NATIONAL CANCER INSTITUTE
NCI Alliance for Nanotechnology in Cancer

Mission to the Inside of a Living Cell

If you are old enough, you remember how excited you were when you learned that Mars might have canals, edible vegetation, and a thin but almost breathable atmosphere. By the 1970s, however, people knew that these were only wishful thoughts because we had traveled there – not in person – but with various sensors and probes. We imaged the planet at high resolution, analyzed its soil and atmosphere for trace elements, and studied how its geology, geography and climate interacted. Many technological developments in space science were necessary to investigate this large and distant planet. Today, researchers are beginning to harness advances in nanotechnology to analyze some extremely small and nearby objects – individual cells. Although this feat won't require traveling great distances through space, it is no less daunting, because it comes with many of the same challenges faced by scientists and engineers who first attempted to investigate the Red Planet using something other than a telescope.

Why go there?

Before discussing the many difficulties involved with sending a functioning probe into a living cell, it is worth considering why one would want to move beyond microscopes and test tubes in the first place. Biochemists have already amassed a great deal of quantitative information about chemical reactions that go on inside cells by studying purified biomolecules that have been extracted from within. However, it has become clear that the environment in a living cellular vessel has a profound effect on the types and rates of reactions that go on there. Biochemical reactions in cells are not at equilibrium conditions, there is a constant supply of free energy and reactants available, and interactions between reactions frequently produce complex signaling networks and pathways.[1]

For example, in the cell many enzymatic reactions are coupled, meaning that the product of one enzyme is passed on for use by the next nearest enzyme to form a complex network, which functions somewhat like an assembly line. In these networks, proper functioning and control is provided by both space and time parameters. Many traditional techniques for analysis require "fixing" cell samples prior to analysis. This process often destroys the precise intracellular architecture governing the network. Thus, the rate of a biochemical reaction occurring in a test tube could be quite different from that observed for the same reaction inside a cell.

DNA, messenger RNA and some proteins exist in low numbers within cells, making them difficult to detect because they often remain hidden among all the other molecules. Measuring gene and protein expression levels may involve detecting a single molecule when an individual cell is used as the reaction vessel. Moreover, because there are so few molecules involved, expression events occur randomly within single cells. Since there is no synchronization of the events between different cells, it is difficult to directly observe these random protein expression events when interrogating a large collection of cells.

But what does single-cell analysis have to do with cancer?

Researchers need detailed information about the biological processes and dynamics in living cells to aid in the detection and diagnosis of cancer. First, a clear "network-level" understanding of signaling and expression in normal cells is required so that scientists can learn to recognize the important changes that occur when cells undergo malignant transformation. Second, the ability to perform single cell analysis can be critical when primary cells from a surgical procedure cannot be propagated due to the type of cell or the low number of cells available.

Third, and most importantly, detection of cancer at an early stage is a critical step in improving cancer treatment. Scientists would like to be able to detect cancer when the earliest molecular changes have occurred within only a small percentage of cells, long before a physical exam or current imaging technology would effectively detect it. Early detection

Courtesy: Xiaohong Xu, Ph.D., Old Dominion University

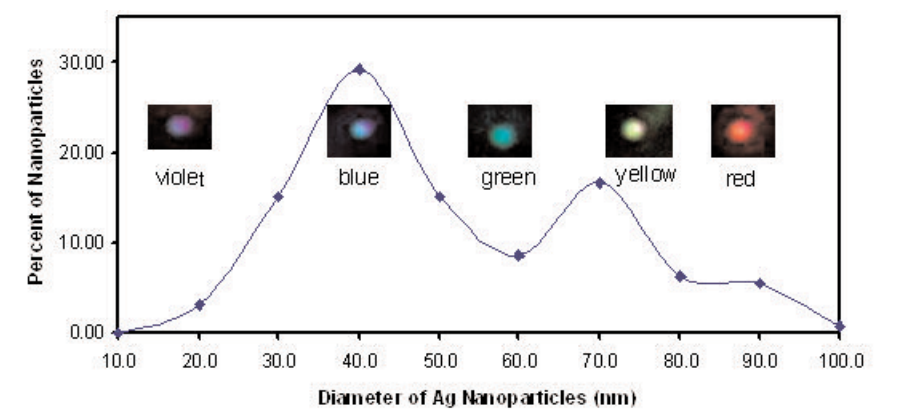

Figure 1. Color vs. size of silver nanoparticles.

will require sensitive methods that can be used to isolate and interrogate individual cells with high spatial and temporal resolution without disrupting their cellular biochemistry.

