ESC progeny cells, the authors observed that more than 30% of injected *miR-34a*–deficient ESCs contributed to the inner cell mass, which gives rise to embryonic cell lineages, and to the trophectoderm, which develops into extraembryonic cell lineages. Thus, *miR-34a*–deficient ESCs had totipotent cell fate potential in vivo. Moreover, injected *miR-34a*–deficient ESCs contributed to multiple, fully differentiated cell lineages in the mouse embryo, yolk sac, and placenta at mid-gestation, demonstrating expanded cell fate potential that is functionally and molecularly distinct from wild-type pluripotent ESCs.

As is the case for 2C blastomeres (*5*), Choi *et al*. observed that induction of transposable element expression in *miR-34a*–deficient ESCs and iPSCs was largely specific to MERVL elements, and that among the most differentially expressed genes between *miR-34a*–deficient and wild-type iPSCs were those proximal to MERVL insertion sites, which generate chimeric transcripts containing MERVL sequences. Overexpression of *miR-34a* in *miR-34a*–deficient iPSCs decreased expression of these chimeric transcripts. This raises the question of whether this miRNA affects cell fate potential by directly or indirectly regulating the activity of MERVL.

"It is not yet known how MERVL affects the process that determines pluripotent and totipotent cell states."

Choi *et al*. delineated a minimal fragment in the MERVL sequence required for its expression and found that it contained no substantial sequence complementary to *miR-34a*. This suggests a model in which transcription of MERVL is by transcription factors or chromatin modification factors that are repressed by *miR-34a*. Bioinformatic prediction identified binding sites for 70 discriminatory transcription factors within the MERVL minimal fragment. Among these factors, only GATA-binding protein 2 (Gata2) exhibits an expression pattern similar to that of MERVL during early preimplantation (2C-stage embryo) development. Mutations in the putative Gata2 binding site within the minimal fragment reduced its activity in *miR-34a*–deficient ESCs. Consistent with this, decreasing *Gata2* expression in *miR-34a*–deficient ESCs abolished the expression of MERVL and MERVL-proximal genes. Thus, Gata2 directly promotes expression of MERVL elements and their proximal genes in *miR-34a*–deficient ESCs. However, Choi *et al*. found no involvement of epigenetic modifications in this process, which is at odds with an earlier report of a histone demethylase as a negative regulator of MERVL in 2C blastomeres (*9*). The *Gata2* transcript harbors potential *miR-34a* binding sites, and Choi *et al*. observed that *Gata2* mRNA amounts increased in *miR-34a*–deficient iPSCs and decreased when *miR-34a* was overexpressed. Furthermore, decrease in *Gata2* in *miR-34a*–deficient ESCs abolished differentiation into both embryonic and extraembryonic cell lineages. Thus, *miR-34a* prevents pluripotent ESCs and iPSCs from entering a totipotent state by repressing Gata2, which otherwise activates the expression of MERVL.

It is not yet known how MERVL affects the process that determines pluripotent and totipotent cell states. It may be that proteins encoded by these elements, which include reverse transcriptase, and fusion proteins between MERVL and proximal genes are the key. Approximately 700 copies of MERVL exist in the mouse genome; therefore, it is one of the most abundant protein-coding genes in the genome. It was recently shown that a minor population of "naïve-like" cells exists among human ESCs that express human endogenous retrovirus type-H (HERV-H) transcripts (*10*). These naïve cells are capable only of embryonic cell fates. In contrast to mouse ESCs, human ESCs normally adopt a "primed" pluripotent state, meaning that they can develop embryonic cell fates but not germline cells. The expression of HERV-H appears to define naïve ESCs; however, it is not known how the HERV-H expression changes between naïve and primed cell states. Nonetheless, the findings of Choi *et al*. suggest that transposable elements have been co-opted to give rise to totipotent cells for early host development. ■

