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Quantitative Techniques for Imaging Cells and Tissues

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Introduction

This special issue focuses on two major aspects of cell biology: imaging and quantitation. The history of cell biology has been tightly interwoven with imaging and in particular with advances in microscopy throughout the past centuries (Wolpert, 1995; Evanko et al., 2009; Milestones Light Microscopy, 2009). The invention of microscopes was instrumental in defining cell theory, centuries ago, and the boundaries of visualization advanced further with electron microscopy, decades ago. Applications of fluorescent confocal microscopy have enabled new ways of observation at the cellular, sub-cellular and even molecular level (still evolving to the current time, Waters and Wittmann, 2014). Less appreciated is the importance of quantitative approaches in cell biology. Quantitation adds an important, often essential new dimension to the scientific process/endeavor, as expressed in the famous quote by Lord Kelvin:

> "In physical science a first essential step in the direction of learning any subject is to find principles of numerical reckoning and methods for practicably measuring some quality connected with it … when you can measure what you are speaking about, and express it in numbers, you know something about it; but when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind; it may be the beginning of knowledge, but you have scarcely, in your thoughts, advanced to the stage of *science*" (Thomson, 1889).

Cell biology benefits in multiple ways from inclusion of appropriate quantitative approaches. This is well established in some areas of cell biology, such as the quantification of cells or synapses in neuroscience (Haug, 1986; Coggeshall and Lekan, 1996; Evans et al., 2004), but less so in other fields of cell biology such as immunocytochemistry, super-resolution microscopy, and nanotechnology. The merging of cell biology and quantitative biology is considered an area in need of greater attention and further development (Liberali and Pelkmans, 2012; Wall, 2012; Shekhar et al., 2014).

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Technical advances in cell biology usually happen first at the qualitative level (which structures and molecules can now be identified or localized at higher resolution?), which is followed with various delays by the exploration and development of quantitative methods to compare different states (how many molecules or particles are present? By how much are they increased or decreased in different conditions? How can the readout be quantified?) The development, testing and refinement of quantitative aspects of novel technologies can take significant lead time, and development of proper applications of quantitative technologies has followed different time courses in different fields of cell biology.

Recognition of novel technical advances in cell biology by recent Nobel prizes

New technologies create new frontiers. Advances have been remarkable in the past two decades in areas such as confocal microscopy, green fluorescent protein, nanotechnology (quantum dots), and super-resolution microscopy. This is reflected by a series of Nobel prizes that were awarded for new technological milestones: structure of macromolecules (in 2002), magnetic resonance imaging (in 2003), green fluorescent protein (in 2008), and, most recently: super-resolution microscopy (in 2014).

The recent surge in new developments in microscopy has opened new avenues of research with intriguing possibilities. A critical “mass” has been reached, in which this research drive poses new demands to advance in the directions just now enabled. The end of this scientific tidal wave is not yet in sight. Amidst these exciting new techniques, we present a special issue that addresses analytical, technical and chemical developments that accompany and/or lie at the basis of the new microscopy applications. Many perceived barriers (spatial resolution, speed, field size, detection limits, probe brightness) have fallen. Never before have we been able to see the microscopic world in which cells and tissues perform their tasks in so much detail, nor have we been able to extract so much information from biological specimens. Qualitative imaging can only provide snapshots of events and states without providing the tools for interpretation. Quantitation is key to our advances as it brings the necessary objectivity to understand rules and causal relationships between observable events.

We congratulate the 2014 Nobel laureates in chemistry, Eric Betzig, Stefan Hell and William Moerner for their achievements in moving beyond the diffraction barrier, allowing light-based imaging on length scales that were, previously, only accessible via electron microscopy – but now in multiple colors, in 3D and in live specimens. Super resolution techniques come in multiple flavors. Those that are based on localizing single emitters even provide information about their number. All of these techniques, because of their dramatically reduced sampling volume, reduce the averaging of events in focus, providing a clearer view of distributions of behaviors. They are, therefore, great tools also for quantitative imaging. Our special issue contains three contributions that directly deal with super-resolution techniques, their possibilities and demands.