Designing brighter probes

Unlike planetary probes, those designed to penetrate a cell and report on the conditions within that cell must be sufficiently small, exceedingly bright, and stable for a long time – material properties that are often mutually exclusive. Probes must also remain stable in the intracellular environment, and not disrupt the cell's normal biochemical functioning. Recently, a research group at Old Dominion University led by X. Nancy Xu, Ph.D., prepared a series of silver (Ag) nanoparticles that meet many of the criteria listed above[2]. Although smaller than 100 nanometers (nm) in diameter, these particles are bright enough to be seen by eye using optical microscopy.

"One of the key advantages is that unlike fluorophores, fluorescent proteins, or even quantum dots, the silver nanoparticles do not photodecompose (fall apart) during extended illumination," explained Xu. Therefore, they can be used as a probe to continuously monitor dynamic events in living cells during studies that last for weeks or even months. Because the color of the scattered light from nanoparticles depends upon their size (Figure 1), they have been used to measure the change in single membrane pores in real time using a technique known as dark-field optical microscopy[3]. Intracellular and extracellular nanoparticles can also be differentiated by the intensity of light scattering (Figure 2). "The primary challenge now is to develop methods for modifying the surface of the nanoparticles to make them more biocompatible, so that more biological processes can be observed without perturbing or destroying the cell's intrinsic biochemical machinery," said Xu.

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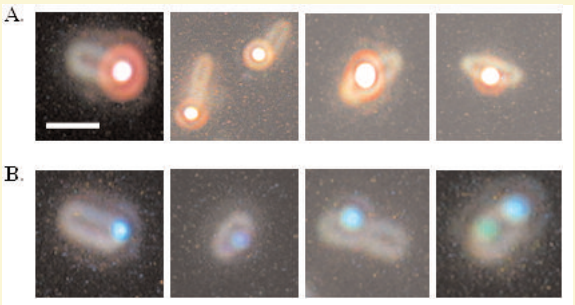

Figure 2. Differentiation of (A) extracellular and (B) intracellular silver nanoparticles using the scattering intensity: optical images of silver nanoparticles accumulated (A) on membrane (outside the cell) and (B) inside single living cells (*P. aeruginosa*). Extracellular nanoparticles in (A) appear to radiate more sharply and brightly, whereas intracellular nanoparticles in (B) look blurry and dim, indicating that the scattering intensity of nanoparticles decreases as nanoparticles enter the cells, owing to light absorption by the cellular membrane.

Amplifying and confining dimmer probes

Less bright, but more biocompatible probes have also been used to measure gene expression in individual living cells by using a microfluidics-based assay. In the past, researchers have been unable to measure the individual events of low-level protein expression because optical techniques lacked the required sensitivity. However, a group of researchers at Harvard University led by X. Sunney Xie, Ph.D., recently studied gene expression in live *E. coli* cells by measuring the expression of an enzyme called β-galactosidase, which researchers often use as a "reporter" of other biochemical events.[4] A single molecule of this enzyme can be used to churn out many fluorescent product molecules (Figure 3) that can be detected optically when excited by a laser. The problem with using this enzyme as an intracellular reporter is that the fluorescent molecules produced do not stay inside the cell. Instead, they are pumped outside and rapidly diffuse away, destroying the potential advantages of signal amplification. What Xie's group did to solve this problem was to trap the *E. coli* cells in closed microfluidic chambers (Figure 3a). Using this approach, the fluorescent product expelled from the cells accumulates in the small volume of the chambers, where the fluorescent signal can easily be measured due to enzymatic amplification (Figure 3b). The fast efflux, or outflow, rate and short mixing time of the fluorescent molecules in the miniature chambers guarantees that the fluorescence signal outside the cells accurately reflects the enzymatic activity inside.

