Probing molecular processes in live cells by quantitative multidimensional microscopy

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Modern light microscopy has become a most powerful analytical tool for studying molecular processes in live cells. Recent advances in sample preparation, microscope design and image processing allow the generation of 'multidimensional' data, simultaneously reporting the three-dimensional distribution and concentrations of several different molecules within cells and tissues at multiple time points with sub-micron spatial resolution and sub-second temporal resolution. Thus, molecular interactions and processes that were approached by biochemical analyses *in vitro* can now be directly monitored in live cells. Here, we address different aspects of multidimensional microscopy and, in particular, image quantification and the characterization of molecular dynamics, as applied to the study of cell adhesion.

This is a high time for light microscopy. Over the past several years, major progress was made in different aspects of fluorescence tagging of cells, microscope hardware development and image processing, which convert the light microscope into one of the most powerful analytical tools in molecular cell biology. These include the synthesis of new generations of bright and more stable fluorophores with a narrow emission spectrum, the improvement of the sensitivity, dynamic range, image size and signal-to-noise properties of scientific-grade digital imaging detectors, and the optimization of optical microscope brightness and resolution. Moreover, a growing number of new spectroscopic approaches have been developed, which provide information not only on the distribution of specific molecules but also on their dynamics, biological activity and specific interactions. Some of these topics were addressed in recent reviews[1–4] and will not be further discussed here. We have chosen to focus on three interrelated topics, namely the scope of multi-dimensional microscopy, image quantification and dynamic analysis. The examples that are featured here were derived from our own studies on the molecular organization of focal adhesions and their attachment to the actin cytoskeleton (see Refs 5–7).

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What is 'multidimensional microscopy'?

By the term 'multidimensional microscopy' we refer to the acquisition, processing and display of multiple, two- or three-dimensional images of the same specimen, each of which depicts a different property. These extra dimensions can include the spatial distributions of several molecular components at one or more time points, as well as additional measured or computed views of the same field, using other imaging approaches (e.g. different transmitted light modes, interference reflection, fluorescence energy transfer and a variety of other spectroscopic methods). How can we extract new information from the analysis of multiple images?

In Fig. 1a we present a rather simple example of such image collection, where we show the distributions of two molecular constituents of focal contacts (phosphotyrosine and paxillin), actin filaments and the DAPI-labeled cell nucleus. In addition, we present the phase-contrast image of the same cell depicting the nucleus, various phase-dense vesicles and other organelles. Choosing a proper color palette contrast and intensity, a 'merged' image can be generated combining information from all five images. Furthermore, additional images can be generated to visualize the spatial relationships between components. In fluorescence ratio images, for example, the color of each pixel represents the ratio value between the intensities of two fluorescent channels in the respective location. Thus, a uniform ratio image represents similarity in the distributions, while variations in ratio values represents heterogeneity in the molecular content of a subcellular structure[5] (Fig. 1b). To quantify the degree of similarity between pairs of components, a correlation coefficient can be calculated, where the value of '1' corresponds to the highest positive correlation and '–1' to strong negative correlation (see Fig. 1c). The calculated ratio images and the correlation coefficients allow not only visualization of molecular inter-relationships but also provides a score for the molecular heterogeneity of the structure of interest (matrix adhesions in the example shown here). Such correlation values shed light on the molecular interactions responsible for the assembly of adhesion sites, help interpret their dynamics and, ultimately, their functions[6] (see below). A comprehensive paired correlation analysis can be highly informative if used to generate dendrograms such as the one shown in Fig. 1d, where a hierarchical tree of similarity between cellular distribution patterns of different molecules

