Imaging of keratin dynamics during the cell cycle and in response to phosphatase inhibition

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**Abbreviations:** CFP, enhanced cyan fluorescent protein; FRAP, fluorescence recovery after photobleaching; GFP, enhanced green fluorescent protein; IF, intermediate filament; KF, keratin filament; LUT, look up table; OA, okadaic acid; OV, orthovanadate; YFP, enhanced yellow fluorescent protein.
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I. Introduction

Among the various intermediate filament (IF) cytoskeletons the keratin network is considered to be particularly stable conferring mechanical resilience onto epithelial tissues. Keratin filament (KF) disruption therefore results in epithelial weakening as is the case in a number of human diseases (Irvine and McLean, 1999; Porter and Lane, 2003; Smith, 2003). Accordingly, mice synthesizing dominant negative keratin mutants or lacking a keratin cytoskeleton in certain cell populations develop various pathologies in the affected epithelial tissues (Coulombe and Omary, 2002; Herrmann et al., 2003; Porter and Lane, 2003). In vitro analyses provide further evidence for the particular mechanical resilience of KFs (Ma et al., 1999; Ma et al., 2001).

It has turned out, however, that KF networks are not simply rigid scaffoldings that are anchored at specific desmosomal cell-cell adhesion sites as has been portrayed in text books but that they are instead highly dynamic cytoskeletal components which are subject to continuous remodeling and rejuvenation (Windoffer and Leube, 1999; Yoon et al., 2001; Windoffer et al., 2004). In addition, considerable motility of KFs and KF precursors has been documented (Windoffer and Leube, 1999; Yoon et al., 2001; Helfand et al., 2003b; Liovic et al., 2003; Windoffer et al., 2004). These properties enable the KF cytoskeleton to respond constantly and quickly to special cellular requirements. The main technology that has helped to identify and analyze these dynamic features is live cell imaging in which cells that synthesize fluorescent keratin polypeptides are monitored by time-lapse fluorescence microscopy. With this technique key questions that relate to basic organizational principles of the KF system are being addressed such as:

- How and where are KFs formed in a living cell? In contrast to the microfilament and microtubule systems almost nothing is known about the biosynthesis and morphogenesis of
KF networks. This lack in understanding is due to the fact that IFs are intrinsically nonpolar since their tetrameric building blocks are symmetric (Parry and Steinert, 1999; Strelkov et al., 2003). This absence of directionality thus precludes vectorial growth as is characteristic for the other cytoskeletal filament components. Furthermore, specific initiation sites that could support the formation of the KF network have not been identified on a molecular level. Live cell imaging of cells that are devoid of an intact KF system but re-build a new network as is the case in certain cells during mitosis (e.g., Horwitz et al., 1981; Franke et al., 1982; Lane et al., 1982; Tolle et al., 1987) now affords the examination of de novo KF network formation in situ.

- How does KF network turnover occur? Two principle models have been proposed: continuous exchange of subunits throughout the entire filament system (Miller et al., 1991; Miller et al., 1993) or usage of certain organizing centers in specific cellular domains (Windoffer et al., 2004 and references therein). Given that the turnover of the KF system exceeds by far the very slow biosynthetic replenishment, live cell analysis is the method of choice to resolve this issue. Photobleach experiments provide powerful means to identify turnover intermediates and to map them to distinct cellular topologies.

- How does the keratin cytoskeleton change during the cell cycle? Prominent cell shape changes are associated with cell division and different phenotypic alterations of the keratin system have been observed in dividing cells (cf. Windoffer and Leube, 2001). Live cell monitoring allows the determination of consecutive steps of the various re-organization events and to relate them to specific cell cycle stages.

- How are keratin dynamics regulated? KFs form spontaneously and very rapidly in vitro without any additional factors (Herrmann et al., 2003). In contrast, living cells regulate this
process tightly. They are able to increase the pool of non-filamentous keratins to almost 100% (e.g., during mitosis). The balance between the different organizational states of keratins (soluble, granular, filamentous) is dependent on the state of cellular phosphorylation (e.g., Omary et al., 1998; Strnad et al., 2001; Coulombe and Omary, 2002; Strnad et al., 2002). By in vivo imaging one can now directly assess the effects of specific enzyme inhibitors on keratin organization. Combinatorial application of different drugs furthermore allows to define complex relationships between morphotype and the action/presence of regulatory factors.

- How do diseases affect the dynamic and organizational properties of the keratin cytoskeleton? Synthesis of fluorescent keratin mutants in cDNA-transfected cells (Werner et al., 2004), exposure of cells producing fluorescent keratins to various stress stimuli (Liovic et al., 2003), and synthesis of fluorescent keratins in pathologically altered cells (Riley et al., 2002) facilitate the examination of dynamic alterations of the keratin system in pathophysiologically relevant contexts. In such model systems the pathogenesis of genetically induced keratin diseases such as epidermolysis bullosa simplex (Irvine and McLean, 1999; Porter and Lane, 2003; Smith, 2003), of environmentally-determined keratin alterations as they occur in toxic liver disease leading to Mallory body formation (Cadrin and Martinoli, 1995), and of various epithelial diseases in the context of cellular stress responses (Coulombe and Omary, 2002) become accessible to close inspection.

These examples shall suffice to point out the potential of live cell imaging to provide novel and unprecedented insights into the dynamic organization of the keratin cytoskeleton. In the following sections we will outline important aspects of this methodology. Emphasis will be on the microscope setup, fluorescence recording and data interpretation that are being used in our laboratory while the reader is referred to excellent reviews that present more general
considerations on the topic (e.g., Rizzuto et al., 1998; Periasamy and Day, 1999; Lippincott-Schwartz et al., 2001; Stephens and Allan, 2003).

II. Materials and Instrumentation

A. Special Reagents

- Okadaic acid (OA): The serine-threonine phosphatase inhibitor (Sigma, St. Louis, MO) is dissolved in DMSO at 10 µg/ml and can be stored at -20°C. It is added to cells at final concentrations between 0.1 µg/ml and 1 µg/ml which should selectively inhibit protein phosphatases 1 and 2A, although higher concentrations are known to also inhibit phosphatase 2B (Cohen et al., 1990; Vandre and Wills, 1992).

- Sodium orthovanadate (OV): The tyrosine phosphatase inhibitor (Aldrich Chemical Corporation; Milwaukee, WI) is freshly dissolved in distilled water at 1M for each experiment. It is either used directly or incubated prior to usage for up to 1 h with 50 mM H₂O₂ on ice to generate the more active pervanadate (Feng et al., 1999). Final concentrations range between 2-50 mM, depending on the cell type, for pervanadate between 0.2 mM and 1 mM.

- Hoechst 33342: The vital DNA stain is from Molecular Probes (Eugene, OR). A stock of 0.733 mg/ml is prepared in H₂O and can be stored at 4°C. Addition of 1 µl stock to 4 ml medium is sufficient to stain chromatin efficiently in living cells in less than 30 min.

