Watching Microscopic Basketball

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Optical imaging techniques allow us to study physical phenomena on the molecular level

"Nothing stands still" – this is how Plato expressed Heraclitus' famous maxim, *Panta rhei*. From the earliest days, motion has fascinated philosophers, natural scientists and artists alike. Celebrating the World Year of Physics in 2005, Wojciech Kilar composed a work he entitled "Sinfonia de motu" ("Symphony of Motion").

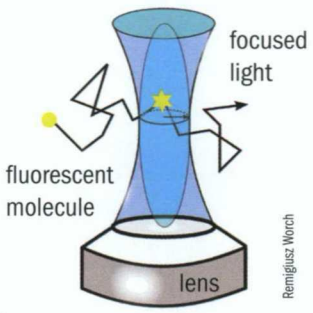
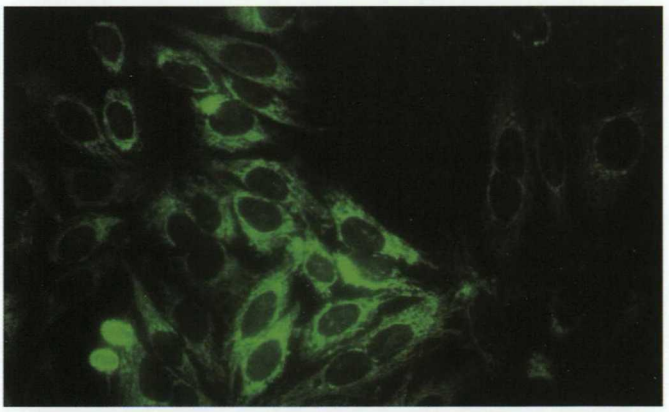


Illustration of the FCS microscopy technique. The laser light focused by the lens is like the "circle" in the center of a basketball court, with fluorescent molecules (the "players") diffusing through it. The arrow shows the shorter semi-axis of the ellipsoid. If molecules are inside the circle, the fluorescence signal F originating from them is registered. The signal at time t is analyzed by calculating the autocorrelation curve G(tau), which provides information on the mean concentration of the molecules and the mean time spent in the focal point

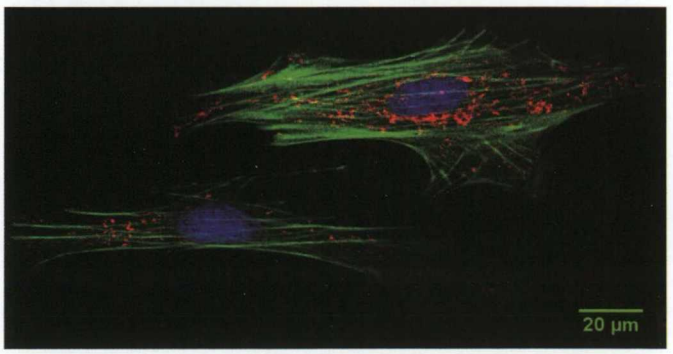
Back in the 1820s, the Scottish botanist Robert Brown was mesmerized by the motion of a grain of pollen suspended in water, observed under a microscope. It seemed incredible to him that the motion of such tiny particles did not seem to have any obvious source; he initially thought it was driven by living processes within the particles. His curiosity drove him to experiment with inorganic matter – he even tested a sample of powdered pieces of the Great Sphinx in Giza, and was shocked to observe the same effect.

We now know that the source of this motion is thermal energy, sufficiently powerful on the molecular scale, while the visible motion of larger objects is the result of ongoing collisions with molecules of the solvent (water). Brownian motion was explained in the early 20th century by Albert Einstein and Marian Smoluchowski.

Although Brown strove to find an example as distant as possible from living matter (leading him to the Egyptian statue), we now know that all particles that comprise matter, including the very smallest, are constantly in motion; we can observe this easily in a drop of milk, where the motion of individual fat molecules a few hundred nanometers in size is clearly visible. Since all physiological processes can be described on the molecular level, it is useful to know the dynamics of molecules in their natural environment.


Opta-Tech

The most sophisticated optical microscopy techniques offer a highest resolution on the level of tens of nanometers, which insufficient for directly observing molecules. Shown here: a fluorescent sample



Opta-Tech

Cell image captured using a confocal microscope (Invitrogen, cat. no. F-36925), a method that offers a highest resolution on the level of tens of nanometers (using superresolution techniques). The imaging equipment used here was purchased through the Polish National Multidisciplinary Laboratory of Functional Nanomaterials – NanoFun, and co-financed by the European Regional Development Fund under the Innovative Economy Operational Programme POIG.02.02.00-00-025/9/

Cellular tumult

If we were able to shrink ourselves to the size of a single micrometer and enter a cell, we would be able to observe myriads of different molecules moving in all directions. Some combine with one another and then disconnect again; some "flow" into individual organelles, while some emerge from within them. We would observe great crowds and commotion, as though in the center of a major city. One thing we would be certain not to find, though, would be a tiny old man with a long, silver beard, as depicted in the French animation "Once Upon a Time... Life". In fact there is no one like him at the helm; no one makes decisions regarding what happens within cells, which are instead determined by chemical and physical laws.