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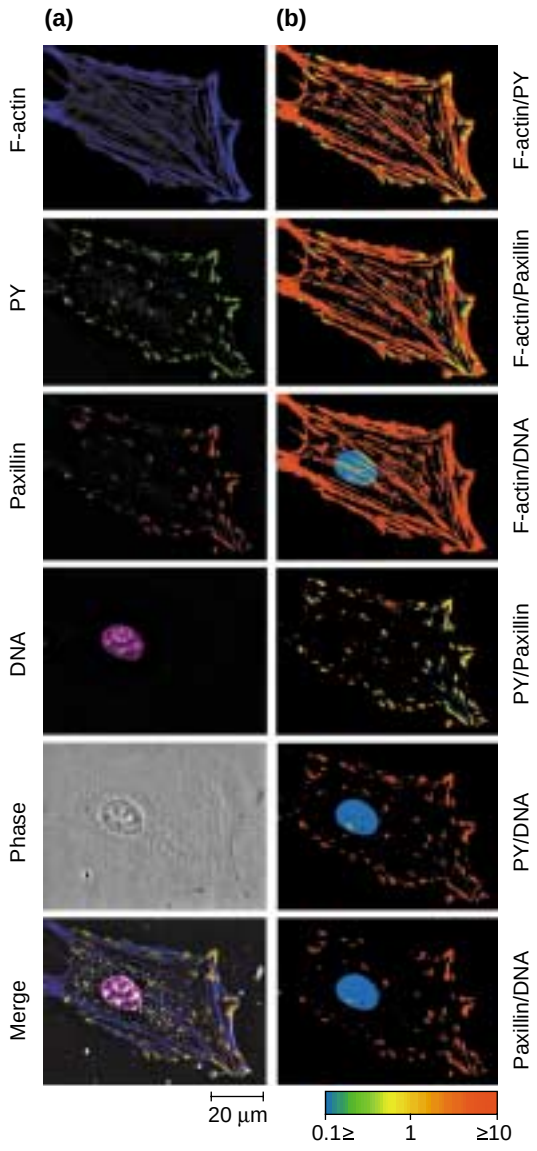

(c)

	PY	F-actin	DNA	Paxillin
PY	1	0.47 ± 0.12	0.28 ± 0.08	0.76 ± 0.04
F-actin		1	0.16 ± 0.08	0.58 ± 0.09
DNA			1	0.28 ± 0.09
Paxillin				1

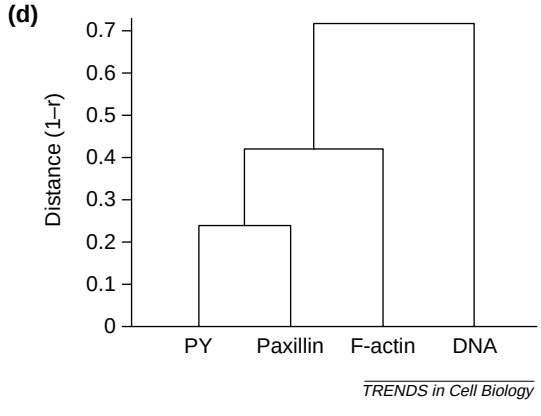

Fig. 1. Multicolor fluorescence microscopy imaging of four different cellular components. REF52 cells were fixed 24 hours after plating, and multistained for F-actin (using TRITC-conjugated phalloidin), phosphotyrosine (PY; using polyclonal anti-phosphotyrosine antibodies followed by FITC-conjugated goat-anti-rabbit antibodies), paxillin (using monoclonal anti-paxillin antibodies followed by Cy5-conjugated goat-anti-mouse antibodies) and DNA (using DAPI). Fluorescent microscopy images, showing separately the cellular distribution of each of the four labeled components within the same cell, were acquired using four different excitation and emission filter sets which are selective to either TRITC, FITC, Cy5 or DAPI fluorescence. (a) Images presenting separately and together ('merge') the distribution of F-actin, PY, paxillin, DNA and the phase-contrast for a single cell using blue, green, red, purple and gray colors, respectively. For merging, the phase image was inverted. (b) Quantitative characterization of the spatial relationships between the six pairs of the four labeled components by fluorescence ratio imaging between components (FRIC), as explained in Ref. 5. Ratio values are presented in a spectrum scale from blue (lowest ratio) to red (highest ratio), enhancing local variations in the component concentrations[5]. (c) Quantitative characterization of the similarity in the spatial distribution between the labeled components based on cross-correlation coefficients between their images, r. Results were averaged (± standard deviation) for 15 cells. (d) A dendrogram obtained using (1–r) as the distance between components (MATLAB functions, LINKAGE and DENDROGRAM). The dendrogram indicates the hierarchy in overlaps of component distributions apparent for the focal contact markers PY and paxillin.

is presented. This approach appears to be rather robust (as it does not depend on interpretation of the images) and might be further applied for analysis of three-dimensional images or dynamic processes in live cells (see below).