B. Cell Lines

Preferably, cell lines are used that are well characterized with respect to their IF complement, present an extended KF network, are easy to maintain, and that are amenable to standard transfection methods. Among the many cell lines that are available from cell culture banks
several have proven to be useful for live cell imaging. They include those that are derived from human adenocarcinomas originating either from simple epithelia such as hepatocellular carcinoma PLC cells (ATCC CRL8024), colon carcinoma CaCo-2 cells (ATCC HTB-37), or from complex epithelia such as mammary adenocarcinoma MCF-7 cells (ATCC HTB-22), and those that originate from multilayered epithelia such as vulvar squamous cell carcinoma A-431 cells (ATCC CRL1555). In addition, immortalized HaCaT keratinocytes (Boukamp et al., 1988) are particularly suited for the analysis of the epithelial cytoskeleton. An important and physiologically quite relevant system is provided by primary keratinocytes that are either available commercially (e.g., from Invitrogen GmbH, Karlsruhe, Germany) or prepared from trunks of newborn mice (Hager et al., 1999). Cells are grown in a 5% CO₂ atmosphere at 37°C and should be propagated in growth medium as detailed by the various suppliers.

When selecting a cell type for live cell imaging various aspects should be considered. For example, a very dense and elaborate network of fine filaments is typical for primary keratinocytes while thick filament bundles are characteristic of PLC cells which will be easier to image but may differ in their dynamic behavior. Flat and spread out cells such as PLC and CaCo-2 cells are well suited for high resolution recordings of the complete cytoplasmic volume, especially in peripheral regions. High efficiency of transfection and stable line formation argue in favor of A-431 cells. Another consideration concerns the different types of keratin cytoskeleton restructuring during the cell cycle. While the KFs of A-431 cells are almost completely disassembled into granular aggregates during mitosis, most other cells exhibit only filament aggregation. Finally, the responsiveness of the keratin system to phosphatase inhibitors differs significantly between different cell types both with respect to drug concentration needed to induce alterations and the kinetics of reorganization. These properties have to be determined experimentally in each case.
To examine keratin dynamics in a non-epithelial environment several cell lines have been used. In some cell types such as 3T3-fibroblasts only very thick filament bundles/aggregates are formed (e.g., Domenjoud et al., 1988; Bader et al., 1991; Werner et al., 2004). In contrast, an extended KF network is observed in cDNA-transfected human small-cell carcinoma SW13 cells that are derived from the adrenal cortex and do not synthesize any IF protein (ATCC CCL-105; Hedberg and Chen, 1986). A similarly complex KF system is also formed in transgenic human H36CE1 lens cells (Liovic et al., 2003).

For imaging cells should be transferred to phenol red-free Hanks' medium, since it is superior to other media such as DMEM for obtaining high quality pictures. The medium contains Hanks' salt solution, 25 mM Hepes, MEM non-essential amino acid solution and MEM amino acid solution, 100 U/ml penicillin, 100 μg/ml streptomycin, 5% fetal calf serum (all from Invitrogen), 4.8 mM N-acetyl-L-cysteine (Sigma), pH 7.4. Addition of ascorbic acid (0.5 mg/ml; Sigma) may improve fluorescence stability in some cases.

C. Microscopes
The methods described here were developed primarily for the following microscope setups:

- *Olympus IX 70* inverse fluorescence microscope (Hamburg, Germany): The microscope is equipped with a Polychrome IV monochromator (TILL-Photonics, Gräfelfing, Germany). Excitation is at 496 nm for enhanced green fluorescent protein (GFP), 498 nm for enhanced yellow fluorescent protein (YFP) and 436 nm for enhanced cyan fluorescent protein (CFP). Emission filter U-M61008 is used for all colors. The microscope is equipped with a shutter (Uniblitz VMM-D1; Vincent Associates, Rochester, NY) to switch between fluorescence and phase contrast microscopy. A piezo-driven z-axis stepper (0.1 μm steps; Physik Instrumente, Karlsruhe, Germany) is attached to a 60x 1.4 N.A. oil immersion objective that is used for
most applications. The entire microscope is encased by a Plexiglas chamber and heated to 37°C. Cells are viewed in γ-irradiated Petri dishes with a glass bottom (Mattek, Ashland, MA). The motorized microscope stage (Märzhäuser, Wetzlar, Germany) is operated with a joystick that is located outside the chamber. Image recording is with an IMAGO slow scan charged-coupled device camera, and the entire system is controlled by TILLvisION software (both from TILL-Photonics). For 3D-delineation of structures, multiple focal planes are recorded at each time point by using the piezo stepper. The resulting picture stacks are either projected on top of each other or are used to prepare 3D-reconstructions with the help of Amira software (TGS; San Diego, CA).

Alternatively, any standard epifluorescence microscope can be employed. Such a microscope should be equipped with appropriate filter sets (excitation, dichroic, emission) to image the fluorescent proteins of interest. For multicolor recording different filter sets are needed. They should be mounted in such a way (e.g., in a filter wheel) that they can be quickly switched with a motorized filter changer. In addition, it is important that the excitation light can be controlled with a shutter to minimize illumination-induced bleaching and phototoxicity. High-sensitivity digital cameras suitable for fluorescence microscopy can be attached to standard microscopes via a C-mount. ImagePro Plus software (Media Cybernetics, Silver Spring, CA), MetaMorph (Downingtown, PA), NIH Image (freeware at http://rsb.info.nih.gov) and/or software provided by the various microscope manufacturers can be used to grab images, to operate the filter changer and to control the shutter. A special culture chamber was designed and constructed by us to examine cells for over a day without any signs of vitality loss (see below).

- **Leica confocal laser scanning microscope** (model TCS SP2; Leica Microsystems, Wetzlar, Germany): For YFP/GFP-detection the 514 nm line of an argon/krypton laser is used in
combination with dichroic DD458,514. For selection of a defined range of emitted light the monochromator is set to 525-625 nm. 100x1.4 NA oil PLAPO objective and a 63x 1.4 N.A. oil immersion objective are useful for high resolution imaging. Other user-friendly confocal laser scan microscopes work equally well for live cell imaging.

D. Culture Chamber

For upright microscopy, a culture chamber was designed that can be mounted directly onto the stage of either an epifluorescence microscope or a confocal laser scan microscope (Fig. 1). The circular chamber is rather small (diameter: 14 mm; height: 1.5 mm) to reduce disturbing vibrations and to allow quick exchange of culture medium. It is embedded in a steel frame with three drill holes (1 mm diameter) for in- and efflux of culture medium and placement of a temperature sensor (Fisher Scientific GmbH, Nidderau, Germany). A peristalsis pump with adjustable flow rates is used to exchange the culture medium either continuously or intermittently. A 16 mm diameter cover slip is placed on a self-made thin silicon ring on top of the culture chamber and a steel plate is screwed on top of the entire assembly to seal it tightly. Note that cells are growing in an inverted position which, however, is of no consequence to adhering cells. Underneath the culture chamber is the larger heating chamber (diameter: 20 mm; height: 8 mm) that is encased by 1 mm thick glass plates at the top and bottom. This chamber is filled with continuously circulating and preheated water to maintain a constant temperature of 37°C in the culture chamber as measured by the temperature sensor that is positioned next to the observation field.

