# Love at 40th sight - take a deep look at your experiment.

Beyond generating stunning images, fluorescence microscopy is one of the favourite work horses of biologists. After all, seeing is believing! As biologists, we use it to study everything from a developing Zebra fish embryo to the localisation of individual proteins in a single human cell. Unfortunately, biological materials, including proteins, rarely fluoresce. To visualise proteins nonetheless, a technique called immunofluorescence is used, by which fluorescently labelled antibodies are added to the studied specimen. The antibodies will then bind to the protein of interest and the fluorescence emitted by the dye attached to the antibody is recorded to generate the microscopy images we are familiar with. Due to the molecular makeup of antibodies and the nature of light, rarely more than 4 proteins can be visualised in the same sample using conventional immunofluorescence. The number of proteins involved in biological processes, however, often exceeds that number, imposing the proverbial blinders on a researcher's ever so elegantly designed experiment.

We set out to develop a novel imaging technique called iterative indirect immunofluorescence imaging -4i- to overcome the traditional limitations of immunofluorescence. 4i enables scientists to probe forty and more proteins in the same specimen, while capturing biologically relevant information from the tissue, cellular and sub-cellular level. We achieve such level of multiplexing by iterating rounds of conventional immunofluorescence followed by imaging and subsequent antibody elution from the sample. With every iteration we add a new set of molecular markers to our dataset.

Organelles are the functional subunits of a cell tasked with performing distinct biochemical and physical functions. Whilst biologists have amassed deep knowledge about these structures, our understanding of organelles' relative position to each other within a cell and in the context of a population of cells with different characteristics such as type, cell cycle phase or cell density, is limited. Chiefly, this was caused by our inability of readily observing more than 4 proteins in the same sample. We thus decided to showcase the capability of 4i, by generating an atlas of the subcellular organisation of organelles in mammalian cells. For this we measured the abundance and localisation of the majority of human organelles and their spatial reorganisation upon drug treatments in hundreds of thousands of single cells using more than 40 antibodies.

Humans see the world in three primary colours, namely red, green, and blue and combinations thereof. When we started analysing the images generated for the organelle survey, we quickly realised that a human brain was indeed made to process 3 and not 40 primary colours. It was simply impossible to remember the relative position of protein 23 to protein 17 to the colocalisation of proteins 34 and 7, let alone studying these relationships in cells from different cell cycle phases or drug perturbations.

We thus decided to develop a machine learning routine to help us make sense out of our experiment. We call it multiplexed protein maps. It quantifies the spatial distribution of multiplexed signals in microscopy images by grouping individual pixels of images into groups based on their multiplexed pixel profiles and then measures the spatial adjacency of these pixel groups to each other. The result is a detailed map of the subcellular organisation of organelles, with which we revealed among others that organelle groups associated with an active metabolism are more abundant in sparsely-growing cells, that cell receptors involved in cancer proliferation are transported differently in cells based on their cell cycle phase and position in a cell islet, and that many drugs used in cell biological research -perhaps unknown to the experimentalist- deeply alter organelle configuration.

We have developed 4i and multiplexed protein maps. 4i enables researchers to generate highly multiplexed images using off-the-shelf antibodies and a conventional fluorescence microscope without degrading their sample in the process. Multiplexed protein maps help researchers to

comprehensively quantify the spatial distribution of multiplexed signals contained in 4i data sets and to link sub-cellular readouts to biologically relevant information from higher spatial scales.

We hope that 4i and multiplexed proteins maps will empower researchers to gain more inclusive results from their microscopy experiments and simplify the study of complex cellular processes such as signal processing, membrane trafficking and cell differentiation. Applications for the new technology aren't restricted to basic research but span from the lab right into the clinics, where 4i and multiplexed protein maps could improve the diagnosis for patients suffering from cancer, autoimmune or neurodegenerative diseases.

By Gabriele Gut