**Slide 1:**

Good afternoon, during this lecture I will introduce state-of-the-art microscopy techniques that have the potential to complement proteomics approaches.

**Slide 2:**

Proteomics techniques such as mass spectrometry or microarrays are powerful to identify components of a biological system. However, complex systems cannot be understood on the sole basis of which protein is present, the “who”; and not even the proteins functions, the “who does what”.

These pieces of information define a “network” structure, where proteins, complexes, or cellular states are the network nodes, and protein interactions or transitions between states are the network connections. But to fully understand how a network functions, we need to know its parameters: how much, where, when the proteins are present and interacting, and how fast they move between their compartments.

Let me use a simple analogy. Consider the Finnish train network.

**CLICK**

A biological function is represented by a well-defined travel between one station and another, in due time.

**CLICK**

To plan this travel, you need to know train departure times, speed, station opening times, regional networks, train capacity, staff holiday schedules … not only the network structure.

**CLICK**

And it is particularly true when some dysfunction blocks the traffic somewhere along the travel path, like in disease where some steps of the biological function are disrupted. In order to pharmacologically correct the disease state and re-establish the connection between the nodes, the knowledge of the network structure is insufficient. We need to know many more network parameters, including dynamical.

**Slide 3:**

Microscopy has always been used to observe biological samples with minimal perturbation. Proteins are too small to be seen with traditional light microscopes, hence researchers started to use the intrinsic properties of some molecules, like chlorophylle or the green fluorescent protein from jellyfish, which emit light in response to their excitation with another source of light. Such molecules are called fluorophores.

In cell biology and biomedicine research, fluorophores are genetically encoded as fusions with proteins of interest. When the fluorescently tagged proteins are naturally expressed by the cells and excited, they can be seen. We can therefore determine their localization patterns, compare protein abundance in different compartments, or use fluorescent tags of different colors to monitor 2 proteins or more, and estimate their co-localization.

When it comes to quantify proteins, standard fluorescence microscopy has two key limitations: first, most cells express naturally fluorescent molecules, that produce a background signal called autofluorescence that adds up to fluorophore signals. Autofluorescence comes from everywhere in the cell, while in contrast proteins of interest often localize to subcellular structures. To optimize the signal to noise ratio, it is therefore required to restrict the thickness delta z of the sample section from which we collect the light to a very thin focal plane. Technically, we achieve such confocal conditions either by using a detection pinhole to collect the light from the sample, or a 2-photon laser to excite only a very thin section of the sample.

A second important limitation of fluorescence microscopy stems from the inherent wave nature of light, that restricts the imaging resolution in the focal plane, often called x-y plane. Through an optical imaging device, an object point becomes a stain, whose size is called point spread function or PSF. In the best-case scenario, the size of this PSF can go down to the so-called diffraction limit, never less. This diffraction limit is typically around 200nm for green fluorophores. Therefore, regardless how small they are, 2 individual proteins fused to green tags will be distinguishable only if they are further than 200nm apart in the focal plane. In real situations, this does not happen, and the signals of individual proteins overlap, making the counting impossible. We’ll see that it is possible to circumvent this issue, but to do so we need to take a step back and look in more details at the fluorescence process.

**Slide 4:**

Light is made of small grains called photons. In short, the fluorescence process happens in 3 steps: first, the fluorophore absorbs one photon from the exciting laser

**CLICK**

and the photon energy brings the fluorophore in an excited state with higher energy. This takes femtoseconds.

**CLICK**

Then, the fluorophore relaxes part of this excess energy through vibrations, changes in conformation, and many other very complex mechanisms. This takes picoseconds.

**CLICK**

Sometimes, not always, the remainder of the excess energy is lost by emitting another photon which contributes to the fluorescence signal. This final step occurs in the relatively long time period of nanoseconds.

**CLICK**

All steps of the fluorescence process can be exploited in order to get information on the proteins of interest and their environment. The energies compatible with the absorption and emission processes are constrained by the molecular structure of the fluorophore, and compose the absorption and emission spectra. Spectral properties are the first tool to distinguish our protein of interest from other sources of fluorescence, and are selected for using adequate filters and fluorophore choices.

Another key parameter of each fluorophore is its molecular brightness: the number of photons emitted per second per molecule under given excitation conditions. As we will see in the next slide, measuring the fluorophore brightness *in cellulo* is key to be able to count molecules.

Finally, photon emission is a stochastic process and the time lag between absorption and emission is a random number for each photon. However, this emission time follows an exponential distribution, characterized by one single parameter called the decay time, or fluorescence lifetime, tau. Tau is a characteristic of the fluorophore, independent from the spectrum and from the brightness.