III. Procedures

A. Generation of Cell Lines Producing Fluorescent Keratin Filaments

1. Preparation of cDNA Constructs
In most instances strong promoters are selected for high level expression of fluorescent fusion proteins and good results are generally obtained with the immediate early promoter elements of the cytomegalovirus (CMV) promoter that is present in many commercially available vectors. The SV40 promoter and the actin promoter work similarly well in many cell types. Preferably, the cDNA coding for the target protein precedes the fluorescent protein-encoding part, although the reverse arrangement works satisfactorily in most though not all instances. Comparison of both construct types helps to exclude position-dependent artifacts of the fluorescent hybrid proteins (see, e.g., Fig. 2). Suitable cloning vectors are available from various companies (e.g., Clontech Laboratories [Palo Alto, CA], Qbiogene [Carlsbad, CA]). These vectors usually include also a polyadenylation cassette at the 3'-end of the hybrid cDNA and contain separate selection cassettes. Among the fluorescence tags that excel in terms of fluorescence intensity and low degree of bleaching, and that do not influence the distribution patterns of chimeras (e.g., by multimerization) are CFP (433 nm excitation; 475 nm emission), GFP (488 nm excitation; 507 nm emission), and YFP (513 nm excitation; 527 nm emission). The CFP/YFP pair is best suited for co-localization and fluorescence resonance energy transfer experiments (Pollok and Heim, 1999; Lippincott-Schwartz et al., 2001; van Roessel and Brand, 2002). Purification of DNA by Qiagen column chromatography (Qiagen GmbH, Hilden, Germany) is sufficient to obtain high quality DNA for transfection.

2. Generation of Stable Cell Lines

Ideally, fluorescent polypeptides should merely act as neutral tags without disturbing the targeted endogenous structures. It is therefore important to establish stable cell lines which can be analyzed by biochemical and morphological methods to show that the properties of the mutant polypeptides are not different from their endogenous counterparts and to exclude that the chimeras do not interfere with endogenous functions and morphogenesis. Calcium
phosphate precipitation and lipofection have worked well as alternative transfection methods in our hands.

In the case of the calcium phosphate precipitation method 5 µg purified plasmid DNA are dissolved in 219 µl 10 mM Tris-HCl (pH 7.5). After addition of 31 µl 2 M CaCl₂ the mixture is pipetted dropwise into 250 µl 2xHBS (1xHBS is 140 mM NaCl, 0.75 mM Na₂HPO₄, 50 mM HEPES, adjusted to pH 7.1 with 1 N KOH) while vortexing. The mixture is briefly incubated at room temperature and then added to the medium of a culture of subconfluent cells. Cells are washed three times with DMEM after 5 h. Depending on the cell type, transfection efficiency is improved considerably by a glycerol shock. In this case, cells are treated with 15% glycerol in 1xHBS for 2-3 min after which they are washed three times with DMEM. Finally, growth medium supplemented with 2.5 µg/ml amphotericin B and penicillin-streptomycin (100 U/ml; Invitrogen) is added. After 1 to 2 days, cells are seeded onto new plates at very low density, and selective medium is added containing up to 1.25 mg/ml G-418 (Geneticin; Sigma) for neomycin resistance-conferring plasmids, up to 300 µg/ml hygromycin (Sigma) for hygromycin-resistance-encoding plasmids, and up to 1 µg/ml puromycin (Sigma) to select for puromycin resistance.

For lipofection 10 µl serum-free medium is mixed with 10 µl hydrated GenePORTER™2 reagent (PEQLAB Biotechnologie GmbH, Erlangen, Germany) prior to addition of 2 µg DNA that is freshly diluted to 50 µl with diluent B provided in the transfection kit. The resulting mix is incubated at room temperature for 10 min and added afterwards drop by drop to subconfluent cells. The medium is replaced after overnight incubation by new medium, and cells can be analysed after 24-48 h to evaluate transfection efficiency. Subsequently, selective agents are added (see above).
Cultures that are subjected to pharmacological selection are kept in the same dishes with occasional medium changes. Single, drug-resistant colonies are picked and transferred individually into multiwell dishes. Cell clones presenting homogenous patterns of fluorescence are selected for further amplification and analyses.

3. Characterization of Cell Lines

For each construct, several stably transfected cell lines should be subjected to extensive analyses including immunofluorescence microscopy, biochemical assays and ultrastructural examinations to ensure that the fluorescent polypeptides do not interfere with the normal physiology and structural organization of the keratin cytoskeleton and its associated cellular components. Ideally, only those clones should be selected for this type of time-consuming scrutiny in which all cells are positive for the transgene as determined by direct fluorescence microscopy and in which the transgene products show an identical distribution pattern in all cells, except for the dividing cells that rearrange their keratin system. The overall morphology of selected cell clones should be compared by phase contrast or Nomarski microscopy to that of the parent cell line to exclude that the transfection and selection procedures singled out cells with altered properties that are unrelated to the production of the transgene. Indirect immunofluorescence microscopy using standard techniques is performed to determine whether the fluorescent transgenes perturb the keratin cytoskeleton and its associated components in any adverse way. It is necessary to demonstrate that the fluorescent keratin chimeras co-localize completely with the endogenous keratin polypeptides whose overall distribution pattern should reflect that observed in the parent cell line. Further analyses should also include components of the desmosomal keratin adhesion sites and other associated polypeptides such as linkers, bundling factors or signaling molecules (cf. Coulombe and Omary, 2002).
Biochemical properties of the transfected cell clones should be examined to clarify two major questions:

- *Is the size of the fusion proteins as expected?* In standard immunoblots using anti-GFP (e.g., from Molecular Probes) and anti-keratin antibodies (e.g., from Progen GmbH [Heidelberg, Germany], Abcam [Cambridge, MA], Sigma, Biomol [Hamburg, Germany]) the size of the fluorescent protein chimeras can be determined. These analyses also provide evidence whether degradation occurs.

- *Are the fusion proteins correctly targeted to the appropriate cell compartment?* To this end high salt pellets which usually contain the majority of keratins are prepared (Achtstaetter *et al.*, 1986). Comparative immunoblotting of the cytoskeletal fraction and the total cell lysate and/or Coomassie Blue-staining of the enriched cytoskeleton will give reliable estimates of correct topogenesis (Strnad *et al.*, 2002; Werner *et al.*, 2004).

Ultrastructural properties of the transfected cell clones should be assessed by electron microscopy. It is important to check whether IF bundles are formed properly, whether granule formation is increased, and whether the association of KFs with their desmosomal anchorage sites is inconspicuous (e.g., Windoffer and Leube, 1999). Additional immunoelectron microscopy will provide evidence for correct targeting of keratin chimeras and their even incorporation into the KF network.

**B. Imaging of Keratin Filament Dynamics in Interphase**

**1. Preparation of Cells**

Stably transfected cell clones that fulfill the stringent criteria of producing an unperturbed fluorescent keratin cytoskeleton are split and transferred either into culture dishes containing cover slips which can be mounted onto the closed culture chamber, or into culture dishes with
a glass bottom depending on which microscope setup is used. Cells should be grown under standard conditions to low confluence which takes ~1-2 days. The culture medium should not contain selective agents to abolish pharmacologically-induced stress. Healthy-looking cultures are then mounted onto the appropriate microscope stage in Hank's imaging medium. The temperature near the imaged cells should equilibrate to 37°C as determined by the temperature sensor. CO₂ is not needed since the Hepes-buffering of the medium is usually sufficient for pH maintenance. Suitable cells for image recording are then selected by fluorescence screening and phase contrast imaging. The "optimal" cell should be flat and should be representative of all cells in a given culture. Giant cells with several nuclei and cells growing in multiple layers should not be used. Short recordings of phase contrast images will give a fairly good indication of the viability of the selected cells. They should exhibit rapid movements of cytoplasmic structures and abundant motility of their free margins.