Image segmentation and quantitative analysis

Quantitative image analysis determines the amount of specific molecules associated with defined subcellular structures as well as the morphological parameters of these sites (e.g. dimensions, shape, orientation and distribution). In order to perform such analysis, the structure must be unequivocally defined – namely, the labeling of the background ('noise') and of the structure ('signal') must be distinguished through a process known as 'image segmentation', leading to recognition of discrete 'objects' in the picture. Ideally, segmentation can be based on a 'binary' decision if an image pixel intensity is well above a certain defined threshold. In reality, quantitative fluorescent microscope image analysis is rarely that simple: a typical fluorescent image of cells has a non-uniform background, usually higher for thicker regions of the cell, or lower near the nucleus if its volume excludes the label. Thus, the use of a single threshold level to segment all the regions of interest does not give an accurate estimate for signal-versus-noise levels.

To illustrate this problem, we again use the labeling for focal adhesion components, based either on immunofluorescent labeling of fixed cells[5,7] or on the expression of fluorescent-fusion proteins in live cells[6]. In our experience, attempts to segment such images are far from trivial: how do we define a *bona fide* adhesion site? Do all structures that stain for vinculin above a certain background

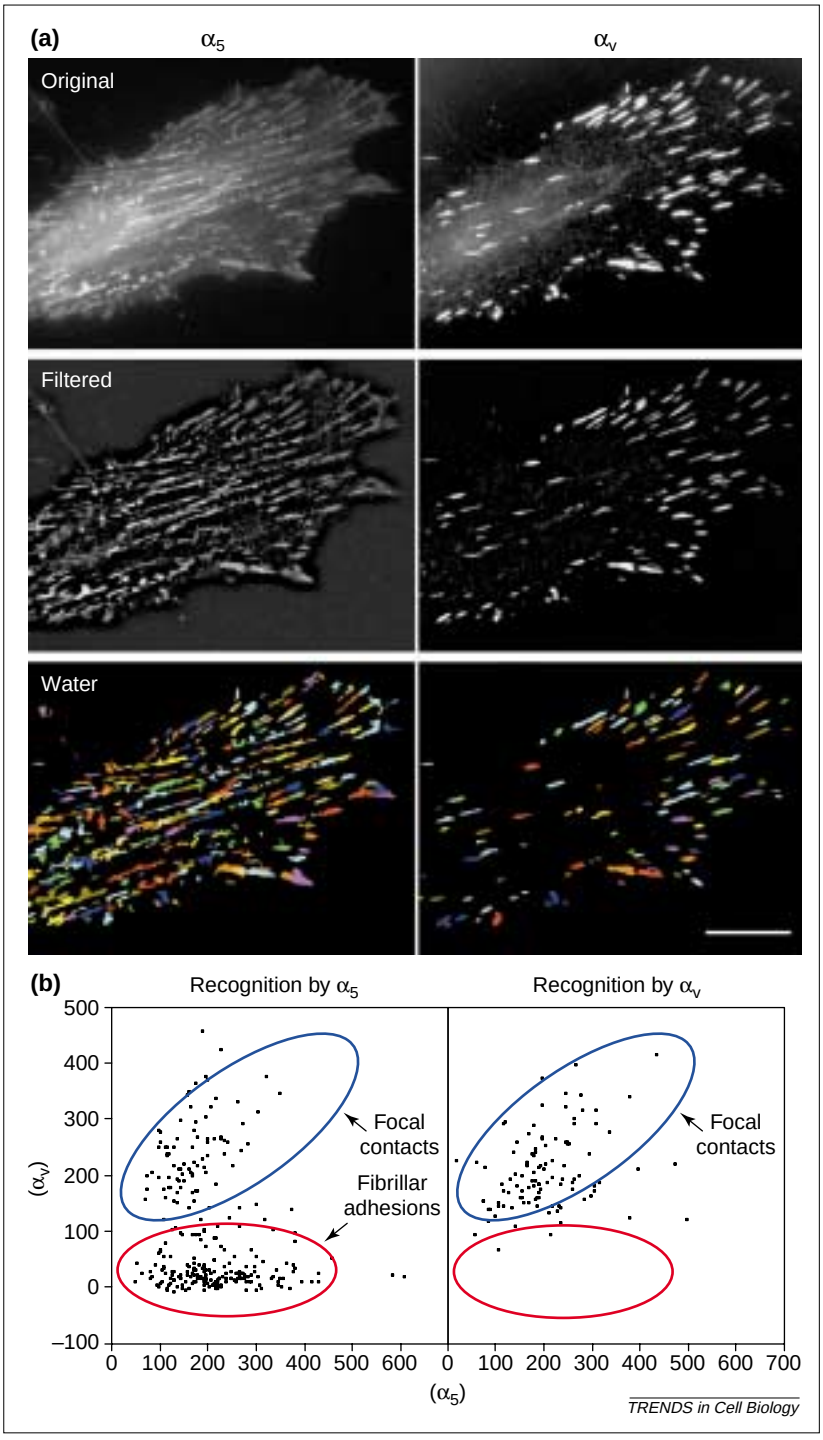

Fig. 2. Quantitative analysis of structures by computerized object-recognition. Human foreskin fibroblasts were fixed 24 hours after plating and double-stained for α_5 and α_v integrins. (a) The original images (top row) shows the typical localization of α_5 and α_v integrins in cell-matrix adhesion sites within the same cell. Note the diffuse fluorescence in the thick region at the center of the cell, which adds a non-relevant and variable contribution to the intensity of cell-matrix adhesions. Middle row: the images after background subtraction, which eliminates diffuse gradients of intensities in the image. Each individual cell matrix adhesion site was recognized by the 'Water' algorithm (bottom row) and is presented by random colors[5]. Bar, 20 μm. (b) Scattergraphs showing the average intensity of α_5 versus α_v integrins in each adhesion site that were segmented according to the α_5 labeling (left) or according to the α_v labeling (right). The α_5-labeled sites (left) could be classified into two different populations: focal contacts, which contain variable levels of α_v integrin, and fibrillar adhesions, with α_v integrin level close to zero. By contrast, α_v-labeled sites (right) appeared to be a uniform population, encompassing the focal contacts.