In the present day, in order to describe living processes, the origins and course of diseases and discover new drugs, it is essential to understand the molecular processes within cells. But is it possible to observe their motion in such a complex environment, considering that we are talking about the scale of a few nanometers – significantly smaller than the droplets of fat in milk? After all, we cannot actually enter cells. The most sophisticated optical microscopy techniques provide imaging with a resolution reckoned in the tens of nanometers at the smallest, which still doesn't make it possible to conduct direct observations on the molecular level. Is it possible to use an optical microscope to study the molecular interactions that underlie all living processes?

Mental game

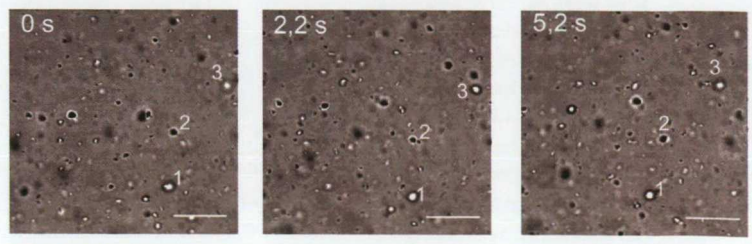
To answer that question, let's engage in a mental experiment, first imagining a basketball court measuring 15 by 28 meters. We focus our attention not on the game itself, but on the players crossing through the central circle with a radius of 1.8 meters. Let's assume that the current positions of the players are defined by random motion in two dimensions. The rules are simple: for each player, we flip two coins. Heads for the first coin means a step to the right while tails means a step to the left; for the second coin, heads means a step upwards and tails means a step downwards. When a player reaches one of the sidelines, they "bounce off" them. Now let's say that someone transmits information on the number of players within the circle in a sophisticated way: when a player is inside, they switch on a light bulb, and when the player leaves the circle, they switch it off. Of course let's assume they have a sufficient number of light bulbs and instantaneous reflexes. At any given moment, the number of light bulbs lit up will then be the same as the number of players in the center of the court. If someone else were unable to directly observe our mental game but instead had a record of the signal at their disposal, how would the intensity of the light fluctuate over time, and what might they be able to learn from it? For example, would they be able to work out the number of players on the court or the speed of the game?

Let's give it a try. If n is the number of players within the circle and k is a constant denoting the intensity of light from each light bulb switched on, our signal $F = k \cdot n$. As shown in Fig. 4, the signal F varies over time in a very irregular manner. There are simple statistical tools we can use to describe these irregularities; for example, for the value of F, we calculate the mean, denoted as $\langle F \rangle$, for any given moment, as well as the variance, *Var (F)*, measuring how individual values differ from the mean. The calculation involves subtracting the mean value from each individual F value, then calculating the mean of the deviations by squaring them first in order to eliminate any problems with negative values. As such, $Var(F) = \langle (F - \langle F \rangle)^2 \rangle$. It turns out that when players move at random, the number of players within the circle during a given time period – and, as such, our signal F – is subject to something known as the Poisson distribution. This distribution is significant because the mean of the values is equal to the variance: $\langle F \rangle = var\ (F)$.

So how is this relevant to our basketball game and light problem? It turns out that if we calculate the relationship between *Var (F)* and $\langle F \rangle^2$, it would be equal to the reciprocal of the mean number of players within the circle ($1/\langle n \rangle$). Note that this result is completely independent of k, the intensity of each light bulb. That means it doesn't matter whether we use fluorescent light bulbs, LEDs or any other light sources to denote the players. What's important is that the signal is proportional to n, so the latter can be deduced. And so, analyzing noise as shown in Fig. 4, we can easily obtain information on the number of players within the circle.

Microscopic court

Does this really work? We can verify the calculations easily using a computer simulation. If analysis of the signal tells us the mean number of players within the circle, then scaling by a factor corresponding to ratio of the surface area of the circle ($S = \Pi (1.8m^2)$) to the surface area of the court (28m x 15m), we should get the number of players on the court. The mean value the present author obtained by running ten such simulations was 10.1± 2.3, which checks out correctly. What is more, the signal also contains information on the dynamics of our game. In this case we can analyze the autocorrelated signal. The formal description of this process is rather complex; in simple terms, it involves investigating how the signal is similar to itself after consecutive steps taken by our players.