2. Image Acquisition

Optimal recording settings have to be determined in each instance. Various aspects are to be considered:

- Size of imaged area: Recordings of large areas are helpful in obtaining a general overview of the various fluorescence patterns in a given culture and to assess the variability of reactivities to specific stimuli. Low magnification imaging will also keep bleaching and phototoxic effects resulting from generation of oxygen radicals at a minimum. Although spatial resolution is rather limited, it will still enable the investigator to decide which cellular domains to analyze further and to determine which recording frequencies are needed to visualize the process of interest. When these parameters are established, high resolution recordings are carried out which will also allow to resolve small structures in restricted cellular compartments and to dissect fast reorganization and transport events. Major
disadvantages of high magnification recordings, however, are the reduced depth of focus necessitating imaging of multiple focal planes and frequent shifts of the documented area out of view due to cell motility or instability of the imaging system, as well as considerable bleaching and increased phototoxicity. In case of the keratin system, e.g., low resolution imaging has provided evidence for oscillating filament motility and overall restructuring of mature filaments (Windoffer and Leube, 1999; Yoon et al., 2001), whereas pictures at highest magnification were needed to delineate keratin turnover stages in the cell periphery (Fig. 3; movie 1; Windoffer et al., 2004).

- **Speed of dynamic process**: The recording frequency has to be adjusted to the speed of the process of interest. Generally, the recording intervals should be such that the motility of the imaged structures can be traced unequivocally between individual frames thus resulting in even motions when viewing the assembled movies. If the imaged fluorescent components undergo rapid shape changes even higher recording frequencies may be necessary. Other factors to be considered are the density of fluorescent elements and their shape heterogeneity. For most purposes of keratin imaging intervals ranging between 30 s and 2 min may provide satisfactory results although resolution of microtubule-dependent movements require much shorter recording intervals (Yoon et al., 2001; Liovic et al., 2003). Similarly, high frequency imaging is needed during times of intense keratin restructuring, e.g., during mitosis and in OV-treated cells.

- **Number of focal planes**: Recording of multiple focal planes is needed to track and visualize structures that, due to intrinsic motility and/or mechanical shift of the imaging system, migrate in and out of the focal plane. Furthermore, structures that extend beyond the optical focus need to be delineated in this way. Most importantly, recordings in multiple planes can be assembled into 3D-reconstructions thereby providing important information on spatial
relationships (see below). Generally, as many confocal planes as possible should be recorded although fewer may suffice in flat cells, as is the case for most interphase cells, than in round cells, which are generated upon entry into mitosis or by treatment with phosphatase inhibitors. Again, bleaching and phototoxicity are limiting factors. The scanning time should be short enough to avoid distortion of structures due to their movement during the recording. We found that the scanning time should be kept below 30 s with recovery intervals from 30 s onward.

- **Image resolution**: A color depth of 12 bit is preferred over the standard 8 bit mode to optimize enable LUT (look up table) adjustment after image acquisition and to optimize quantification of gray values when measuring fluorescence intensity for various applications (see below). The image resolution in confocal recordings is 1,024x1,024 pixel for low frequency recordings and 512x512 pixel for high frequency recordings.

- **Confocal laser scan microscopy**: Settings that have worked well in confocal microscopy are recording intervals between 30 s and 2.5 min, laser power at 6% of minimum laser power, a medium scan speed, a line average of 4, a photomultiplier gain of 800, a standard pinhole size as given by the software, imaging of up to ten focal planes, and a resolution of either 1,024x1,024 pixel or 512x512 pixel.

As an example, Fig. 3 and corresponding movie 1 depict time-lapse fluorescence micrographs of the peripheral region of a hepatocellular carcinoma cell of line PK18-5 that was stably transfected with a cDNA construct coding for a human keratin 18-YFP fusion protein. Only a selected region is shown to highlight morphological details of fluorescence patterns in the cell periphery which contains presumptive KF precursors. To monitor these precursors, which move with a speed of 100-300 nm/min, images were acquired at 30 s intervals. Note that it is
difficult to deduce the continuous inward-movement which is, however, readily detected in the corresponding movie 1. Due to the thin and extended cell periphery and the high focal depth of epifluorescence microscopy the entire fluorescence distribution at the cell edge could be recorded in a single focal plane, whereas the fluorescence in the thicker, more central area (top) was not fully resolved. Adjustment of the LUT in the squared middle area helped to visualize further details although the limits of the current imaging technique is still apparent.

C. Special Aspects of Recording in Mitotic Cells

For time-lapse fluorescence recordings of dividing cells it is usually sufficient to provide exponentially growing cultures that were seeded at low density. 1-2 days after plating, mitotic cells can be easily identified as they round up and elongate during this process. To further guide in the identification of dividing cells, addition of vital DNA stains such as Hoechst 33342 or transfection with fluorescent histone cDNA constructs is of use. The histone H1-EGFP-encoding plasmid (Rolls et al., 1999) works well to monitor chromatin distribution throughout the entire cell cycle (Fig. 4; movie 2). A particularly challenging problem in the high resolution analysis of keratin alterations is, that disassembly of the KF network occurs at the onset of mitosis, i.e., before shape changes of cells, nuclear envelope breakdown, and chromatin condensation are detectable.

The rounding of cells during mitosis necessitates imaging by confocal laser scan microscopy for optimal resolution. Multiple focal planes should be recorded for subsequent 3D-reconstruction (Fig. 5; see also below), as this is the best way to identify domain-specific keratin assembly forms and to delineate their true spatial configuration and respective arrangement(s).
A major problem of imaging mitotic cells is their high degree of sensitivity toward intense illumination. Very often cells arrest in late metaphase without re-entry into the cell cycle, yet cytoplasmic motility and membrane movements continue for several hours without any further changes in cell shape and keratin morphology. Although addition of ascorbic acid ameliorates the sensitivity of cells, it is a constant battle to find a compromise between satisfactory temporospatial resolution needed for complete monitoring of keratin rearrangement and cell cycle arrest. Therefore, low resolution images are first taken to obtain a general overview while high resolution recordings are done to delineate specific aspects of keratin behavior during individual stages of mitosis.

**D. Imaging in the Presence of Phosphatase Inhibitors**

An important feature of live cell imaging is the possibility to directly monitor the effects of various drugs on the dynamic behavior of fluorescently labeled structures. In the case of the KF system, substances that alter levels of phosphorylation are of particular interest (Omary *et al.*, 1998; Coulombe and Omary, 2002). Since the phosphatase inhibitors OA and vanadate (ortho- as well as per-vanadate) are known to induce considerable reorganization of the keratin system in several epithelial cell types (e.g., Kasahara *et al.*, 1993; Yatsunami *et al.*, 1993; Blankson *et al.*, 1995; Feng *et al.*, 1999; Strnad *et al.*, 2001, 2002), we will limit our description to these two agents. Other drugs may be applied and tested in an analogous fashion.