qualify to be defined as 'focal adhesions' irrespective of their size, shape and subcellular distribution? What should be done when the labeling intensity of defined adhesion sites in one region of the cell is lower than the background in some other regions? The solution for such a dilemma is to apply segmentation after determination and subtraction of local background levels, yielding flattened background with zero average, over which high-intensity 'mountains' define the regions of interest[5]. Development of suitable procedures for 'background filtration' is crucial for accurate image processing, particularly when background levels are high, relative to the signal. Often this is not sufficient, mainly when the regions of interest are closely packed and the separation between structures is difficult. To address such problems, additional image-processing tools must be developed. For 'patchy' structures such as focal adhesions or cytoplasmic vesicles, we have used the 'watershed' segmentation algorithm, which separates neighboring structures according to the intensity 'valley' between them[5]. In the example shown in Fig. 2a, the raw images of cells, double-labeled for two different integrins, are shown (top panel), as well as the filtered images (after local background subtraction), which do not contain the diffuse cytoplasmic labeling. The 'water' segmentation defines the individual adhesion sites and color-codes them. Once segmented, additional information can be obtained about the adhesion sites, their dimensions and composition.

In Fig. 2, the segmentation was carried out for two molecules, namely α_5 or α_v integrin subunits, which clearly have only partially overlapping subcellular distributions. Comparing the integrin composition of the segmented structures, as shown in the scattergraphs (Fig. 2b) reveals two distinct subpopulations of structures (focal contacts and fibrillar adhesions, in this case) that differ greatly in their integrin composition. Such analyses can generate statistical data on the morphological properties (e.g. size, orientation, elongation) and molecular composition of the segmented objects (Fig. 2b). The importance of comprehensive and precise image segmentation should not be underestimated as molecular processes in cells occur in specific compartments that can often be heterogeneous and differ greatly from the 'average' structure studied biochemically.

