# Shape and function from motion in biomedical imaging: part 3.

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Structures, shapes and functions are intimately linked in living systems. Their quantification requires unravelling the multidimensional relations they have at multilevel and multiscale, the multiphysics expressions they take, and the intricated spatial and temporal patterns they share. The understanding of dynamic, complex molecular and cellular systems call for the improvement of observational devices among which imaging tools have a major role. We have emphasized here [1] for different objects and objectives how it was important, at the same time, to design new paradigms for information processing and to establish sound mathematical models in order to integrate and control the increasing amount of data and knowledge. The main goal was to propose a lecture grid while revisiting some aspects of image processing, and to stimulate research in the area. In a first paper devoted to motion analysis, some basic and generic issues were reviewed [2] like motion estimation, object tracking, segmentation and motion coupling. A second paper [3] dealt with motion in medical macro-imaging with the idea to propose new windows on its relations with the image capture and image reconstruction. This third and last paper in this series is devoted to biological imaging, e.g nano and micro imaging. Although a number of concerns are shared when going to these scales, the conditions of observation, the objects under study and the problems to address make this research very challenging by the cautions that must be taken to control the experiments, a mandatory condition to get conclusive results and to infer sound conclusions.

***Sample preparation and conditioning***

This is certainly a major difference with the other imaging application areas. In remote sensing, robotics, etc., although objects and scenes may be difficult to analyse (large number of entities, multiple appearances, variations with environmental conditions, etc.), there are only a few elements that have to be controlled beforehand. Even in medical imaging, the clinical process to acquire images remain relatively simple (e.g calibration, parameterization of the modality, injection mode of contrast medium, etc.).

In biology, whatever the object to be examined and before any observation, the preparation of the samples is a critical step: all subsequent image derivations fully depend on its quality. For instance, and beyond the extraction procedure to get it in a proper way, the optical properties of a sample rely on the all components that will be used to fix it: the glass coverslip, the refractive index of the immersion medium, the temperature have an important impact in the image formation and the presence of distortions and aberrations. We should expect at this level more standardization in the experimental protocols in order to significantly reduce the variations between sample properties. In addition, and we will see that below, these properties are directly enhanced by extrinsic markers, which are themselves time-varying (degradation of the fluorescent probes over time, or photobleaching). Tagging techniques for 3D visualisation, for instance, of structures and functions of subcellular entities and processes, made a real breakthrough in biology. They use probes, or markers attached to a targeted protein: they assume that they will not impact the protein and the cell in vivo functions when introduced into the cells.

Emphasizing these aspects does not mean at all that other classical problems are solved: the limitations of the imaging devices (noise, nonstationnary point spread function (PSF), lack of real-time focusing for acquiring image stacks, etc.) as well as the intrinsic variabilities of biological elements and assemblies.

***The biological objects***

They gather molecules, proteins, cells and all the compounds/assemblies to which they can lead, ranging from nanometers to micrometers. Let us first concentrate on cells [4] [5]. A cell may contain some 100000 proteins, each protein representing a functional unit. Functional complexes are in the range of tens of nanometers. The high diversity of cell shapes and motility profiles has been shown in many studies, still most often performed in 2D. For shape extraction, ill-defined boundaries, cell overlapping and touching, background and cell non stationarity are among the difficult problems to tackle. With respect to motility, understanding the migration mechanisms has major medical implications. Embryo development involves cell migration to distant sites where differentiation into specific types occurs and shapes different organs. Migration of tumour cells leads to metastatic cancer. Cell division (and fusion) tracking allows the definition of cell lineage trees and analysis of cellular phenotypes. Accordingly, differentiation of live cell types can be expected through typing motion paths and motion changes. Both aspects are inter-related: morphological changes (membrane protusion, retraction and contraction) and cell-cell interactions are involved in motility pattern and cell shapes may influence the cell fate in embryo. The disambiguisation of these overall relations and the capability to control the underlying mechanisms should lead to new preventive and therapeutic methods. Mechanical stresses induced by movements can control the expression of developmental genes [6]. Biomechanical models should bring important insights for such purpose [7]: they require, to be properly identified, highly prolonged image acquisitions (without altering cell physiology), accurate segmentation algorithms (in particular for cluster formation), efficient tracking methods dealing with multiple and shape-changing targets. These methods will also increase our ability to analyse the reactions of cells, isolated or not, to external stimulations.

These slow processes contrast with others which require high temporal sampling rates. For instance, calcium signalling is a major element in almost all cellular mechanisms. A Ca signal can be sustained, transient or oscillatory and, in the latter case, Ca controls cellular processes such as apoptosis, gene transcription and cell proliferation. Intracellular calcium ion elevations (or propagation waves) can be detected by means of fluorescence tagging methods and highlight their possible implication in the openings of some ryadonine receptor Ca release channels.

Many other fascinating areas are to be explored like dynamics of protein membrane machines, aggregation of biomolecules or protein folding mechanisms [8] [9] [10] which are far from being elucidated. For instance, a recent view based on the concept of 'energy lanscape' emphasizes that the folding reaction follows different pathways. This motivates an active research on folding trajectories of single protein with the difficulty problem of convenient trapping.

***Cellular and subcellular sensing and tagging techniques***

Many solutions are today available to get insights into biological objects (refer to a recent special issue [11]). *Atomic Force Microscopy* (AFM), with the tapping mode in fluid, allows accessing individual biological molecules and thus makes possible the study of their dynamics and their interactions. The time resolution remains however low without reducing the scan area and correction of lateral drift is required. Tracking algorithms over a long time horizon (with the assumption of no sample damage) provide a way for quantitative descriptions of their stochastic movements. *Electron microscopy* (EM) is another mean to explore the structures of biological

specimens over a wide range of scales, from cellular structures to single macromolecules. *Cryo-electron tomography* (CET), with a resolution of about 4-5 nm, offers the means to observe assemblies of multiple proteins with positions, shapes and even conformational changes. However, the radiation of biological samples supposes special caution for preserving their native conformations.