**Slide 5:**

To access fluorescence brightness and lifetime, we need fast scanning confocal microscopes, to control spectra with adequate optical filters, detect and count single photons, measure and record their emission times with respect to femtosecond exciting laser pulses repeated during a dwell time of 20 to 60 microseconds, using ultra-fast electronics (<0.1ns response time, e.g. TCSPC, FLIMbox…). Finally, we will develop custom data analysis software to fit the needs of particular experiments.

**Slide 6:**

Let’s start with the beginning: counting molecules.

**Slide 7:**

The problem with counting proteins from fluorescence data stems from the fact that it is generally hard to know if a high intensity comes from many dim particles or a few bright ones. This is also true in single photon counting mode, where the number of photons coming from each pixel called the pixel intensity F is on average the product of the number of fluorescent particles n by their molecular brightness e. I use the word “particles” on purpose, to distinguish from individual molecules. Indeed, in real cells fluorescently tagged proteins can associate and form dimers, trimers or even higher order oligomers depending on their biological functions. And these fluorescent “particles”, encompassing one or more fluorescent proteins, move between pixels leading to fluctuations of the pixel intensity.

Those fluctuations due to the motion of fluorophore oligomers yield variance in the pixel intensity, when we repeatedly scan and image the SAME sample. The second term is the contribution of the detector to the signal variance. Thus, if we can measure both the mean intensity F, and the variance sigma at each pixel, we now have 2 equations to compute separately the 2 parameters, the number and brightness of the fluorescent particles. I stress that these numbers are for the particles that can, in principle, encompass multiple molecules if the latter form oligomers.

So we can count fluorescent particles within the excitation volume. To know if they are monomeric, dimeric or larger clusters, we have to compare the measured molecular brightness e with that of the monomeric fluorophore imaged in the same conditions, e\_monomer. Indeed, the brightness of a dimer is twice that of a monomer. Therefore, the number of fluorescent molecules present in one particle, also called the stoichiometry, is just the ratio of the particle brightness to the monomer fluorophore brightness. And the number of proteins now is the ratio of the fluorescence intensity to the monomer fluorophore brightness. Hence, measuring pixel intensity and variance in single photon detection mode provides at the same time protein concentration and stoichiometry at the pixel level in live cells.

Let me illustrate these concepts with two different samples: on the top row, yeast cells where a nuclear protein showed as blue hexagons is tagged with GFP shown as green dots, and on the bottom row cells expressing free GFP. Both samples have 8 GFP molecules within the laser excitation volume shown in grey here. However, on the top sample, the proteins form dimers and hence the GFP molecules move as “fluorescent particles” made of 2 GFP molecules. The same samples are excited and scanned many times and the measured fluorescence intensity fluctuates over time. Because on the top sample, the GFP molecules move by groups of 2, intensity fluctuations are stronger on the top sample than on the bottom sample, and the measured brightness is twice that of the bottom sample. And because there are only 4 dimers in the top sample, we count n=4 particles in contrast with bottom sample where we count n=8, 8 monomers.

In conclusion, applying this technique to our sample of interest and in parallel to cells expressing the monomeric fluorophore, we can get maps of proteins number, or concentration, and stoichiometry. We can do the same for mRNAs, and also quantify heterologous protein complex formation using sN&B in 2-color mode. Supplementary slides are provided for those of you who are interested.

**Slide 8:**

Measuring absolute proteins or mRNA concentrations rather than relative expression levels is key to understand complex systems. This slide briefly summarizes how sN&B measurements of the absolute concentration of transcription factors (TFs) indicated a novel mechanism of size control in budding yeast. We correlated the concentration of the transcription factors that trigger the commitment to the cell division cycle at the G1/S transition with the size of single yeast cells. With absolute measurements, we discovered that there are not enough TF proteins in small cells to saturate the target gene promoters, making the G1/S transition impossible. We also showed that the TF concentration increases with cell size, leading to a saturation of the targets in larger cells, allowing the commitment to the cell division cycle. Strikingly, in cells grown on poor carbon sources such as glycerol shown in red here, those transcription factors were upregulated, allowing a premature G1/S transition at a smaller size. This discovery would have not been possible without absolute measurements of the number of TF proteins in single cells.

**Slide 9:**

Because it contains both space and time components, sN&B raw data can be re-analyzed to extract information about protein motion. The method is called Raster Image Correlation Spectroscopy or RICS.

**Slide 10:**

The laser scans the sample along an horizontal line, acquiring photons during the dwell time at each pixel, then starting over the next line along the vertical direction. Hence, horizontal and vertical neighbor pixels of the SAME scan of the sample are imaged a few dozens of microseconds, and a couple of milliseconds apart respectively.

