Multidimensional Live Cell Imaging of Cancer-Mediated Events

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Progress in structural biology, advances in optical technologies and hardware, and developments in image analysis software have heralded a new era for multidimensional live-cell biomolecular microscopy. Collectively, these techniques allow fast dynamic imaging of the fine structure of living cells at high spatial and temporal resolutions. Indeed, recent studies of cells, tissues and microorganisms have illustrated the extraordinary possibilities for, and value of, visualising dynamically fine structures across different time scales (t) and positions, and within a relative large sample volume (X, Y and Z). Producing such multidimensional data (X, Y, Z & t) demands high-end live-cell-imaging microscopes as well as tools for sample labelling, accurate sample-relocation stages, and finally dedicated live cell image processing software. In this contribution we provide an overview of the latest generation of state-of-the-art multidimensional live cell light/laser optical microscopes. We present also a short survey of our latest research data with this type of imaging technique on various cancer cell lines. Finally, we address the most common problems and artefacts we encountered during live-cell imaging when subcellular structures of live biological samples were visualized over prolonged periods of time.

Key words: Anti-cancer drug studies; Biomolecular markers and labelling techniques; Cell-cell interactions; Combined and correlative microscopic imaging; Electron microscopy; Fluorescence Microscopy; Image Analysis; Micro-Membrane Blebbing; Organoid-like cell cultures; Pre-Apoptotic Signs; Tumour biology; Morphomics

1. Introduction

Multidimensional live-cell imaging or time-lapse microscopy allows to record dynamic events within a sample under physiological conditions over prolonged periods of time. Typically, light and/or laser data are captured simultaneously on given time points and played back later on at speeds higher than the recording speed. By doing so, ‘slow’ real-time processes can be overseen in a matter of seconds instead of minutes, hours or days. This has the obvious advantage that biological processes can be studied in a much more straightforward way and hence facilitates the understanding of the dynamic behaviour of tissues and cells and their complex associated inter- and/or intracellular pathways. For example, assume that one image is recorded every 60 sec over a total recording time of 2 hrs and later played back at a rate of 12 images per sec, then the entire process can be watched over in 10 sec instead of 7,200 sec.

Recent progress in new optical technologies and software, has created the extraordinary possibilities to image and analyse the fine structure in fluorescent-labelled live biological samples at high spatial and temporal resolutions. Presently top-end dedicated live cell microscopes can perform high-throughput multidimensional (X, Y & t [time]) imaging on a large set of biological samples at different positions (for statistical rigour), at high depth (Z) resolution (typically between 250-275 µm), and at (patho)physiological relevant temperatures (T); i.e. in five dimensions (5-D) (Fig. 1). The majority of these platforms are operating in a near full-automated way by using fast soft- and hardware components, and often the job of the live-cell microscopist is limited to hitting pre-programmed image recording scripts for particular standard ready-to-go applications.
Fig. 1. Scheme depicting the spatial (i.e., volume) and temporal (i.e., time) operation scale of dedicated live-cell imaging platforms. Live cell imaging allows to operate under different length coordinates (X, Y & Z) over time (t) and at variable temperature settings (T). This is therefore often called ‘multidimensional (i.e., X, Y, Z, t & T) live cell imaging’. Depending on the sample source (e.g., tissue slices, organoid-like in vitro models or 3-D cell cultures) and the labelling approaches applied (i.e., advanced sample preparation) dynamic structural and molecular information on the cells’ behaviour can be collected up to the supramolecular resolution level. Nowadays, high-end multidimensional live cell imaging systems also allow the collection of large sets of data over multiple positions (i.e., different cells within the same experiment) thereby directly contributing to the statistical rigour of the observations.

Note, multidimensional live light or laser optical imaging can be integrated via advanced sample preparation methods with electron microscopy imaging (grey dotted box). This concept is generally defined as ‘correlative light and electron microscopy’ and allows one to image specifically labelled targets in living samples and then record dynamic processes by light or laser imaging technology (left), and finally investigate the same location on the same sample by electron microscopy [1]. For more information on this cross-correlative imaging technique we refer to our previous contribution in the *Formatex Book* series [2] and Fig. 6E-F of this paper.

Typical examples of broad research areas to which multidimensional live cell imaging can be applied in the life sciences are: dissecting the fine-structure and dynamic changes of membrane-mediated events, including receptor dynamics and protein folding; dynamic visualisation of GFP-labelled proteins at the supramolecular level; in situ observation of protein interaction or polymerisation processes; studying time-lapse microbial (e.g., bacteria, viruses) translocation, replication and budding processes; imaging temperature-dependent cellular processes, such as cold-induced microtubule depolymerisation or cold-arrested intra-, inter- or transcellular transport or heat-induced stress effects that are of key importance in cell survival and/or ageing studies; and elucidating drug- or nanoparticle-mediated cellular processes and their subcellular targets.

A range of materials, methods and notes have been described for imaging cultured cells using time-lapse live cell imaging microscopy [3-4]. Tight control of the physiological environment is one of the most critical factors and largest challenges in successful live-cell imaging experiments. In general, maintenance of steady-state culture conditions involve a high degree of thermal stability (37°C ± 0.5), continual renewal of the culture medium (osmolarity of ± 320 mOsmol/kg/H₂O) and a neutral pH (7.4 ± 0.3), stabilised by the use of 20 mM HEPES buffer and/or a flow of CO₂ through the time-lapse live cell microscope cell chamber or tissue cabinet to maintain a steady-state concentration of 5%. It is well known that fluctuations in temperature, osmotic pressure and pH have severe affects on cell viability and structure making consistent time-lapse imaging difficult. Commercial liquid cells for studying biological samples under controlled conditions are available, but only useful for a limited number of standard applications. In addition, numerous special designs to cope with thermal, and pH stability during imaging are also on hand each with their own pro’s and con’s. Therefore, biologists often designed their own set-ups and as a consequence a variety of home-made systems exists and differs from laboratory to laboratory when reviewing the literature. In the next sections, we discuss and review our personal experiences by comparing home-built vs. commercially available live-cell imaging solutions. In addition, we illustrate the performance of both configurations with our research findings on colorectal cancer cells. Often, basic straightforward (home-built) configurations allow to address the majority of live-cell imaging research quests.
2. Home-built single-position live-cell imaging microscopes