**1. Determination of Suitable Drug Concentration**

The sensitivity of different cell types to either OA or OV varies greatly. Dilution series should therefore be prepared to determine drug levels with the strongest effects on the organization of the keratin system. Precise titration of the lowest possible drug amount needed to elicit these effects is important to avoid alterations of the other cytoskeletal filaments (see, e.g.,
Strnad et al., 2001, 2002). For the determined "optimal" concentration time series have to be prepared. Keep in mind that in the case of OA keratin reorganization takes hours whereas for vanadate KF network breakdown occurs within minutes (Figs. 6, 7).

2. Examination of Reversibility

Ideally, one would like to define conditions under which re-organization of the keratin system can be switched on, arrested and reversed at will. To find such conditions turns out to be rather tricky, since drug-induced morphological changes often occur only after significant lag periods. In the case of OA-treatment (Strnad et al., 2001), a short 30 min-treatment is sufficient to induce the formation of aggregated KF bundles and small, long-lived granular keratin aggregates after ~2 hours. Furthermore, restitution of a normal keratin cytoskeleton is not observed after extended periods of time. On the other hand, removal of the drug or energy depletion after occurrence of the first morphological signs for keratin reorganization prevent progression of filament network disruption thus resulting in a stationary phenotype. In contrast, OV-induced alterations are - at least in part - reversible (Strnad et al., 2002). If the drug is removed after a 10 min-incubation, at which time extensive granular aggregates have formed in most cells, a filamentous keratin network is re-established in less than an hour (Fig. 7). There is, however, a distinct threshold of drug exposure above which rapid KF re-formation is not possible any more. Instead, only a slow re-organization is observed. Both types of re-organization differ not only with respect to their kinetics but are probably dependent on different factors and cellular topologies (Strnad et al., 2002).

3. Light Dependency

Recent findings suggest that the responsiveness of the keratin cytoskeleton can be modulated by light exposition. It was initially observed that, depending on the type of recording protocol and the type of medium, vanadate action differed considerably (Strnad et al., 2002).
Systematic investigations revealed (Strnad et al., 2003) that a 1-10 min exposition of cells to normal room light of less than 200 Lux as measured with a digital luxmeter (Mavolux 5032B from Gossen Foto- und Lichtmeßtechnik GmbH, Nürnberg, Germany) is sufficient to inhibit vanadate-induced keratin re-organization (see also Fig. 7E). The protective effect appears to be wavelength-independent and is reversed within 1-2 hours. Interestingly, a similar inhibitory effect on OV-action is also observed in cells that are preincubated with the p38 kinase inhibitor SB203580 (Strnad et al., 2003). The maximal protective effect of light is elicited in cells grown in Hanks' medium while DMEM antagonizes light-dependent KF network protection. Although these observations offer a new experimental inroad into the regulation of keratin dynamics, they also add another layer of complexity onto the experimental design of live cell imaging of the keratin system. It is necessary to perform control experiments in the dark for each experimental setting to assess interference of light with a particular dynamic phenomenon under investigation. This may often be a challenging task to accomplish, since manipulations in the dark are rather cumbersome. On occasion, we have even used professional night vision gear.

E. Fluorescence Recovery after Photobleaching

Fluorescence recovery after photobleaching (FRAP) is a powerful technique to determine protein turnover in a given cellular compartment or structure and to visualize motility of fluorescently labeled structures in and out of defined cellular domains (e.g., Lippincott-Schwartz et al., 2001). A confocal microscope has to be used for these experiments, since it allows user-defined bleaching of circumscribed areas of interest with different geometries. A disadvantage of bleaching very small regions is that cell motility often distorts these areas and results in overlap with non-bleached parts of the cell. This may in part explain some of the differences in keratin turnover determinations of the other labs that only bleached small bar-shaped segments across filament bundles (Yoon et al., 2001) and our own lab that bleached...
larger, trapezoid cell segments extending from the cell periphery to the perinuclear region (Windoffer et al., 2004). On the other hand, phototoxicity becomes a problem if larger regions are bleached. Therefore, additional transmitted light images should be recorded in this instance to continuously monitor cell viability.

For most experiments, a wide pinhole size (setting of 500) is advantageous to obtain a high focal depth. In this way, a strong fluorescence signal is maintained enabling short scanning times and minimizing photobleaching, and artificial fluorescence alterations due to focal shifts are avoided. For higher spatial resolution, the pinhole size can be reduced (a setting of 90 worked well in several experiments), although it is then necessary to increase the time of the recording intervals and to image multiple planes. For bleaching 100% of medium laser power is applied in the defined area of interest for a total of 20 scans. Immediate postbleach recording is done to confirm complete loss of fluorescence in the area of interest. All focal planes should be examined to exclude that focal shift or cell motility contribute erroneously to rapid fluorescence recovery. Further images are recorded at prebleach settings and at low frequency to minimize bleaching to facilitate full fluorescence recovery.

To monitor the net inward-directed motility of keratins (Windoffer and Leube, 1999), cell segments extending from the plasma membrane toward the perinuclear region are bleached. In these bleached areas not only gradients of recurring fluorescence are revealed but also keratin conformations are delineated that contribute to the formation of KFs and KF bundles. The method thus allows to unequivocally define precursor-product relationships. "Young" filaments are detected as a fine mesh in the cell periphery that mature into thick KF bundles that are located in the central cytoplasm (Windoffer et al., 2004). Three time points of a typical FRAP experiment are depicted in Fig. 8 demonstrating that new KFs are formed in the
cell periphery while only little turnover of pre-existing filaments occurs in the more central cellular domains.

F. Data Analysis

1. Preparation of Movies

The LUT of the recordings should be optimized for black and white contrast (see, e.g., Fig. 3 and movie 1 for comparison of different LUT settings in adjacent areas). The correction should be identical for all images of a given series to avoid misrepresentation of fluorescence patterns. In addition, the size of the original recordings should be reduced by altering the color depth from 12 bit to 8 bit. The clarity of visualization can be improved in some instances by image inversion. It is also important to crop the original recordings by using ImagePro Plus routines to focus on the process of interest and, even more importantly, to reduce the movie size. Self-made ImagePro Plus macros can be prepared to add time stamps and annotations to the movie frames. Finally, the resize routine can be used to shrink the number of pixels per frame to the required final size.

To present movies in the internet, the uncompressed movies are converted into QuickTime movies using "video" compression. Alternatively, movies can be converted into MPEG-1 files that are more compatible with different computer systems and programs.

2. 3D-Reconstructions

Recordings of z-stacks as those shown in the top row of Fig. 5 can be processed to gain information on the spatial conformation and arrangement of fluorescent structures:

- Projection of all images of a z-stack into a single image (Figs. 4, 5; movies 2-4): This method is quick and often sufficient for conversion of 3D-stacks into an interpretable format.
Software of confocal microscopes and ImagePro Plus provide different projection routines. Best results are usually obtained with the maximum projection method. It should be kept in mind, however, that valuable 3D-information is lost in this type of presentation.

- **Surface view of 3D-reconstructions** (Figs. 5, 6, 9; movies 5, 7): Surface visualization provides virtual 3D-models of the fluorescent structures that can be displayed from any angle. To enable this type of presentation, a threshold of fluorescence intensity has to be defined for the entire data set. Particular phenomena are highlighted by manually defining the border of the structures of interest which, however, is extremely time-consuming and can only be applied in specific instances.