Overview of the contributions to the Special Issue

For this special issue of *Cell & Tissue Research*, we have asked experts in the fields of imaging, image analysis, and quantitation of cells and tissues to provide an update on the Status of quantitative methods. These methods range from older methods, introduced decades ago, such as autoradiography and immunocytochemistry, to the most recent, such as super-resolution microscopy. Tried and tested quantitative techniques that were established long ago can and will be reinterpreted and adapted to meet the new measurement possibilities offered by the latest technologies. Heterogeneity in refinement of the quantitative methods is reflected in the various contributions of this issue of *Cell & Tissue Research*. For example, as elucidated in the contributions of Geuna and Herrera-Rincon (2015), Kubinova and Janáček (2015), and Mayhew (2015), proper counting of particles in sectioned tissues has been worked out over several decades, culminating with the design of the tools of stereology (Haug, 1986; Coggeshall and Lekan, 1996; Evans et al., 2004), but it took nearly a century and considerable effort by a large number of investigators to perfect the design-based approaches after the first cells were counted under the light microscope in the late 1800s (Blinkov and Glezer, 1968; Haug, 1986). Currently, the key stereological method papers enjoy exceptionally high citation rates, although the actual usage still lags behind, as revealed in the contribution by Geuna and Herrera-Rincon (2015).

Our special issue starts with the century-old problem of how to count particles in sectioned tissues. Geuna and Herrera-Rincon (2015) provide an update of the current status of the stereological tools, as applied to sections at the light-microscopic level, including an update of current usage of different counting techniques over the past decade. This is followed by the contribution of Kubinova and Janáček (2015) who examine the specific challenges and opportunities when applying stereological tools to confocal microscopy. The next review, by Herculano-Houzel et al. (2015) presents a novel alternative method of counting cells, the isotropic fractionator, which basically homogenizes tissue to a “soup”, collects the nuclei, and samples and distinguishes neurons from non-neuronal cells by antibody-labeling. The advantages and disadvantages of this simple, yet elegant approach are compared with stereology, including practical hands-on issues. We complete the “particle counting tool kit” with the contribution by Mayhew (2015). He explains how stereological methods are used for tissues at the ultrastructural level, again including several hands-on, practical examples that guide the reader through each of the steps.

Staying with the important aspect of correlating light-microscopic and electron-microscopic levels of analysis, Giepmans and colleagues (Kuipers et al., 2015) present a novel genetic tool (FLIPPER) for labeling (“painting”) and examination of molecules in cultured cells at the ultrastructural level that allows for optimal fixation and preservation of morphology, thus expanding the toolkit of researchers who wish to examine specific molecules and processes at the ultrastructural level. Such analyses can make use of nanotechnology, e.g. for tagging antibodies and other proteins. In this context, the current status and the emerging use of the quantification of nanoparticles (quantum dots) is covered by the contribution co-authored between the labs of Tania Vu and Diane Lidke (Vu et al., 2015). Nanotechnology is an emergent field that has a huge intersection with cell biology and promises fascinating

progress not only in basic cell biology, but also applied biology, including clinical areas such as diagnostics and therapeutics.

The decades-old field of autoradiography and in particular drug distribution is covered from a pharmaceutical/drug development perspective that reviews how autoradiographic approaches are essential to verify drug distribution and kinetics in animals and humans for the testing of new drugs (Solon, 2015). Several decades after the introduction of immunocytochemistry, histopathologists are still in the process of defining and developing proper procedures for true quantification of immunolabels. With the current possibilities of visualizing single emitting fluorophores and achieving nano-scale localization, these questions are becoming very acute again. Likewise from a clinical perspective, we have an opportunity to hear the vision as laid out by a clinical pathologist, and specifically how immunocytochemistry can and must become more quantitative in the future (Taylor, 2015). Digital data analysis and increased computing power now allows for much more efficient and advanced neuromorphometry, but to optimize data documentation and interpretation in this new field, standardization is required, as explained in the “at a glance” article by Giorgio Ascoli and his colleagues (Parekh et al., 2015). This paper reviews how databases have become important tools in the dissemination and access to neuronal tracings of different cell types in different species.