This is just one example of image segmentation that allows us to obtain quantitative information about a specific subcellular structure. There are additional examples of image segmentation studies including analyzing endocytotic and exocytotic vesicles[8], mitochondria and Golgi[9], chromosomes,

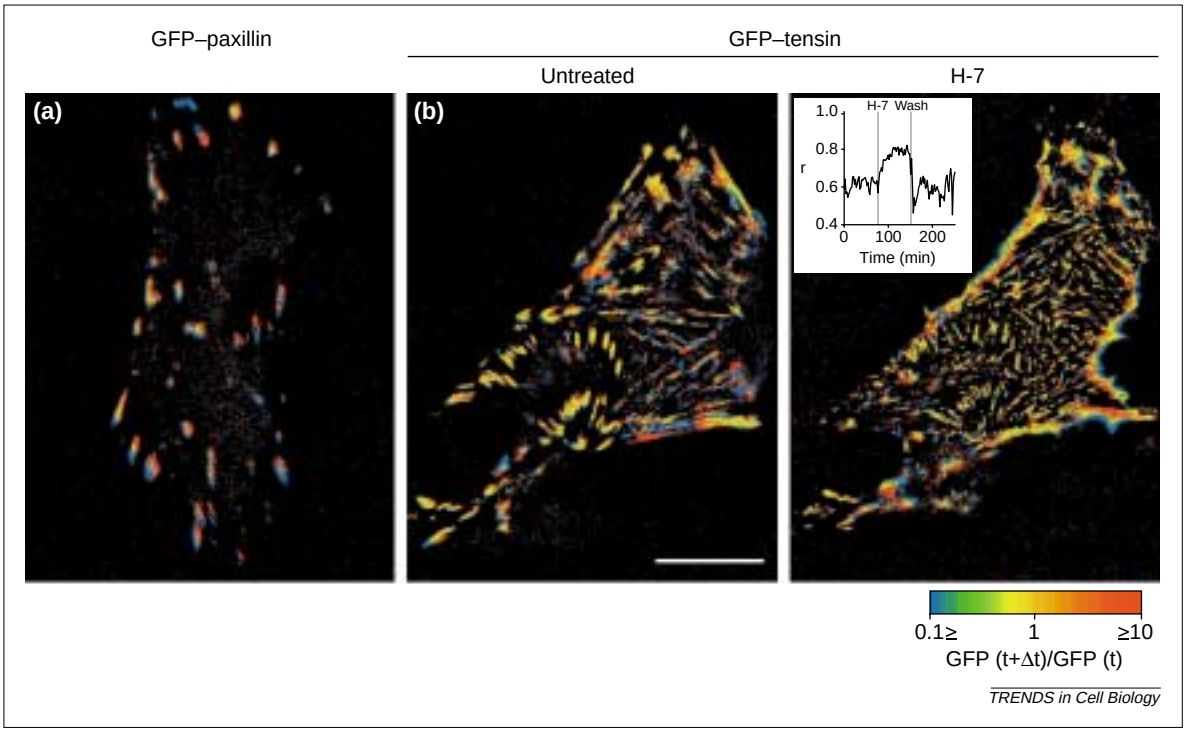

Fig. 3. Analysis of dynamic changes in the location and organization of cellular components. Human foreskin fibroblasts were analyzed by time-lapse fluorescence recording starting 24 hours after transfection, with GFP–paxillin (a) or GFP–tensin (b), and plating. The dynamics of the GFP-tagged proteins is demonstrated by fluorescence ratio imaging in time (FRIT) for pairs of images taken at 10-min intervals, as described in Ref. 6. Thus, a red color indicates a new structure, a blue color indicates a structure that had disappeared, and a yellow color indicates a structure that exists in the same location at the two time points. GFP–paxillin is located only in focal contacts and thus reveals their dynamics (a). Note the centripetal movements of many focal contacts as reflected in the FRIT image by a peripheral blue structure adjacent to a more centrally located red structure. GFP–tensin is located both in focal contacts and fibrillar adhesions (b). Fibrillar adhesions are dynamic structures, as indicated by their blue and red colors in the FRIT image. However, upon inhibition of actomyosin contractility by H-7, their movements are inhibited, as indicated by their yellow FRIT color. The insert shows a correlation analysis of the dynamics of fibrillar adhesions before the treatment, during the incubation with H-7, and after washing away the drug. For that purpose, the correlation-coefficient (r) was calculated between pairs of subsequent time points (with an interval of 2 min) for the duration of the movie in a constant region that contains only fibrillar adhesions. Note that, during the incubation with H-7, the correlation coefficient was higher, indicating the inhibition of fibrillar-adhesion mobility. Bar, 20 μm.
Abbreviation: GFP, green-fluorescent protein.

centrosomes and nuclear substructures[10]. It should be emphasized that for each system (namely, antigen and cell type) specific segmentation and quantification procedures have to be developed or at least adapted as, unfortunately, there are no general solutions for image interpretation.