- **Voltex representation of 3D-data sets** (Fig. 5, movie 6): In this case, each voxel of an image stack is displayed transparently in its 3D-position. Adjustment of LUT and of transparency results in a realistic representation of fluorescence in a given volume and may thus be superior to the surface view.

To obtain real 3D-images, surface and voltex reconstructions can be presented as anaglyphs which have to be viewed with special red-green glasses (e.g., Fig. 5; movies 5, 6). Custom-made Amira scripts are used for automatic generation of the various types of 3D-reconstruction from image stacks, and the individual reconstructions from each time point are then assembled into movies.

A major limitation of all 3D-reconstructions is their low z-resolution which is orders of magnitude lower than that in the x-y directions. The technically attainable z-resolution is further compromised by phototoxic effects due to extensive exposition of cells due to repetitive scanning of the same cellular domains. As a consequence, reconstructions often
have to be prepared from only a few sections and are therefore prone to many artifacts. This is especially true for surface views, since the manual threshold setting may either result in joining of structures that are actually separate or, conversely, or in separation of structures that are actually connected. Careful examination of the un-edited images of all focal plane recordings is therefore a prerequisite prior to any digital data processing. Yet, after careful and responsible evaluation the disadvantages are clearly outweighed by the advantages of 3D-reconstructions which offer an excellent way to visualize fluorescent structures in 3D-space and to delineate the complete keratin cytoskeleton of a given cell (Fig. 6; Windoffer and Leube, 2001). In this way, it was possible, e.g., to decide that the fluorescent cytoplasmic keratin dots in mitotic cells are granules and not filaments/rods in cross section (Windoffer and Leube, 2001). Even more importantly, the transition between granules and filaments can be elucidated in 4D-movies (Fig. 5; Windoffer and Leube, 2001).

3. Quantification of Soluble Pool

Although the soluble, non-filamentous keratin pool is only very small, it is probably this keratin fraction that is of utmost physiological relevance, since it is highly dynamic by rapid diffusion throughout the entire cytoplasm and is in an exchange equilibrium with the KF network that is most likely subject to precise regulation by phosphorylation (Omary et al., 1998). Most notably, the soluble keratin pool increases significantly during mitosis (e.g., Chou et al., 1993) where it appears as an increased diffuse fluorescence in the cytoplasm of keratin-GFP-labeled cells that can be extracted with Triton X-100 (Windoffer and Leube, 2001). Quantification of the soluble pool works best in mitotic cells with their sparse and/or absent keratin network and the few granular aggregates. Using ImagePro Plus, gray values are determined for each pixel within a given area. The sum of all gray values of less than 255 is calculated for each time point from the 12 bit images with Excel (Microsoft). White pixels (gray value of 255) are excluded assuming that they correspond to aggregated and/or
filamentous keratins all of which exhibit very strong fluorescence. The mean gray value is then determined for the entire area of interest at each time point and used for graphical representation of time-dependent changes (see, e.g., Windoffer and Leube, 2001).

4. Quantification of Fluorescence Recovery

A suitable area for measurements of fluorescence recovery has to be defined in FRAP experiments within the bleached area. It should be restricted to the centre of the bleached region, since the sharp boundary between bleached and unbleached regions is usually lost within a few minutes and cells tend to move thereby distorting and translocating the bleached region within the recording field. In this defined central area all gray values are summed up using the recorded 12 bit image data. The prebleach value is defined as 100%, the postbleach value as 0%. With the help of Excel spreadsheet routines diagrams are prepared. From these the t1/2 of fluorescence recovery within the analyzed area is determined. In the case of keratins one should keep in mind that recovery times differ depending on intracellular topology, and that other factors such as illumination, phosphorylation, or cell cycle stage may effect turnover rates.

5. Diagrammatic Representations of Time-Dependent Fluorescence Patterns

a. Mobility Diagrams

Given the vectorial movement of keratin fluorescence toward the cell center, a line is selected from fluorescence recordings that is perpendicular to the cell edge and extends from the outside of the cell toward the nucleus to analyze centripetal KF mobility. Along this line, the movement of the cross-sectioned circumferential KFs and peripheral KF precursors are followed between successive images. Amira software is used to depict the fluorescence motility during time and the results are plotted in diagrams of position along the line versus time. Velocity of the inward-translocation can be easily determined in these diagrams. The
motility is greatest in the cell periphery where KF are formed and integrated into the pre-existing KF network. In this area, the inward-directed keratin movement results in oblique and parallel lines in the diagrams in a Christmas tree-type pattern (Windoffer et al., 2004). The trunk of the tree corresponds to the peripheral KF network, the branches to the inward-moving keratin particles prior to their integration into the network. Mobility diagrams are also useful for characterization of the dynamics of granules that are present in cells producing epidermolysis bullosa simplex-type keratins or cells that are treated with OV. Such diagrams revealed in both instances that granule production occurs in a submembraneous compartment, followed by vectorial centripetal movement of comparable speed, and disassembly at a distinct circumferential zone of the cytoplasm (Werner et al., 2004).

b. Time-Space Diagrams

Time-space diagrams are prepared from movies. Instead of assembling z-stacks of pictures as is done for 3D-reconstructions time-stacks are generated from corresponding regions recorded at consecutive time points. Image processing is analogous to 3D-reconstruction to obtain surface views of time traces (Fig. 10; Windoffer et al., 2002; Windoffer et al., 2004). Such reconstructions can be used to directly determine the life-time of fluorescent particles, to depict their direction of movement, to calculate their speed of motility and to visualize their shape changes during their life cycle. In the example depicted in Fig. 10 for OV-treated cells the life-time of the imaged keratin aggregates is ~20 min, they move consistently from the cell periphery toward the cell center with a speed of ~250 nm/min, and they first grow to a certain size which is maintained for most of their life-time prior to their rather sudden disassembly.

c. Intensity Diagrams
Diagrams of re-appearing fluorescence in relation to time and intracellular topology are prepared from time-lapse fluorescence image series that are recorded in FRAP experiments. In this case, 12 bit gray values are quantified in the central areas of bleached regions using Image-pro Plus. The fluorescence intensity values along this single line are then plotted against time and transformed into a surface view with color-coded intensity peaks with the help of Amira software (Fig. 11; movie 8; see also Windoffer *et al.*, 2004).

**IV. Pearls and Pitfalls**

**A. Transient versus Stable Transfection**

A principle problem of any transgenesis is the introduction of mutant polypeptides that may result in non-physiological perturbations. This is particularly apparent in transient transfection experiments in which various phenotypes are observed using exactly the same construct. Fig. 12 presents examples of fluorescent keratin distribution patterns, in which either the characteristic IF-type cytoskeleton (A), an intermediate phenotype with aggregated filament bundles and variously shaped aggregates (B), or a predominantly granular appearance is seen (C). A similar diversity of IF-morphotypes was also described for cells producing neurofilament-GFP fusions (*Szebenyi et al.*, 2002). Therefore, transient transfection assays can only provide an overview of the entire spectrum of possible phenotypes but are not useful for analyses at the single cell level which may lead to misrepresentation of the morphology and dynamics of labeled entities. Thus, stable cell lines are needed as reproducible and reliable model systems that can be examined in detail and in which all cells equal each other. It should be kept in mind, however, that the kinetics of the labeled filaments may still differ considerably from that of the endogenous counterparts. In the case of keratin polypeptides, however, half life determinations suggest that the fluorescent keratin hybrids are, similar to the endogenous molecules, rather long-lived and may not differ so much in their kinetic behavior (*Windoffer et al.*, 2004).
B. Epifluorescence Microscopy versus Confocal Laser Scan Microscopy