REFERENCES

1. K. Takahashi, S. Yamanaka, *Development* **142**, 3274 (2015).
2. Y. J. Choi, C.-P. Lin, D. Risso, S. Chen, T. A. Kim, M. H. Tan, J. B. Li, Y. Wu, C. Chen, Z. Xuan, T. Macfarlan, W. Peng, K. C. Kent Lloyd, S. Y. Kim, T. P. Speed, L. He, *Science* **355**, eaag1927 (2017).
3. N. V. Fedoroff, *Science* **338**, 758 (2012).
4. S. Schlesinger, S. P. Goff, *Mol. Cell Biol.* **35**, 770 (2015).
5. T. S. Macfarlan, W. D. Gifford, S. Driscoll, K. Lettieri, H. M. Rowe, D. Bonanomi, A. Firth, O. Singer, D. Trono, S. L. Pfaff, *Nature* **487**, 57 (2012).
6. Y. J. Choi *et al*., *Nature Cell Biol.* **13**, 1353 (2011).
7. H. Siomi, M. C. Siomi, *Mol. Cell* **38**, 323 (2010).
8. A. Mehta, D. Baltimore, *Nat. Rev. Immunol.* **16**, 279 (2016).
9. T. S. Macfarlan *et al*., *Genes Dev.* **25**, 594 (2011).
10. J. Wang, G. Xie, M. Singh, A. T. Ghanbarian, T. Raskó, A. Szvetnik, H. Cai, D. Besser, A. Prigione, N. V. Fuchs, G. G. Schumann, W. Chen, M. C. Lorincz, Z. Ivics, L. D. Hurst, Z. Izsvák, *Nature* **516**, 405 (2014).

10.1126/science.aam6589

BIOPHYSICS

Flipping nanoscopy on its head

Fluorescent imaging with minimal photon flux can achieve single-nanometer resolution

By **Jie Xiao**[1] *and* **Taekjip Ha**[1,2,3,4]

About the smallest object we can see with the naked eye is our own hair. With a magnifying glass, we can see about 10 times better, and light microscopy, until relatively recently, could resolve features about 300 times thinner than human hair (~250 nm). Recent developments in fluorescence "nanoscopy" made it possible to routinely image cellular structures at 20- to 30-nm resolution (*1*), but a gap remained at the molecular scale: Most proteins are smaller than 5 nm across. On page 606 of this issue, Balzarotti *et al*. (*2*) report a new concept in nanoscopy, termed MINFLUX, that achieves the true molecular resolution (2 to 3 nm) and dramatically reduces the number of photons required by "flipping" a common wisdom in nanoscopy on its head.

In traditional optical imaging, even a very tiny object such as a single fluorophore (<1 nm) becomes blurred because light diffraction makes it appear much larger—about half the wavelength of the light used. Nonetheless, the center of the imaged fluorescence spot can be determined with extremely high precision (down to 1.5 nm if 10,000 photons are used) (*3*). The Abbe diffraction limit can be overcome by determining the position of, or localizing, one molecule at a time, with single fluorescent molecules that can be switched on or off stochastically (*4*, *5*). This localization-based strategy, together with other nanoscopy approaches (*6*, *7*), ushered in the "resolution revolution" that enabled breakthrough biological discoveries in the past decade (*1*).

A common wisdom in nanoscopy is to localize a molecule near where the signal is the strongest, which requires high-emission photon flux that is often limited by the emission rate of the fluorophore. In MINFLUX, Balzarotti *et al*. devised the opposite strategy, in which they localize a molecule near

the signal minimum (see the figure, top left). Imagine an incident beam pattern that excites a molecule maximally at position x_0 along its profile (signal = N_0 photons). In order to confidently declare that the molecule has moved by Δx to a new nearby position x_1 (N_1 photons), the associated signal change ($N_0 - N_1$) should be larger than the Poissonian noise ($N_0^{1/2}$). For example, if N_0 is 100, Δx has to be large enough to reduce the signal by 10 photons. But if we flip the excitation profile so that we get zero signal at position x_0 (signal $N_0 = 0$), much smaller Δx can be detected as long as $N_1 > 1$ (see the figure, top right).