Because fluorescently tagged proteins move, a spike in protein concentration at a given pixel will spread out over time, leading to correlations between signals recorded at increasingly distant pixels during the SAME scan, that contain information on how fast proteins from the spike have moved. Those correlations decrease as a function of an increasing pixel shift.

In RICS we examine two type of intensity correlations: correlations between the signals at any pair of pixels separated by a pixel shift delta\_j along the horizontal direction, and pairs of pixels separated by a pixel shift delta\_i along the vertical direction. Those correlations are shown here for the Smc4 condensin subunit tagged with GFP, in WT yeast cells in blue and cdc48 mutant cells in red. cdc48 is the yeast homolog of the VCP/p97 ATPase in human. GFP fused to a nuclear localization signal NLS was used as a control for free nuclear diffusion.

In this example, the horizontal correlations shown on the left represent the point spread function of the microscope. When this happens, it means that there is no protein motion across pixels at the timescale of horizontal scanning. In contrast, the vertical correlations decrease faster than the PSF, showing that proteins move on the millisecond timescale sampled by vertical correlations. Moreover, correlations of Smc4-GFP decay slower than that of freely diffusing NLS-GFP fluorophore. This shows that the motion of Smc4-GFP is constrained, in line with condensin often sitting immobile on DNA. Interestingly, Smc4GFP correlations in the cdc48 mutant decay even slower than in WT, indicating that Cdc48 contributes to condensin cycling.

**Slide 11:**

Correlations represent the RICS raw data. To get more quantitative insight, correlations curves are generally fitted with motion models. In this purpose, we use either existing software like SIMFCS that generally test free diffusion models, or custom models if more complex motion models are relevant to a particular dataset or experiment.

Here we show the histogram of diffusion coefficients fitted from such data in individual cells, showing that RICS is good enough to distinguish subtle changes in protein mobility, like for instance condensin that is more DNA bound during metaphase compared to other cell cycle phases, or in absence of Cdc48 ATPase,

**Slide 12:**

So far, we have used fluorescence brightness and photon counts. Let’s now use the information relative to the time lag between the excitation pulses and photon detections, which we call the emission time delta\_t.

**Slide 13:**

In FLIM, we accumulate millions of photons, recording their emission times delta\_t, until we can reconstruct the fluorescence decay curve, represented as a scatter plot here. As discussed in the introduction, in a given chemical environment a given fluorophore has a characteristic decay time tau also called fluorescence lifetime. If we are in presence of a single fluorophore, the decay curve is perfectly exponential and the lifetime tau is obtained by fitting the curve to an exponential model.

The fluorescence lifetime is an interesting parameter, in particular because it does not depend on the fluorophore concentration and therefore FLIM provides an additional contrast that can discriminate between different fluorophores with overlapping spectra, for instance low expression proteins from autofluorescence.

In real life samples, we usually have multiple sources of fluorescence, and therefore the fluorescence decay curve cannot be fitted with one single parameter.

**CLICK**

In this situation, we generally perform sine and cosine Fourier transforms of the decay curve and get 2 numbers at each pixel. Then, we represent each pixel in 2D with these numbers as coordinates, using dedicated software. We get what is called a “phasor plot”.

**Slide 14:**

Originally, FLIM has been used to distinguish multiple fluorescent species of the same color. Let’s see in practice how it looks like. This slide shows 4 phasor plots, on which each pixel of an original FLIM image is shown at coordinates that correspond to the sine and cosine transforms of the fluorescence decay curve at this particular pixel.

The phasor plot shown on the top left was computed from images of saccharomyces pombe fission yeasts expressing no fluorescent tag. So this spot correspond to autofluorescence in this cell type. The bottom left phasor plot was computed from images of the same fission yeasts, but also expressing a mNeongreen tagged version of the histone deacetylase Mst2. The top right phasor plot was computed from images of the fluorescein dye in solution. And the bottom right plot combines pixels from all samples for comparison. All these sources emit green fluorescence, and would be hard to distinguish just using filters for instance. However, using the lifetime data, we can see that those sources give different signatures on the phasor plot. The fluorescein and mNeonGreen fluorophores appear as tightly focused spots along the circle here, while autofluorescence is a broad “spot”, often not even on the circle, indicating that it mixes all sorts of lifetimes. Because it is more on the left side, fluorescein has a longer lifetime than mNeonGreen.

**Slide 15:**

How can this be used in practice? Let’s look at the yeast G1 cyclin Cln3, the functional homolog of Cyclin D. This protein is so rare that it has never been properly observed or quantified at endogenous levels. Its concentration could be as low as a couple of nM. Cells expressing a GFP-tagged version of the Cln3 protein cannot be distinguished from untagged cells which emit autofluorescence only just by looking at signals.