Courtesy: Sunney Xie, Ph.D., Harvard University

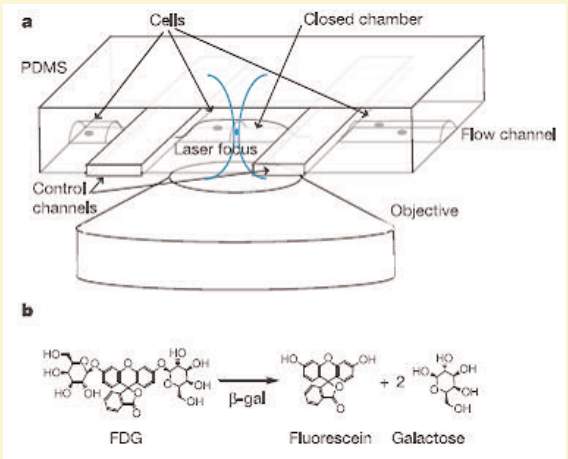

Figure 3. Single reporter molecule sensitivity in a microfluidic device. (a) Schematic diagram of the microfluidic chamber used for the enzymatic assay. Cells are trapped inside a volume of 100 picoliters, formed by compression of a flow channel by two control channels. (b) Enzymatic amplification: hydrolysis of the synthetic substrate fluorescein-di-beta-D-galactopyranoside (FDG) by the reporter enzyme b-gal yields a fluorescent product, fluorescein.

What the research team observed were abrupt changes in the levels of fluorescent products being formed in chambers containing dividing cells of *E. coli*. These step-wise increases in the rates of fluorescein generation were used as an indirect measure of the random burst-like expression of new β-galactosidase molecules. The method also allowed the researchers to quantitatively characterize intracellular protein expression with single-molecule sensitivity from a specific gene by determining the average number of bursts per cell cycle (0.11), as well as the average number of β-galactosidase molecules produced per burst (approximately 5). From a basic research point of view, such measurements represent an important first step in the quantitative understanding of a fundamental biochemical processes in living cells.

"From a technological point of view, the microfluidic device can also be used to detect low copy number proteins or mRNAs," noted Xie. Many proteins thought to be diagnostic or prognostic for cancer are expressed at very low levels and make up only a small fraction of the total protein present in cells. These low abundance biomarkers are difficult to detect by traditional genomic and proteomic techniques due to limited sample quantities, and the low dynamic ranges exhibited by the current methods.

"Techniques such as cDNA microarrays for mRNA detection, and mass spectrometry for protein detection, require analysis of many

cells due to their low sensitivities," explained Xie. "The dynamic range of protein expression ranges from 1 to around 100 million copies per cell. Mass spectrometry can detect at best around 1,000 copies per cell, so our microfluidic-based assay device fills the gap between 1 to around 1,000 copies," stated Xie. Thus, this method may one day be able to extend many of the current proteomic techniques and open up the possibility of system-wide characterization of proteins expressed at low copy numbers.

Advanced imaging systems

Some of the most widely used fluorescent reporters for monitoring gene expression in live cells are green fluorescent protein (GFP) and the derivative yellow fluorescent protein (YFP). However, imaging a single molecule of YFP being produced in a single cell is difficult because the fluorescent signal diffuses throughout the entire cytoplasm, where it is overwhelmed by the cell's own background fluorescence. This diffusion happens faster than the time it takes to acquire an image. Xie's group solved the diffusion problem in this system by joining together a variant of YFP (Venus) with the membrane protein known as Tsr.[5] When this Tsr-Venus fusion protein is produced, it assembles into the inner membrane of *E. coli* cells due to the presence of Tsr. Therefore, the fluorescent protein is confined to the membrane and can be detected with single-molecule sensitivity using a fluorescence microscope. When the researchers counted the fusion proteins as they were generated *in vivo*, they again found that the protein Tsr-Venus was expressed in random bursts (Figure 4). As in the previous example from the Xie group, quantitative details about the random fluctuations in gene expression were provided by the detection-by-immobilization assay strategy.