As such, higher resolution is achieved with much reduced photon flux, but the signal may also be zero or minimal because no molecule is present. Thus, MINFLUX requires some a priori knowledge of where the molecule is. However, this level of imaging requires very few photons and can be done with conventional microscopy. In other words, emitted photons from the molecule do not pay the main cost of determining its position, as in localization-based approaches, but to merely confirm its presence and fine-tune its position estimation within the excitation pattern. In addition, because the localization accuracy is determined with excitation modulation, MINFLUX has the additional benefit of polarization-independent accuracy, an occasional problem in existing nanoscopy.

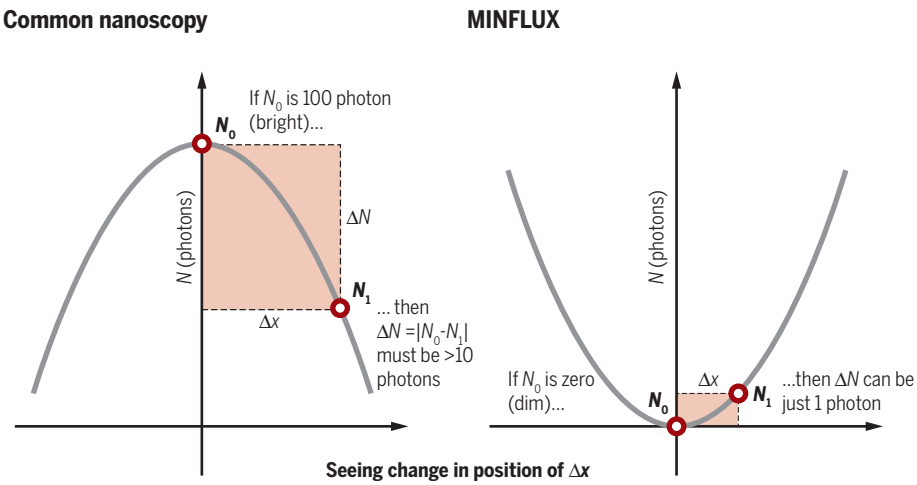
Once the position of a molecule in a view field is roughly located, the excitation pattern is serially moved to multiple positions around the molecule with a small displacement (50 to 150 nm) (see the figure, bottom). The observed fluorescence signal of the molecule at each position is then compared with the expected signal based on the known intensity profile and placement of the excitation pattern to estimate the position of the molecule. In the current work, a donut-shaped excitation pattern with an intensity zero at center was used, but in principle, any excitation pattern should work.

Using MINFLUX, Balzarotti *et al.* resolved fluorescent molecules spaced only 6 nm apart from each other on a DNA origami structure, with only 1000 photons per molecule in ~2 min imaging time. In comparison, these molecules could not be resolved with the same number of photons in existing localization-based nanoscopy even under the most ideal conditions of no background and a perfect-detection camera.

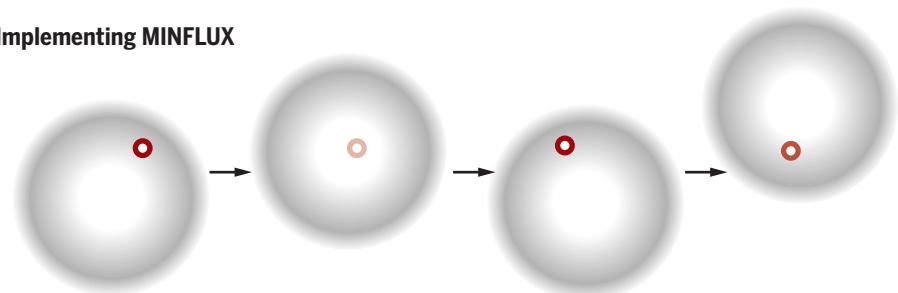
[1]Department of Biophysics and Biophysical Chemistry, Johns Hopkins University, Baltimore, MD 21205, USA. [2]Department of Biophysics, Johns Hopkins University, Baltimore, MD 21205, USA. [3]Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21205, USA. [4]Howard Hughes Medical Institute, Baltimore, MD 21205, USA.
Email: xiao@jhmi.edu; tjha@jhu.edu

Seeing better in dimmer light

The MINFLUX method uses minimal excitation to resolve changes in molecular position within an excitation pattern. In common nanoscopy, a molecule's position is determined near the maximum of the excitation intensity profile whereas in MINFLUX, it is determined near the minimum, requiring much fewer photons.



