Akar, M., Mettetal, J. T. & van Oudenaarden, A. Stochastic switching as a survival strategy in fluctuating environments. Nature Genet. 40, 471–475 (2008).

33. van Zon, J. S., Morelli, M. J., Tanase-Nicola, S. & ten Wolde, P. R. Diffusion of transcription factors can drastically enhance the noise in gene expression. Biophys. J. 91, 4350–4367 (2006).

34. Courtois, F. et al. An integrated device for monitoring time-dependent in vitro expression from single genes in picolitre droplets. ChemBioChem 9, 439–446 (2008).

35. Karig, D. K., Jung, S.-Y., Srjantcev, B., Collier, C. P. & Simpson, M. L. Probing cell-free gene expression noise in femtoliter volumes. ACS Synth. Biol. 2, 497–505 (2013).

36. Sokolova, E. et al. Enhanced transcription rates in membrane-free protocells formed by coacervation of cell lysate. Proc. Natl Acad. Sci. USA 110, 11692–11697 (2013).

37. Shim, J.-u. et al. Simultaneous determination of gene expression and enzymatic activity in individual bacterial cells in microdroplet compartments. J. Am. Chem. Soc. 131, 15251–15256 (2009).

38. Paulsson, J. Summing up the noise in gene networks. Nature 427, 415–418 (2004).

39. Dunlop, M. J., Cox, R. S., Levine, J. H., Murray, R. M. & Elowitz, M. B. Regulatory activity revealed by dynamic correlations in gene expression noise. Nature Genet. 40, 1493–1498 (2008).

40. Ellis, R. J. Macromolecular crowding: obvious but underappreciated. Trends Biochem. Sci. 26, 597–604 (2001).

41. Ge, X., Liao, D. & Xu, J. Cell-free protein expression under macromolecular crowding conditions. PLoS ONE 6, e28707 (2011).

42. Gillespie, D. T., Petzold, L. R. & Seitaridou, E. Validity conditions for stochastic chemical kinetics in diffusion-limited systems. J. Chem. Phys. 140, 054111 (2014).

43. Vargas, D. Y., Raj, A., Marras, S. A. E., Kramer, F. R. & Tyagi, S. Mechanism of mRNA transport in the nucleus. Proc. Natl Acad. Sci. USA 102, 17008–17013 (2005).

44. Kim, S., Mlodzianoski, M., Bewersdorf, J. & Jacobs-Wagner, C. Probing spatial organization of mRNA in bacterial cells using 3D super-resolution microscopy. Biophys. J. 102, 278a (2012).

45. Stogbauer, T., Windhager, L., Zimmer, R. & Radler, J. O. Experiment and mathematical modeling of gene expression dynamics in a cell-free system. Integr. Biol. 4, 494–501 (2012).

46. Ozbudak, E. M., Thattai, M., Kurtser, L., Grossman, A. D. & van Oudenaarden, A. Regulation of noise in the expression of a single gene. Nature Genet. 31, 69–73 (2002).

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Author contributions
M.H., L.M. and W.H. conceived and designed the experiments. M.H., L.M., R.M. and W.H. performed the experiments. M.V.R. performed the experiments. M.H. and L.M. analysed the data. E.S., J.G. and H.H. provided reagents and resources. M.H., L.M. and W.H. conceived and designed the experiments. M.H., L.M., R.M. and W.H. performed the experiments. M.V.R. performed the experiments. M.H. and L.M. analysed the data. E.S., J.G. and H.H. provided reagents and resources. The authors thank R.Y. Tsien for kindly donating the genes encoding for YFP and CFP, F.H.T. Nelissen and D. Foschepoth for assisting with cloning work, E. Dubac for designing the molecular beacon, and J. Thiele for designing the masters for the fluidic devices. This work was supported by a European Research Council (ERC) Advanced Grant (246812 Intercom), a VICI grant from the Netherlands Organization for Scientific Research (NWO), and funding from the Ministry of Education, Culture and Science (Gravity programme, 024.001.035).