However, the lifetime signature is different, and the phasor spot for Cln3GFP cells seem to be stretched towards this point here, close to the middle of the circle. This point corresponds to the pure GFP fluorophore. This example illustrates an important concept in FLIM: pixels containing a mix of 2 fluorescent sources do not appear on the circle anymore, but along the chord of the circle arc joining the 2 spots. Likewise, pixels containing a mix of autofluorescent molecules and GFP-tagged proteins appear in the phasor plot along the straight line joining the autofluorescence spot and the pure GFP spot, like I have indicated here with a white arrow. The position of the pixels along this line depends on the relative fraction of autofluorescent photons to GFP photons at the pixel.

In FLIM software, it is generally possible to visualize pixels according to their position on the phasor plot, so according to their lifetime. Doing so on our Cln3GFP cells and untagged control cells clearly identifies the pixels containing the GFP signal in addition to autofluorescence, they are shown in yellow here, or red pixels where GFP dominates, while pixels containing autofluorescence only are shows in purple and present mostly in the untagged cells.

Thus, here the Cln3 protein becomes visible, based on lifetime data. The ability of FLIM to identify fluorescent species can be used to demonstrate for instance the nature of a recovered signal in Fluorescence Recovery After Photobleaching or FRAP experiments, as briefly shown in Supp slides.

**Slide 16:**

FLIM can also be used to probe physico-chemical properties of cellular media.

**Slide 17:**

Indeed, lifetime naturally depends on pH, oxygen concentration, the presence of other ions, or other changes in the chemical neighborhood. Particular fluorophores can be engineered to increase this sensitivity and serve as local physico-chemical probes.

This is for instance the case of the pH. pHLuorin2 is a variant of GFP that has been optimized for pH sensing, so that in the physiological pH range from 5 to 7, the lifetime changes from 2.3 to 2.7 which can be measured accurately. In this example, the authors have fused pHLuorin to protein markers of the different compartments of the secretory pathway, and measured the lifetime of pHLuorin in live HeLa cells. These measurements provided pH maps inside the cells, with a resolution of a single lysosome.

**Slide 18:**

The sensitivity of fluorescence lifetime to chemical perturbations of the fluorophore makes it also an excellent readout for Förster resonance energy transfer, or FRET.

**Slide 19:**

What we call FRET is a loss of brightness due to short range molecular interactions of the fluorophore with another molecule. By short range, I mean 1-10nm, so FRET happens when the fluorophore, which we’ll call a FRET donor, and the other molecule which we’ll call a FRET acceptor, are within the same molecular complex. Then, part of the energy acquired by the donor fluorophore upon laser excitation is rapidly transferred to the acceptor, with two consequences: there is less fluorescence emission, so donor brightness decreases. But also, the relaxation to the fundamental state of the donor happens sooner than in the absence of the FRET acceptor, leading to a decrease in the fluorescence lifetime.

Therefore, biochemical probes using FLIM as a readout are increasingly developed to probe short range interactions, including protein-protein interactions, but also posttranslational modifications, or PTMs. For instance, in this work Doll and coworkers have tagged proteins of interest with GFP, and oligosaccharides with a dye that works as a FRET acceptor for GFP. Hence, when the protein of interest becomes glycosylated, the two fluorophores are so close to each other than FRET happens and the lifetime of GFP drops.

**Slide 20:**

In short, quantitative bio-imaging is a versatile toolbox to complement -omics data, and access cellular parameters in live cells that are inaccessible to multiomics, or with greater accuracy, though with lower throughput at this stage.

**Slide 21:**

The analysis of fluorescence fluctuations overtime allows to count molecules and map their concentration, oligomeric state, and diffusion coefficient at the subcellular scale. These techniques can, in principle, be implemented with analog detectors that do not count single photons, with additional controls and analysis steps. Going further, the analysis of the emission time of single photons allows to reconstruct the entire fluorescence decay curve at each pixel and therefore to probe a fundamental feature of fluorescent molecules: the fluorescence lifetime. FLIM imaging allows to distinguish fluorophores of the same color, detect very small amount of fluorophore even drown in cellular autofluorescence, and therefore get a much higher contrast. FLIM can be coupled to virtually any other microscopy techniques, allowing to also measure protein diffusion, synthesis rates, but also to probe subtle changes in the local environment of the fluorophores. Such changes can be caused by physico-chemical properties like pH or local oxidation, but also by protein binding, PTMs or conformational changes, allowing to quantitatively estimate these protein alterations in live cells.