Implementing MINFLUX



The position of a molecule (red circle) is probed by watching it brighten and dim as the donut-shaped excitation profile is moved around it. Dark gray is brightest excitation.

Similar resolution was achieved of DNA origami previously by using a method called DNA PAINT, but with 50,000 photons and an image acquisition time of 2 hours (*8*).

The minimal photon flux feature of MINFLUX is particularly advantageous for single-molecule tracking experiments that

"...emitted photons from the molecule do not pay the main cost of determining its position..."

are often limited by rapid photobleaching of fluorescent proteins. Individual 30*S* ribosomal molecules labeled with photoactivatable fluorescence protein diffusing in live bacterial cells were followed for orders-of-magnitude more time points. The average length reached ~750 time points per trajectory compared with ~5 to 10 in standard tracking experiments, making it possible to detect temporal changes in diffusion coefficient. The tracking duration was only ~150 ms, however, in part because of fluorescent intermittency at the millisecond time scale.

With all its stunning performance, MINFLUX still operates under the fundamental limit of all optical nanoscopy methods—that is, temporal resolution must be traded off in order to improve spatial resolution because of the sequential nature of estimating molecule positions (*9*, *10*). In its present form, four probing positions of the donut beam must be serially sampled to localize a molecule. Furthermore, in order to achieve molecular resolution, these beams must be placed within 50 to 150 nm of the molecule or so, which limits the effective field of view. Although each localization cycle takes only a few microseconds aided by hardware-based modulation of excitation profile, the serial scanning format and small field of view would require ~100 hours to image an area with the size of a human cell. Although there is still much to improve, it should be noted that the original single-molecule localization nanoscopy images took as much as an overnight acquisition 10 years ago (*4*) but can now be done in less than 1 min or so.

GRAPHIC: C. BICKEL/*SCIENCE*

What can we expect of MINFLUX, and more broadly, superresolution and single-molecule imaging? Because MINFLUX can now reach a resolution less than 5 nm, single-molecule fluorescence resonance-energy transfer, which can determine distances of up to ~7 nm at 0.3-nm resolution with only about 100 photons (*11*), may be combined to obtain dynamic structural information continuously covering from the length scale of single amino acids to the cellular scale or larger. A considerable challenge would be to extend the molecular resolution to three-dimensional imaging, which most certainly would require interferometric methods (*12*). Moving toward multicolor imaging is likely to be more straightforward because the precision in position determination is largely wavelength-independent in MINFLUX and because more fluorescent reporters become eligible because of the reduced photon budget.

"The minimal photon flux... is particularly advantageous for single-molecule tracking experiments..."

Ultimately, the true spatial resolution of an image is going to be limited by how densely the sample can be labeled, However, the greater resolving power achieved at molecular distances that has been enabled by MINFLUX is likely to stimulate further developments in probe and labeling technologies. MINFLUX also requires more hardware engineering as compared with other localization-based nanoscopy. Nevertheless, rapid commercialization, pending further developments necessary for cellular imaging, may make it available to biologists in the not-too-distant future. ■

REFERENCES

1. C. Eggeling, K. I. Willig, S. J. Sahl, S. W. Hell, *Q. Rev. Biophys.* **48**, 178 (2015).
2. F. Balzarotti *et al.*, *Science* **355**, 606 (2017).
3. A. Yildiz *et al.*, *Science* **300**, 2061 (2003).
4. E. Betzig *et al.*, *Science* **313**, 1642 (2006).
5. M. J. Rust, M. Bates, X. Zhuang, *Nat. Methods* **3**, 793 (2006).
6. T. A. Klar, S. Jakobs, M. Dyba, A. Egner, S. W. Hell, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8206 (2000).
7. M. G. Gustafsson, *J. Microsc.* **198**, 82 (2000).
8. M. Dai, R. Jungmann, P. Yin, *Nat. Nanotechnol.* **11**, 798 (2016).
9. S. W. Hell, J. Wichmann, *Opt. Lett.* **19**, 780 (1994).
10. E. Betzig, *Opt. Lett.* **20**, 237 (1995).
11. T. Ha *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6264 (1996).
12. G. Shtengel *et al.*, *Proc. Natl. Acad.Sci. U.S.A.* **106**, 3125 (2009).