Quantification of molecular dynamics

The study of molecular dynamics is naturally performed with live cells. Despite being experimentally demanding, and usually limited to small number of examined cells, the use of live cells for imaging work has been a source of new and exciting information. Direct measurement of dynamic cellular processes in live cells has major advantages over analyzing cell populations that are fixed at various time points and analyzed statistically. The inherent variability between individual cells, does not only require an analysis of a large number of cells, to narrow the statistical error, but can often totally obscure significant differences, which might be readily apparent by following, in time, a single cell. The most common raw material for dynamic analysis are time-lapse movies of cells containing inherently fluorescent molecules expressed by the cells or specific sets of fluorescent molecules, including chemically labeled proteins, lipids or carbohydrates. Obviously, the time resolution of such movies is crucial and should be adjusted to the rate typically displayed by the molecular process studied. Thus, imaging of ion fluxes or diffusion of small molecules requires millisecond resolution, slower processes such as vesicular transport or cytoskeletal reorganization can be monitored over seconds, and processes such as fibroblast motility or cell division can be recorded at somewhat longer intervals. Recording data at both high sensitivity and high temporal and spatial resolution is difficult with current equipment. Video-rate and special fast charge-coupled device (CCD) cameras can capture images at millisecond rates. However, their sensitivity is usually lower compared with slow-scan digital CCD cameras. The latter can record fast dynamics only for small subregions of their area. It appears that there is still an urgent need for improved fast cameras with high (close to 100%) quantum efficiency and very low internal noise.

Time-lapse movies are often spectacular and very informative – yet how can one quantify dynamics

based on them? Dynamic parameters are approached by a variety of methods such as image-correlation analysis, feature tracking, photobleaching and photoactivation. Use of image correlation for comparing the distribution of a fluorescent reporter molecule at different time points is based on the principle that the more dynamic a process is, the lower will be the correlation between images taken at different time points. It should be appreciated that correlation analysis can, in principle, be applied to any set of images provide that noise levels are reasonable. In the example shown in Fig. 3, we have produced 'temporal ratio images' of GFP–paxillin or GFP–tensin, from images recorded at two different time points (10 min apart). To generate these images, the ratio between the intensity values recorded at each pixel at the two time points is calculated and represented by a color, ranging from red (new appearance) to blue (total disappearance of labeled site). Paxillin-associated adhesion sites, in the stationary cell shown here, were quite dynamic and apparently translocated centripetally, as manifested by the directions of the red and blue adhesion colors (Fig. 3; see also Ref. 6). Tensin, shown in Fig. 3b, is associated with both focal contacts and fibrillar adhesions, and while the former did not change much in consecutive time samples (represented by the yellow color), the fibrillar adhesions appear to be highly dynamic. Addition of the kinase inhibitor H-7, which blocks actomyosin contractility, inhibited the movement of fibrillar adhesions and abolished focal contacts, as manifested by the yellow color of the fibrillar adhesions in the ratio image (compared with their red-blue colors before treatment; see also Ref. 6). As mentioned above, the dynamic reorganization of the structure (adhesion sites, in our case) can be quantified by the temporal correlation coefficient that reports on the degree of similarity between the images of the same field at two time points. In the graph insert to Fig. 3, the changes over time in the correlation between GFP tensin patterns is shown, pointing to the increase in correlation (namely reduction in mobility) upon drug treatment. It is noteworthy that spatial and temporal correlation analyses can be extended to a wide variety of images, thus providing new insight into molecular processes in live cells.

Object tracking is another powerful quantitative tool that can shed light on dynamic processes in live cells. Tracking involves image segmentation (usually, three-dimensional) and tracing of the changes in the location and shape of each labeled structure over time. Tracking algorithms can define the center of mass of the objects of interest, as well as the rate and direction of their movement, their persistence, changes in volume and surface area, etc. Examples for tracking of migrating leukocytes in culture over time[11] or individual nuclei in developing *Drosophila* embryo[12] were described, but use of the approach, as a whole, for studying subcellular dynamics is very demanding and rarely used.