Epifluorescence microscopy and confocal laser scan microscopy complement each other. Epifluorescence microscopy is superior in detecting the curvilinear nature of the extended KFs and KF bundles. In addition, the speed of image acquisition is unrivaled. The use of a monochromator further increases the versatility of epifluorescence to image structures that are labeled with many different types of fluorophores. To compensate for the lack of confocality, one can record images in different focal planes with the help of a piezo-stepper and use special deconvolution programs (Amira or any other standard program) to obtain 3D-space information. Therefore, for many applications of live cell imaging of keratins, epifluorescence microscopy is the method of choice for recording and preparation of high-quality images and movies. Only in cases, where cells are not sufficiently flat as is the case, e.g., during mitosis or after extensive drug treatment, and in bleaching experiments a confocal laser scan microscope is needed. Furthermore, for optimal 3D-resolution confocal recording is clearly the better method.

C. Effects of Light

Imaging is intrinsically coupled to light exposition of cells which, however, interferes not only with the fluorescence signal itself due to photobleaching but has also toxic side effects resulting in altered cell behavior, and endows the keratin cytoskeleton with specific properties affecting its responsiveness to certain drugs. Remarkably, KFs are protected against the action of the tyrosine phosphatase inhibitor OV by light. While OV induces extensive granule formation within minutes in the dark, a brief light exposition is sufficient to prevent keratin disassembly almost completely (Strnad et al., 2003). It is currently unknown, how this effect is mediated. But the observation that similar protective mechanisms can be elicited by pre-treatment of cells with the p38 inhibitor SB20358 suggest that signaling pathways may be
involved. The emerging general caveat for all imaging experiments of the keratin system is that its properties may be altered considerably by light exposition and that possibly other, yet unknown factors exert similar effects.

V. Discussion and Concluding Remarks

A principle problem of cell biological research has been the interpretation of insights that have been gained from analyses of dead cells, fixed cells, or certain fractions thereof all of which are prone to multiple types of artifacts. The characterization and development of autofluorescent proteins, most prominently of the green florescent protein, that was isolated from the jelly fish *Aequorea victoria*, have provided tools to label cellular structures such that they can be examined in living cells (Tsien, 1998). Analyses of stably-transfected cell lines that produce normal-appearing fluorescent keratin hybrids have yielded completely new concepts about the unresolved mysteries of the keratin cytoskeleton as formulated in the Introduction. Thus, we find that *de novo* keratin formation originates predominantly from the cell cortex progressing toward the cell interior at the end of mitosis (Windoffer and Leube, 2001) and that continuous keratin turnover occurs preferentially in a specific submembraneous compartment (Windoffer *et al*., 2004). Further live cell imaging experiments suggest that the driving force behind the vectorial and dynamic keratin distribution patterns relies both on microtubules and microfilaments and their associated factors (Windoffer and Leube, 1999; Yoon *et al*., 2001; Liovic *et al*., 2003; Werner *et al*., 2004). Finally, it has been demonstrated in keratin-GFP-labeled cells that changes in cellular phosphorylation have a significant impact on keratin organization and dynamics in a temporally and spatially defined manner (Strnad *et al*., 2001, 2002, 2003). These insights on keratin network modulation have been extended onto disease states by usage of cell lines that produce fluorescent dominant negative keratin mutants (Werner *et al*., 2004).
The studies on the dynamics of the keratin cytoskeleton have been complemented by exciting analyses of the other IF types and systems. In these instances, an astounding variety of dynamic behaviors has been described in living cells producing fluorescent IF protein chimeras. These behaviors include diverse types of motility of individual filaments and filament bundles in the absence of cell shape changes (Ho et al., 1998; Yoon et al., 1998; Martys et al., 1999; Roy et al., 2000; Wang et al., 2000; Wang and Brown, 2001), which may correspond - at least in part - to the reported KF movements (Windoffer and Leube, 1999; Yoon et al., 2001). Furthermore, these analyses have shown that IFs are an integrated part of the cytoskeleton and that its motile properties are predominantly determined by microtubules and its associated motor proteins (Prahlad et al., 1998; Chou and Goldman, 2000; Chou et al., 2001; Helfand et al., 2002; Helfand et al., 2003a, 2004). Some of these principles may also be shared by keratins, although clear differences appear to exist (Windoffer and Leube, 1999; Yoon et al., 2001). Interestingly, highly motile, non-filamentous structures that are referred to as squiggles, IF particles and/or round/ovoid structures and are transported along microtubules have been identified for vimentin (Prahlad et al., 1998; Yoon et al., 2001; Helfand et al., 2002), peripherin (Helfand et al., 2003b) and neurofilaments (Yabe et al., 1999; Prahlad et al., 2000; Yabe et al., 2001; Chan et al., 2003). It is assumed that they are IF precursors (Prahlad et al., 1998; Yabe et al., 1999; Chou and Goldman, 2000; Chou et al., 2001; Yabe et al., 2001; Chan et al., 2003; Helfand et al., 2004). In addition, larger filamentous structures have been reported to be transported along axons in living neurons (Roy et al., 2000; Wang et al., 2000). It remains to be shown how and whether these various transport particles are related to keratin squiggles (Yoon et al., 2001), to keratin precursors that were recently identified in the periphery of epithelial cells (Windoffer et al., 2004), and/or to the highly motile particles previously described (Liovic et al., 2003). Finally, it has been shown by imaging of fluorescent IF-chimeras that the balance between granular and filamentous IF forms is dependent on phosphatase activities and the phosphorylation status of
IFs (Yabe et al., 2001; Chan et al., 2003) as is the case for the keratin system (Strnad et al., 2001, 2002).

Given the rapid advances in imaging technologies, it can be expected that further exciting findings about the complex dynamics of the keratin cytoskeleton will soon be reported. By using multicolor imaging, it is possible to simultaneously monitor the relative distribution of various components of the keratin system (e.g., Windoffer et al., 2002). Furthermore, molecular interactions can be visualized by fluorescence resonance energy transfer (FRET; e.g., Periasamy and Day, 1999; Pollok and Heim, 1999; Lippincott-Schwartz et al., 2001; van Roessel and Brand, 2002), and fluorescence correlation spectroscopy (FCS) will afford the investigation of multimerization states of soluble cytoplasmic keratins (e.g., Lippincott-Schwartz et al., 2001; Bacia and Schwille, 2003). This is clearly just the beginning of a new era of in situ experimentation with many more improvements and refinements yet to come to enlarge our knowledge about the dynamic integration of the keratin system into the entire functional and structural cellular assembly.

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Figure Legends

Fig. 1 Photograph (oblique view of top) and schematic drawing (cross section) of a self-designed culture chamber that is used for live cell imaging. Adhesive cells growing on the inverted cover slip are placed on top of a small culture chamber that is accessible through drill holes for exchange of culture medium and for placement of a temperature sensor. Proper temperature is attained by a heating chamber at the bottom that is connected to tubes containing circulating, pre-heated water. A clear optical path is maintained by glass plates to enable recording of transmission phase contrast or Nomarski images. The objective is immediately adjacent to the inverse growing cell monolayer for transmission and epifluorescence microscopy. For further details see text.