Additional information
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Competing financial interests
The authors declare no competing financial interests.
Methods

Device fabrication. The design of the microfluidic devices was produced using AutoCAD. The wafers contain a negative relief in SU-8 photoresist on a silicon wafer substrate. The channels for the droplet production are 25 µm in width. To make the microfluidic devices, crosslinker and polydimethylsiloxane (PDMS) were added together in a ratio of 1:10 and the solution was poured on the wafers (cleaned with isopropanol). Air bubbles were removed using a desiccator. Thereafter, the devices were put in the oven at 65 °C for at least 2 h. After preparing the PDMS layer, the device was bonded on a glass slide by activating the PDMS and glass surfaces using plasma cleaner (Femto), after which the surfaces were bonded together. Air between the surfaces was removed by applying pressure gently. The device was incubated for at least 3 h at 100 °C, after which the device was coated with a 2% silane solution.

Device and set-up operation. Liquids were pumped into microfluidic devices using adjustable pumps (Harvard apparatus, PHD 2000 infusion) connected to a syringe via polytetrafluoroethylene tubing (inner diameter of 0.056 mm; outer diameter of 1.07 mm). Droplets in the microfluidics were stabilized using a 2% biocompatible Krytox-based tri-block copolymer surfactant in Fluorinert FC-40 oil (Sigma-Aldrich) or hydrofluoroether (HFE).

Data acquisition and analysis. The devices were mounted on an inverted microscope (Olympus IX81) equipped with a motorized stage (Prior, Optiscan II). Fluorescence images were taken with a sensitive-electron multiplying charge-coupled device camera (iXon, Andor) using illumination from a mercury lamp. Analysis of the images was performed using a home-written Matlab routine.

Plasmids. pET plasmids with CFP and YFP sequences at multiple clone sites were a gift from R.Y. Tsien. The sequences for CFP and YFP production in the pET plasmids were inserted into pRSET vectors (Life Technologies) with Nco-I at the 5′ end of the coding sequence (CDS) and a Xho-I restriction site at the 3′ end. The plasmids were purified, and the purity was analysed using gel electrophoresis and sequencing analysis (GATC Biotech). The concentration of plasmids was determined using a Nanodrop N1000 spectrophotometer. The pRSET vector had T7 RNAP promoter and terminator regions.

Lysate preparation. *E. coli* Rosetta2 cells were grown at 37 °C to OD600 = 1.5 in 2YTPG broth. After cell growth, all subsequent steps were conducted over ice. The cells were collected (3,000g, 10 min, 4 °C), thoroughly dissolved in ice-cold 20% sucrose solution (16 ml for 3 g wet pellet weight) and incubated on ice for 10 min. Cells were then collected (3,000g, 10 min, 4 °C), resuspended in ice-cold milli-Q (MQ) (4 × wet pellet weight) and immediately spun down (3,000g, 10 min, 4 °C). Cells were resuspended in ice-cold MQ (4 × wet pellet weight), allowed to incubate on ice for 10 min and spun down (3,000g, 10 min, 4 °C). The pellet was then carefully washed twice with ice-cold MQ (1.5 × volume). The spheroplast pellet was stored at −80 °C.

The spheroplasts were thawed and resuspended in ice-cold MQ (0.8 × volume). Cells were lysed with 10 cycles of sonication (10 s at 10 µm amplitude followed by 30 s on ice). Cell debris was collected (30,000g, 30 min, 4 °C) and dialysed ×1 against 50% dialysis buffer (5 mM Tris, 30 mM potassium glutamate, 7 mM magnesium glutamate, 0.5 mM dithiothreitol, DTT) and 3 × 100% dialysis buffer (10 mM Tris, 60 mM potassium glutamate, 14 mM magnesium glutamate, 1 mM DTT).

*In vitro* transcription/translation mixture. For transcription–translation systems, the reaction mixtures consisted of one-third cell lysate from BL21 (DE3) host strain (∼25 mg ml−1) and two-thirds reaction buffer. The final reaction mixture contained 50 mM Hepes (pH 8.0), 2.4 mM guanosine triphosphate, 1 mM each of adenosine triphosphate, cytidine triphosphate and uridine triphosphate, 0.66 mM spermidine, 0.5 mM cyclic adenosine monophosphate, 0.22 mM nicotinamide adenine dinucleotide, 0.17 mM coenzyme A, 20 mM 3-phosphoglyceric acid, 0.045 mM folic acid, 0.13 mg ml−1 transfer ribonucleic acid, 1 mM of each amino acid, 10 mM magnesium glutamate, 66 mM potassium glutamate, 130 U T7 RNA polymerase and 8.3 mg ml−1 cell lysate, contributing an additional 5 mM magnesium glutamate and 20 mM potassium glutamate. Plasmids were added last to initialize transcription.