10.1126/science.aam5409

STATISTICS

Measurement error and the replication crisis

The assumption that measurement error always reduces effect sizes is false

By **Eric Loken**[1] *and* **Andrew Gelman**[2]

Measurement error adds noise to predictions, increases uncertainty in parameter estimates, and makes it more difficult to discover new phenomena or to distinguish among competing theories. A common view is that any study finding an effect under noisy conditions provides evidence that the underlying effect is particularly strong and robust. Yet, statistical significance conveys very little information when measurements are noisy. In noisy research settings, poor measurement can contribute to exaggerated estimates of effect size. This problem and related misunderstandings are key components in a feedback loop that perpetuates the replication crisis in science.

It seems intuitive that producing a result under challenging circumstances makes it all the more impressive. If you learned that a friend had run a mile in 5 minutes, you would be respectful; if you learned she had done it while carrying a heavy backpack, you would be awed. The obvious inference is that she would have been even faster without the backpack. But should the same intuition always be applied to research findings? Should we assume that if statistical significance is achieved in the presence of measurement error, the associated effects would have been stronger without noise? We caution against the fallacy of assuming that that which does not kill statistical significance makes it stronger.

Measurement error can be defined as random variation, of some distributional form, that produces a difference between observed and true values (*1*). Measurement error and other sources of uncontrolled variation in scientific research therefore add noise. The latter is typically an attenuating factor, as acknowledged in various scientific disciplines. Spearman (*2*) famously derived a formula for the attenuation of observed correlations due to unreliable measurement. In epidemiology, it is textbook knowledge that nondifferential misclassification tends to bias relative risk estimates toward the null (*3*). According to Hausman's "iron law" of econometrics, effect sizes in simple regression models are underestimated when the predictors contain error variance (*4*).

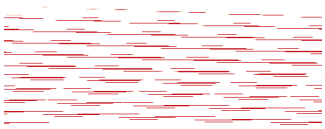
It is understandable, then, that many researchers have the intuition that if they manage to achieve statistical significance under noisy conditions, the observed effect would have been even larger in the absence of noise. As with the runner, they assume that without the burden—that is, uncontrolled variation—their effects would have been even larger (*5*–*7*).

The reasoning about the runner with the backpack fails in noisy research for two reasons. First, researchers typically have so many "researcher degrees of freedom"—unacknowledged choices in how they prepare, analyze, and report their data—that statistical significance is easily found even in the absence of underlying effects (*8*) and even without multiple hypothesis testing by researchers (*9*). In settings with uncontrolled researcher degrees of freedom, the attainment of statistical significance in the presence of noise is not an impressive feat.

The second, related issue is that in noisy research settings, statistical significance provides very weak evidence for either the sign or the magnitude of any underlying effect. Statistically significant estimates are, roughly speaking, at least two standard errors from zero. In a study with noisy measurements and small or moderate sample size, standard errors will be high and statistically significant estimates will therefore be large, even if the underlying effects are small. This is known as the statistical significance filter and can be a severe upward bias in the magnitude of effects; as one of us has shown, reported estimates can be an order-of-magnitude larger than any plausible underlying effects (*10*).

In a low-noise setting, the theoretical results of Hausman and others correctly show that measurement error will attenuate coefficient estimates. But we can demonstrate with a simple exercise that the opposite oc-

[1]*Department of Educational Psychology, University of Connecticut, Storrs, CT 06269-3815, USA.* [2]*Department of Statistics and Department of Political Science, Columbia University, New York, NY 10027-6902, USA. Email: gelman@stat.columbia.edu*



Editor's Summary

Flipping nanoscopy on its head

Jie Xiao and Taekjip Ha (February 9, 2017)

Science **355** (6325), 582-584. [doi: 10.1126/science.aam5409]

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.