Still shots from a confocal microscope, showing Brownian motion of fat droplets in milk

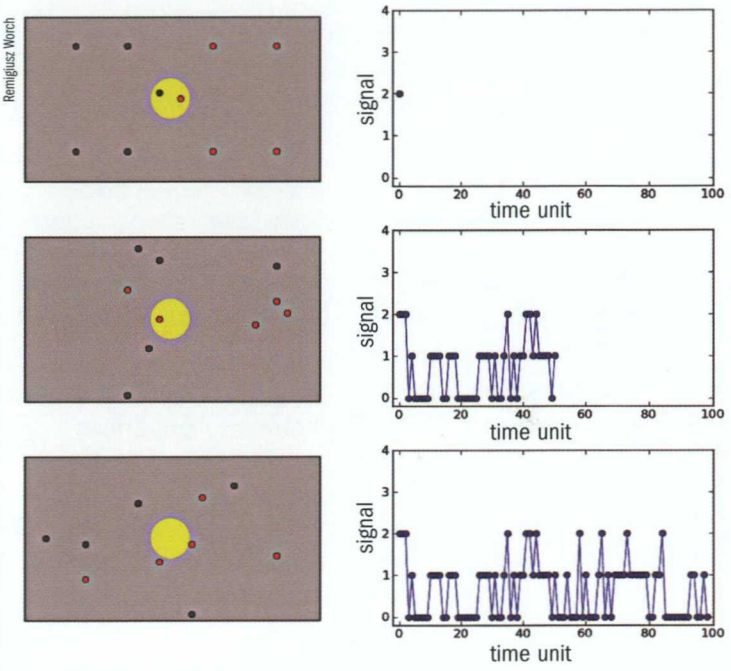
Such analysis makes it possible to capture the specific time spent by individual players within the circle. Knowing the radius of the circle allows us to obtain information about the diffusion factor, which corresponds to the speed of random motion, such as our players' Brownian motion.

What's the point of this mental exercise? Well, it turns out we can study the motion and concentration of molecules in the same way. Our conceptual basketball game is actually a macroscopic representation of the microscopic technique known as fluorescence correlation spectroscopy (FCS). The role of the circle on the basketball court is played by laser light focused by the microscope's lens, which effectively "cuts out" an ellipsoid in space with a shorter semi-axis of approx. 200 nm. As we noted previously, that's still rather large on a molecular scale. Even though we cannot directly observe molecules carrying special fluorescent markers (the "players" we want to track), it seems that analysis analogous to that described above for the basketball court can still provide information about the number of molecules (and as such their concentration) and the dynamics of their motion. Since this is a microscope-based technique, such motion can be studied in solution as well as within living cells, cellular membranes and artificial systems imitating such structures.

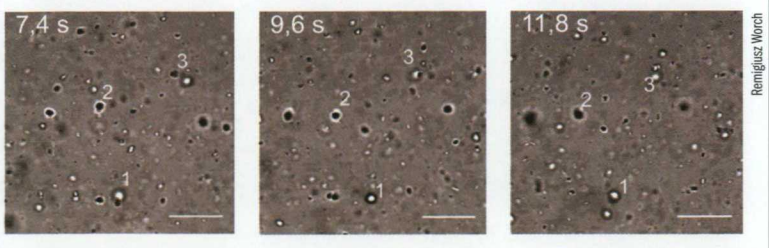
FCS can also be expanded to two colors: if we wish to study how two kinds of marked molecules (for example green and red) interact with one another, we can use a second laser that stimulates or "sees" the second type of molecules. If the molecules occur as a red-green complex, they will move as a single object, otherwise their motion will be independent. This is visible in the registered signals, with the motion resembling people strolling round a park, observed from above. An individual might arrive, sit down on a park bench, then get up and move on again. This is represented by a green signal. If a dog arrives at the park, we get a red signal. If it isn't a four-legged friend on a leash, forming a complex with his owner, the green signal (the person) does not resemble the red one (the dog), resulting in a low cross-correlation between the two signals. If the dog is on a leash, the similarity between the signals will be high. This forms the basis for fluorescence cross-correlation spectroscopy (FCCS).

Beyond the the nanoworld

When I first heard of these techniques back in 2005, I immediately knew that I wanted to study them. Thus far I have used them to study interactions between fragments of proteins passing through cellular membranes (transmembrane helices), as well as for researching the mobility of lipids and proteins in artificially-made structures resembling those occurring in nature. The results allow us to draw conclusions about the influence of cellular membrane structures on the motion and interaction betweens their constituents. In particular, we have discovered that the presence of a cytoskeleton – a cellular scaffolding – has a different effect on the mobility of membrane proteins than that of lipids. Using FCS in its two-color version has allowed us to disprove existing theories by demonstrating that complexes of certain receptors (interleukin 4) are actually formed within cells rather than on their surfaces. My aim for the next few years is to create a laboratory researching the dynamics and interactions between the biomolecules involved in a range of processes, such as early stages of infection with the flu virus, model chemical reactions taking place under cellular conditions, and changing structures and dynamics of lipid membranes in the presence of proteins that bind to them. Even in the molecular nanoworld, everything flows! ■



Frames from an animation depicting a game on a basketball court. The recorded signal is proportional to the number of players within the circle at the center of the court



Example molecules being "traced" are marked with 1, 2, 3. Scale strip equivalent to 10μm

Further reading:

Worch R., Bokel Ch., Hofinger S., Schwille P., Weidemann T. (2010). Focus on composition and interaction potential of single-pass transmembrane domains. *Proteomics*, 10: 4196-4208.