A variety of other imaging and point-probing methods shed light on molecular dynamics in live cells. For example, fluorescence photobleaching recovery (FPR or FRAP[13]) can provide direct information on diffusion rates of specific molecules, active transport or directional flow and detect changes due to interactions and assembly of complexes. In this procedure, labeled structures in live cells are locally photobleached using an intense laser beam, and the rate and isotropy of the recovery of fluorescence into the bleached area is measured. A complementary method for marking specific sites is the photoactivation of caged fluorophores, creating labeled spots or complex patterns, which can be followed over time[14]. The finding that GFP increases its red fluorescence when irradiated[15] extends the photoactivation methodology to inherently fluorescent cell lines for time- and space-resolved microscopic analysis of spatially resolved sites in live cells. Probing the fluorophore environment is possible by FRET[16] and FLIM[17,18]. Fluorescence correlation spectroscopy[19,20] analyzes the dynamics of changes, without introducing laser spot bleaching, by exploiting the fluctuations in fluorophore number. The improvement of sensitivity and noise characteristics of new imaging detectors enables the translation of even small intensity differences in the acquired images into an actual number of labeled molecules. This, for example, is the basis of speckle microscopy analysis[21] applied to record movement and assembly of single molecules in specific subcellular compartments.

Concluding remarks

Quantitative microscopy, and in particular examination of molecular processes in live cells, is going through a phase of major progress. A small number of new (as well as some more traditional) approaches, were overviewed here, but the field of advanced light microscopy as a whole is currently going through an unprecedented period of development. There are still major gaps between the current state of the technologies of light microscopy and the ambitious goal, to be able to visualize and quantify multiple molecular processes at near-molecular resolution and at very high temporal resolution, yet these gaps are constantly being narrowed and bridged. The traditional spatial resolution limit was crossed[22] using structured illumination[23] and other approaches[24], and a growing numbers of high-resolution spectroscopic approaches are adapted for light microscopy. These developments will certainly open new avenues for studying molecular events in the context of the live cell.

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Mechanisms that regulate mechanosensory hair cell differentiation

Ulrich Müller and Amanda Littlewood-Evans

Hair cells of the vertebrate inner ear are mechanosensors that detect sound, gravity and acceleration. They have a specialized cytoskeleton optimized for the transmission of mechanical force. Hair cell defects are a major cause of deafness. The cloning of disease genes and studies of model organisms have provided insights into the mechanisms that regulate the differentiation of hair cells and their cytoskeleton. The studies have also provided new insights into the function of receptors such as integrins and protocadherins, and cytoplasmic proteins such as Rho-type GTPases and unconventional myosins, in organizing the actin cytoskeleton.

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Hair cells derive their name from the array of actin-rich finger-like protrusions – the stereocilia – that project from the apical cell surface (Fig. 1). Stereocilia contain mechanically gated ion channels that open or close upon deflection of the stereocilia. The resulting changes in ion flux lead to changes in the rate of neurotransmitter release from hair cells onto innervating neurons. In this way, a mechanical signal is converted into an electrochemical neuronal signal[1].

Hair cells are distributed throughout several sensory epithelia (Fig. 2). In the cochlea, the organ that perceives sound waves, they are organized as one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs). In the vestibule, the sensor for acceleration and gravity, they are segregated into five patches[2]. Hair cells are attractive models for the analysis of mechanotransduction at the cellular, molecular and biophysical levels, and intense efforts are under way to identify the still-elusive mechanically gated ion channel in vertebrates[3]. Hair cells are also of medical interest because defects in these cells lead to hearing loss. Although hair cells can be regenerated in avian hearing organs, humans cannot regenerate cochlear hair cells[4]. It is therefore important to elucidate the mechanisms that govern the genesis and regeneration of hair cells in order to help develop rational strategies for therapeutic intervention. Recent years have brought substantial progress in defining the molecules that regulate hair cell differentiation and the assembly of the hair cell cytoskeleton. Here, we summarize some of these findings and point out several open questions.

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