Of course, the application of these techniques comes with technical constraints. But all these constraints are now easily implemented, and technical progress in some respects, like the performance of fluorophores is achieved every year.

I hope you enjoyed this brief overview, more lectures will be developed soon and posted on OERcompbiomed github, so stay tuned!

**Supp. Slide #1:**

It is in principle possible to also use sN&B to count mRNAs. Indeed, sequence-specific RNA-interacting proteins, such as the bacteriophage MS2 or PP7 coat proteins, can be fused to fluorophores such as GFP. These coat proteins recognize specific RNA sequences with picomolar affinity, allowing to visualize single mRNA molecules that have been extended genetically to include the bacteriophage sequence. On this example, the Whi5 cell cycle inhibitor in yeast was tagged with the PP7 stem loop sequence, in cells ectopically expressing the PP7 coat protein fused to YFP. On these videos, we can see the YFP-tagged coat proteins clustering to bright dots only in the presence of the PP7 stem-loop-tagged Whi5, demonstrating their binding to single Whi5mRNA molecules, that can be manually counted.

Such RNA labeling system can also be coupled to sN&B, to evaluate the mRNA concentration. In this preliminary dataset, the brightness generated by autofluorescence (blue dots) was already quite strong, making it difficult to evaluate the brightness of single YFP molecules (in grey). But yet, the measured brightness in the sample expressing both the PP7-YFP fusion and the Whi5-PP7 stem loop mRNA was clearly larger than in the sample expressing only the PP7-YFP. Each Whi5mRNA molecule is supposed to contribute 24x the brightness of a single YPF molecule, once the contribution of autofluorescence is removed, making in principle possible the quantification of the concentration of Whi5mRNA molecules.

**Supp. Slide #2:**

The principle underlying sN&B, that fluorescence signal fluctuation is a readout of the oligomeric state of proteins, can be readily applied to quantitatively characterize heterologous protein interaction.

The idea is simple: the proteins 1 and 2 for which we want to quantify the interaction must be tagged to spectrally distinct fluorophores, for instance one green and one red like GFP and mCherry, and repeatedly imaged like we do in sN&B. The photon counts in the green and red channels are recorded separately at each pixel j, and at each scan k.

From this data, we compute the mean and variance in photon counts in each channel over all scans, and deduce the number and brightness of the type 1 and 2 proteins within the laser excitation volume.

In parallel, we compute the cross variance that measures how fluctuations in green and red photon counts are correlated. One copy of protein 1 leaving the excitation volume between 2 consecutive scans, yields a negative value for the fluctuation term Fk,j-Fj. If proteins 1 and 2 form a complex, then at the exact same scan one copy of protein 2 will also leave the excitation volume, also yielding negative fluctuation in the red channel Ck,j-Cj. The product of the two contribution will be positive. The same is true if a protein1-protein 2 complex enters the excitation volume. Hence, when averaging over all scans, the remaining cross correlation sigmacc will only sum up positive terms, and thus will be positive.

If proteins 1 and 2 are not bound, fluctuations in the signals of protein 1 and 2 are independent, and over all repeated scans the cross correlation sigmacc averages out to 0.

When normalized to the mean intensity in the green and red channels, sigmacc yields a Bcc coefficient that quantitatively measures how much proteins 1 and 2 are bound, irrespective of their relative expression. With adequate calibration, it is possible to compute the fraction of proteins 1 and 2 that are bound in the same complex, and thus to estimate the Kd of the interaction, at each pixel in live cells.

**Supp. Slide #3:**

The ability of FLIM to identify fluorescent species can be used to demonstrate for instance the nature of a recovered signal in Fluorescence Recovery After Photobleaching or FRAP experiments.

In short, in a FRAP experiment we excite fluorescently tagged proteins too much so that the fluorophores are destroyed. This is called photobleaching. Then, you wait until the signal recovers. If you have photobleached just a tiny part of your cell, the recovered signal is made of protein-fluorophore fusion that come from other parts of the cells, and thus have not been photobleached. In this case, the speed of signal recovery is a measure of protein motion. On this example, we have photobleached all the Whi5mNeonGreen pool of some cells, shown with white arrows, and then the recovered signal in those cells shown as red and blue arrows here comes from freshly synthesized Whi5 proteins (Whi5: G1/S transcription inhibitor in yeast, Rb homolog).

One issue with FRAP experiments can be that the nature of the recovered signal is unclear. Coupling FRAP to FLIM measurements of the lifetime in the recovered cells, we could identify the mNeonGreen lifetime, and therefore be 100% sure that the recovered signal comes from de novo Whi5 protein synthesis.