Fig. 2 Epifluorescence microscopy of a methanol-acetone fixed hepatocellular, carcinoma-derived PLC cell (A) and living mammary adenocarcinoma-derived MCF-7 cells (B) expressing human keratin 14 hybrids which contain an YFP tag either at the carboxyterminus (HK14-YFP; A) or the aminoterminus (YFP-HK14; B). Note the detection of an extended filament network in both instances. Bar, 10 μm.

Fig. 3 Epifluorescence images depicting a peripheral region of a living hepatocellular carcinoma-derived PK18-5 cell synthesizing fluorescent keratin hybrid HK18-YFP. Note that changes in keratin distribution are difficult to discern from the series of still images but that they are easily recognized in the corresponding time-lapse series that is provided as movie 1 revealing a continuous inward flow of fluorescent material. The LUT was adjusted in the square region in the middle to enhance low fluorescent structures. The cell edge is demarcated by a red line. Its position was determined from phase contrast images that were recorded in parallel. Bar, 5 μm.
Fig. 4  Projected fluorescence images that were obtained by confocal laser scan microscopy of vulvar carcinoma-derived AK13-1 cells in anaphase co-expressing keratin chimera HK13-EGFP and a histone H1-EGFP chimera (encoded by plasmid VLP51; (Rolls et al., 1999). Note that there is no apparent correlation between keratin dynamics and chromatin movements. Bar, 2.5 μm.

Fig. 5  Different types of presentation of a time series of z-stacks of confocal laser scan fluorescence images that were recorded in a mitotic AK13-1 cell producing HK13-EGFP. The top part of the panel (stack) depicts three confocal fluorescence micrographs of a z-stack at four different time points. The corresponding movie 3 was assembled from a single focal plane (recording intervals, 2.5 min). The next row of pictures presents projection views of the entire z-stacks at the different time points. The complete series of projection images was combined into movie 4. Below, stacks were reconstructed into 3D-anaglyph pictures that should be viewed with red-green glasses for complete 3D-visualization. For comparison, surface and voxel reconstructions are shown. The complete time series of the 3D-images are provided as movies 5 and 6, respectively. Bar, 5 μm.

Fig. 6  3D-reconstruction (surface view) of fluorescence images recorded in 32 confocal planes at 1 024 x 1 024 pixel of a single AK13-1 cell producing fluorescent HK13-EGFP fusion proteins. The cell was treated with 0.1 μg/ml OA for 4 h and was fixed with methanol/acetone prior to imaging. Keratin granules are seen together with residual perinuclear KF aggregates. Bar, 5 μm.

Fig. 7  Reactivity of fluorescent keratins in AK13-1 cells in response to OV. Epifluorescence micrographs depict control cells in (A), cells after incubation with 10 mM
OV for 2 min (B) and 10 min (C), and 25 min after a 3 min treatment with 10 mM OV in (D). The cells shown in (E) were exposed to monochromatic light (440 nm) for 5 min at 4 mW/cm² prior to incubation with 20 mM OV for 10 min. Bars, 10µm.

**Fig. 8** Fluorescence micrographs taken from a FRAP experiment showing a section of an PK18-5 cell before bleaching (prebleach), immediately after bleaching (postbleach; box demarcates bleached area) and 150 min after bleaching. Note the recurrence of HK18-YFP fluorescence predominantly in the cell periphery. Bar, 5 µm.

**Fig. 9** 4D-analysis of the keratin fluorescence in a dividing AK13-1 cell producing HK13-EGFP. Images were recorded in multiple focal planes with a confocal laser scan microscope every 1.6 min and were used to generate the 3D-reconstructions (surface view). Four consecutive reconstructions are depicted highlighting re-distribution of granular keratin. The complete time series is provided as movie 7.

**Fig. 10** Preparation of time-space diagrams. (A) shows three epifluorescence images (inverse presentation) that are taken from a time-lapse recording of a small region in the cell periphery of an AK13-1 cell synthesizing HK13-EGFP after a short treatment with 10 mM OV. Surface views were prepared from the time series as indicated in (B). For clarity's sake, only a few representative traces are depicted. These surface views (C) reveal a life time of ~20 min and a consistent inward-directed movement from the subplasmalemmal region at right to the cell interior at left of ~250 nm/min.

**Fig. 11** Time-intensity diagram depicting fluorescence alterations that were observed in a FRAP-experiment of a PK18-5 cell synthesizing fluorescent HK18-YFP chimeras. The diagram was derived from the image series presented in movie 8. Four images from the movie
are shown at right presenting the prebleach keratin cytoskeleton, the location of the bleached area together with the line (in red) that was selected for intensity measurements and two time points during fluorescence recovery (24 min and 76 min postbleach). The diagram at left displays the values of fluorescence intensity that were measured along the red line (distance) in relation to time. Note that the vast majority of fluorescence recurs in the cell periphery originating beneath the plasma membrane that moved gradually outside the imaged area during recording. Bar, 5 μm.

**Fig. 12** Fluorescence micrograph of cells shortly after cDNA transfection with expression constructs coding for fluorescent HK13-EGFP fusion proteins. Note the different phenotypes ranging from a typical IF cytoskeleton (A) to a mixed filamentous/granular morphotype (B) and an exclusively granular appearance (C). Bars, 5 μm.

**Legends to Movies**

**Movie 1** Time-lapse recording (30 s intervals) of the epifluorescence in the periphery of a PK18-5 cell producing HK18-YFP. Note the continuous inward flow of fluorescent material. The LUT was adjusted in the central square area to highlight details of fluorescence changes.

**Movie 2** Simultaneous fluorescence recording of the distribution of histone H1-EGFP and HK13-EGFP in a dividing AK13-1 cell during cytokinesis. The projection images were obtained from recordings in multiple focal planes and were taken every 2.27 min.

**Movie 3** Recording of keratin HK13-EGFP fluorescence distribution in a single focal plane of an AK13-1 cell (middle) that has entered mitosis. Recording intervals, 2.5 min.
Movie 4  Projection views of fluorescence images that were obtained in five focal planes of a mitotic AK13-1 cell (middle) synthesizing HK13-EGFP fusions. Recording intervals, 2.5 min.

Movie 5  Time series of 3D-reconstructions (surface views as anaglyphs) derived from a live cell fluorescence-recording of an AK13-1 cell at the onset of mitosis depicting the distribution of HK13-EGFP hybrids. The image series should be viewed with red-green glasses for 3D-visualization. Recording intervals, 2.5 min.

Movie 6  3D-reconstructions (voxel-reconstructions as anaglyphs) of a time series that was recorded at 2.5 min intervals in an AK13-1 cell upon entry into mitosis delineating the distribution of HK13-EGFP chimeras. The image series should be viewed with red-green glasses for 3D-visualization.

Movie 7  Surface view of 3D-reconstructions derived from time-lapse fluorescence image stacks that were recorded at 1.6 min intervals in an AK13-1 cell at the end of mitosis depicting altering patterns of HK13-EGFP distribution.

Movie 8  Time-lapse fluorescence image series (2 min recording intervals) taken during a FRAP experiment that was performed in a hepatocellular PK18-5 cell producing HK18-YFP chimeras.

VI. References


Figure 01
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