What about the possibility of imaging a lone protein molecule freely tumbling in the cytoplasm? It turns out that it is possible, if one can take the picture fast enough. Strobe photography can be used to image a bullet going through an apple without any blur (Figure 5C).[1] The sharpness is due to the short duration of the flash. The bullet simply doesn't travel very far while it is illuminated and imaged. This same technique can be applied to "freeze" proteins in the cytoplasm. By using a laser as an extremely bright and short (~300 microseconds) flash, an individual protein can be imaged. Xie's research group has already used this technique to detect single red fluorescent proteins in *E. coli* cytoplasm with a high signal-to-background ratio (Figure 5D).[1] "The method could also be used, for example, to determine the cellular concentration of weakly expressed proteins without calibration," remarked Xie. Further improvements on this method, however, will require the development of reporters with high photostability and better-controlled photochemistry.

Courtesy: Sunney Xie, Ph.D., Harvard University

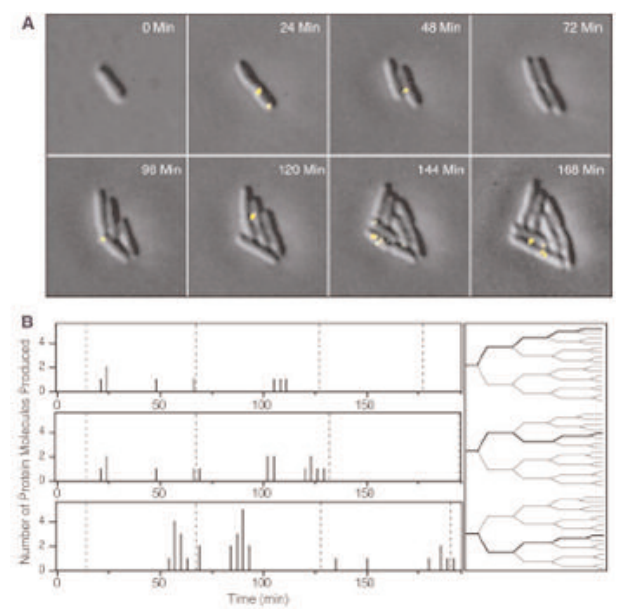

Figure 4. Real-time monitoring of the expression of Tsr-Venus fusion. (A) Sequence of fluorescent images (yellow) overlaid with simultaneous differential interference contrast (DIC) images (gray) of *E. coli* cells expressing Tsr-Venus. The eight frames are from time-lapse fluorescence movie taken over 195 min. (B) Time traces of the expression of Tsr-Venus protein molecules (left) along three particular cell lineages (right) extracted from the time-lapse fluorescence-DIC movie of (A). The vertical axis is the number of protein molecules newly synthesized during the last three minutes. The dotted lines mark the cell division times. The time traces show that protein production occurs in random bursts, within which variable numbers of protein molecules are generated. Each gene expression burst lasts ~3 to 15 min.

C: Courtesy: Harold and Esther Edgerton Foundation, 2006, courtesy of Palm Press, Inc
D: Courtesy: Sunney Xie, Ph.D., Harvard University

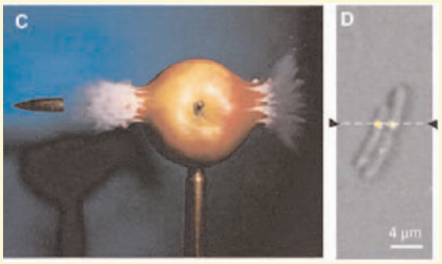

Figure 5. (C) Image of a bullet passing through an apple, obtained using strobe photography. (D) Fluorescence/DIC overlay image of three *E. coli* cells, with two containing single cytoplasmic red fluorescent protein molecules.

The next frontier

Ultimately, the probes developed by Xu's group and the techniques pioneered by Xie's group may be combined to produce highly sensitive assays with high spatial and temporal resolution. This advance will allow researchers to study the interactions of multiple genes in the same cell simultaneously by using different colored reporter molecules. In addition to transcription and translation, similar live-cell single molecule assays will offer the prospect of studying more complex cellular processes, such as cell signaling. Continuous advances and evolution along these research fronts is necessary to unravel biochemical processes *in vivo*, and to develop tools that can be used to detect and diagnose cancer using only a single patient cell.

—David Conrad

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