Fluorescence detection is inherently more quantitative than the classical precipitation-based immunohistochemical stainings. Its linearity with concentration, its exceptional sensitivity with modern detectors – allowing single molecule detection which has been one of the pillars of the modern super-resolution techniques, and its possibility for multiplexing multiple labels with different spectral separation characteristics in the same sample, puts fluorescence imaging at the foremost front of modern microscopy. A recent reinvention of a century-old idea for optical tomography, i.e. the selective imaging of a thin plane in the three-dimensional object, is called light sheet-based fluorescence microscopy (LSFM) (Pampaloni et al., 2015). By the orthogonal arrangement of illumination and emission paths, multiple advantages that are important for minimally invasive “deep imaging” of living specimens are gained: it provides optical sectioning, penetration depth is increased, illumination is restricted to the detection volume - which reduces probe destruction and phototoxicity. This method is now being used for a variety of applications, including “microtome-free histology.”

The first paper on super-resolution techniques is by Bianchini and colleagues (Bianchini et al., 2015) who provide an overview of the current state of the stimulated emission depletion (STED) technique. Here, the effective excitation volume is “squeezed down” from what can be achieved by lenses (“diffraction-limited”) by overlaying the spot with an equally diffraction-limited beam that switches fluorescence off. The off beam is structured in the shape of a ring with zero illumination at its center. Pumping this beam closes the ring down, reducing the excitation spot down to the remaining zero-hole of the off beam. Progress has been made in resolution, achieving multi-color imaging and the required power of the off beam.

A second method to achieve super-resolution is discussed by Dedecker and colleagues (Vandenberg et al., 2015). In the so-called “localization methods”, the fluorophores are made to glow sparsely enough to be imaged individually. A single emitter will not be able to be imaged as a point because, as in excitation, lenses also cannot focus point images perfectly. However, the individual nature of the blurred spots allows the determination of their center, i.e. it is possible to point at the origin of the spot, which is the single emitter. Repetitive imaging of the sparsely fluorescing scene will thus deliver lists of coordinates for the single emitters, from which a synthetic image can be reconstructed. The methods for making fluorophores light up non-simultaneously, and the challenges in obtaining images from spots are discussed – this is inherently an issue of quantitation, and the possibilities are considered also in comparison to STED.

These applications demand unique properties from the fluorophores used. Higher resolution demands higher photon output. In STED, the fluorophore must be able to be switched off efficiently by appropriate illumination with the off beam. The fluorophore must be photostable at this wavelength, as high powers are needed to squeeze the off ring down. In localization microscopy, fluorophores must be switchable by light or must be made to “blink” at the appropriate interval to generate inhomogeneously sparse images of the single emitters. This requires chemical tricks. These properties are not always found in the fluorescent proteins. Bruchez and his colleague (Yan and Bruchez, 2015) discuss the current state of the field of chemical labeling in biological tissues. Using chemical handles introduced to targets of interest in cells, chemical dyes can be introduced. These methods combine the precision of genetic targeting that the fluorescent proteins also possess, but with virtually limitless options for the actual fluorophore and its properties. These chemical approaches have been instrumental for the advance of super-resolution microscopy of cells and can also be used to encode, in cells, quantitative assays for biochemical events.

The editors are confident that this collection of reviews covering the intersection of imaging, image analysis and quantitative approaches will be useful for cell biologists who wish to introduce or expand quantitative cell biology in their research and strive to better understand some of the new quantitative techniques related to imaging of cells and tissues – and delve into the “real *science*” as defined so befittingly by Lord Kelvin.

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