Optical 3D image forming devices are among the most quickly evolving techniques. They have the advantage to achieve dynamic studies on hydrated, living cells [12] [13]. *Fluorescence Resonance Energy Transfer* (FRET) leads to track change in protein conformation, to co-localize and detect protein-protein interaction. *Fluorescence Recovery After Photobleaching* (FRAP) allows studying the intracellular dynamics of proteins within cells (diffusion and mobility of the fluorophore, binding and dissociation rates of labelled proteins). They are both based on intensity measurements and depend on the label concentration. The occurrence of autofluorescence or the use of several fluorophores with close emission spectra lead to signal mixtures difficult to separate. In *Fluorescence Lifetime Imaging Microscopy* (FLIM), the key feature is that the image contrast is related to the lifetime of fluorophores (each one having a unique lifetime). The acquired data depend on extrinsic variables such as oxygen, pH and calcium concentration which, when controlled, may provide highlights on the environmental impact. New advances in sensing are expected with faster scanning (higher than 100 frames/s) and better depth resolution in multiphoton imaging, multiple objective imaging [13] and selective plane illumination or stimulated emission depletion (STED).

These technological advances are accompanied by the design of protein probes. They allow the understanding of the cellular and subcellular mechanisms involved in the synthesis and the delivery of specific macromolecules. Many fluorescent labels have been developed and will continue to be. Beyond quantum dots (made of a nanometer-sized semiconductor crystal core and an external protective shell) which have a broad absorption and a narrow emission spectra, resulting in brighter fluorescence, a very active research is devoted to labelling of recombinant proteins and photocontrollable proteins (photoactivable, photoswitchable).

This short overview is far from covering the all modalities already at our disposal and the exceptionally fast evolution they have. It would have been of interest to mention variants such as Fluorescence Correlation Spectroscopy (FCS), Image Correlation Spectroscopy (ICS), etc. One of the most promising technique is certainly represented by Multifocal Multiphoton Microscopy (MMM) for imaging in 3D the living cell [14], overcoming the diffraction barrier set by Ernst Abbe in 1873. Another example is given by Secondary-Ion Mass Spectrometry (SIMS) and the new Multi-isotope imaging mass spectrometry (MIMS) which offer a way to investigate and quantify tagged molecules in subcellular volumes [15]. In all cases, combination of these techniques will occur that will lead to a new generation of instruments.

***Signal and image processing challenges***

If these technologies are crucial to study spatial and temporal properties in cells and cell organizations, they are not enough. They all suffer from limitations that must be corrected before any analysis. Instrumental noises (photon shot, background, thermal agitation, etc.) and distortions (out-of-focus, illumination fluctuations, non-stationary attenuation due to self absorption, ...) are first to be reduced. Photobleaching impacts the image intensity and the observation time when absorption and scattering effects limit the excitation and detection depth (the refractive index is space and time dependent). Sophisticated deconvolution, denoising and calibration solutions are required in order to extract reliable quantitative measures: to be efficient, they should make use of physical characteristics of the devices. In the same vein, registration or mosaicing has to be applied when multiple fields of view are acquired.

Segmentation and tracking operations remain however the most challenging tasks to achieve. They must deal with large time-varying shapes, high image intensity variations, objects moving in and out, time-dependent object features (fluorophore or chromophore propagation and disparition), photobleaching, etc. Deformable models are confronted to low contrast boundaries and region-based segmentation may be only valid on subparts of the objects. To take one example, tracking multiple molecules (or compounds) in living cells (which can move themselves) relies on the detection of single elements that will be further linked over frames. If the former is already difficult, the latter is even more demanding due to object density and detection errors, object fusion and splitting. Spatial proximity, similarity in appearance and motion, priors on motion modes (these modes may change over time) and object types can fail to establish the time-stamped correspondence (performed either locally on successive images or globally over the image sequence) and to derive sound individual path features (possibly in 3D) and to set motion classes (path signatures) in subsets of objects.

Any quantitative assessment requires multiple sample analysis and reproducible confrontation. Solutions to these problems call for revisiting and improving the all available schemes at our disposal and the classical assumptions onto which they rely. The present methods, with often empirical parameter tuning, are still unable to face this complexity. In-depth validation of preprocessing, segmentation and tracking algorithms is mandatory for opening windows to live cell functions and subcellular processes.

***Conclusion***

The understanding of multilevel mechanisms, with the all complexity they have, from a basic and disease-oriented perspective, is a major challenge and will mobilize a large part of biomedical research in the future. The study of protein and cell assemblies, in such conditions that their intricated cascades of behaviours will be preserved, can not only bring answers to biological and medical questions but also lead to unsuspected issues. Image analysis, applied to joint observations at nanometre to millimetre scales, with its potential to extract, quantify structures and shapes and to track proteins and cells, should bring new insights on mechanisms of life. Significant breakthroughs at experimental and methodological levels are still required to achieve this goal. From an image processing viewpoint, there is a demand for fully innovative algorithms that will be independent of image intensity variations, shape and topology changes and any priors on movements. Cutting edge technologies for in-vivo explorations (e.g. the only natural environment), beyond the endo-microscopes already available, will merge with the all image-guided world developed in computer-assisted medicine.

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