It is not unusual to walk into a research laboratory and to discover high-quality performing home-built live-cell imaging microscopes (Fig. 2). The motivation to pursue this route can be diverse but is generally driven by specific research needs or budget restrictions. Microscopists become very inventive when it comes to designing imaging platforms and often their know-how and development skills spark the interest of microscopy companies, resulting in the availability of commercial quality performing microscopes soon after (see, section 3 and Fig. 4). I can lively remember that one achieved thermal stability of their live-cell imaging microscope via the aid of two hair-dryers continuously blowing hot air towards the entire microscope to reach the ‘desired’ temperature. Later a more elegant approach could be found back in one of the commercial available platforms (Fig. 4B).

![Fig. 2. Home-built live-cell imaging platform. (A) Depicting the essential components needed to perform single-position live-cell time-lapse imaging over prolonged times. In this set-up an inverted microscope (1) equipped with Hoffman modulation contrast objectives (ranging between 20 and 63×) is placed on a recovered anti-vibration balance table in order to ensure isolation from environmental (vibration) noise. This is a necessity when stable and drift free time-lapse data have to be generated. The top C-mount port of the microscope is used to hold a low-noise basic CCD camera (2) which is connected via a split-connection to a TV monitor (3), allowing to observe real-time changes during imaging and a time-lapse recorder (4). In this set-up, the temperature of the sample stage was maintained at physiological temperatures using a heated microscope sample stage insert (5). (B) Typical result when no precautions are taken to block-out the ‘toxic’ light rays that emanate from the light source. This image shows two clusters of colon carcinoma cells that display typical signs of apoptosis as early as 12 minutes after starting imaging. Arrows denote large membrane blebbing. Every time-lapse experiment should begin with pre-recording a time sequence of at least 10-15 minutes first (i.e., control situation) before the start of the experiment in order to check and hence validate whether the imaging platform is able to capture genuine artefact-free live-cell data. (C) A typical home-built live cell chamber to be used in conjunction with the set-up described under A. In this, a standard 35-mm plastic petri dish was modified and optimised for live-cell imaging.

Our group started to get involved in live-cell time-lapse microscopy after we continued to generate ‘effector to target’-cytotoxicity data with a high degree of variability between experiments. To increase our understanding and to find an explanation for the variability observed by using biochemical assays, we decided to return to the ‘essential basics’ by studying the live interactions between the effector cells (i.e., liver natural killer cells) and target cells (i.e., colorectal cancer cells) first. For this, we brought together the essentials components needed to perform basic time-lapse light microscopy [5], as depicted in Figure 2: i.e., an inverted microscope (Leica DM IRBE) equipped with a 40× Hoffman modulation contrast objective; a CCD-camera (Pulnix, Cat. No. TM-300 CCD miniature, USA); a video monitor (Philips, Cat. No. CM8833 Personal Monitor, The Netherlands); and a video recorder (JVC, Cat. No. SR-5970E time-lapse SVHS-VCR, UK). In order to maintain the cells at 37°C, we bought a thermostat-controlled stage plate (Linkam Scientific Instruments, Cat. No. CO 102, UK) that was directly mounted on the stage insert of the inverted microscope. Being aware that thermal stability is crucial (see further) we kept the room temperature at 30°C to limit the temperature difference between the cell chamber and the environment. Furthermore, in order to avoid light damage to cells during recording as much as possible (Fig. 2B), a green interference bandpass filter centered at 546 nm was placed between the illumination source and the objective [6].
Figure 3. Example of the outcome of a typical live-cell imaging experiment obtained with a home-built live-cell imaging platform as outlined in detail in Figure 2. This image sequence was recorded over 25 minutes (A = 0 min; F = 25 min) at a capture rate of one image every five seconds (total of 300 images). In this in vitro study, CC531 rat colon carcinoma cells were seeded on a 35-mm petri dish (see, Fig. 2B & 2C) and allowed to adhere and grow for 24 hours. Next, freshly isolated primary liver-associated natural killer (NK) cells were added to the dish and live-cell recording started immediately (A). For full materials & methods regarding the experimental cell culture conditions see reference [7]. The black arrow follows the position of a crawling NK cell over the image sequence and the white double arrow denotes the long axis of a CC531 cell in time. From this sequence it becomes clear that after the NK cell formed contact with the CC531 cell (i.e., image B) the cancer cell start to become rounded and blebbing is evident (F). Note the cancer cells in the top left corner of the image sequence which retain their normal morphology: i.e. well adhered and stretched cells. Furthermore, after the NK cell made its ‘kiss-of-death’ (B) the cell moves towards another cancer cell target (C) illustrating that one NK cell can kill more than one cancer cell. Interestingly, on its way to another CC531 cell, two NK cells appear to seek contact (dotted line box under D) and finally entangle for about 5 minutes (E) before proceeding their way. This is a very intriguing observation which suggests NK cross-talk that allows updates between cells on the number of cancer cells they destroyed or maybe this just resembles a check of the ‘self vs. non-self’ cellular immunity concept. (F) At the end of the time-lapse sequence, the NK cell that we have followed is closely interacting with another CC531 cell.

Note, if you combine this type of imaging with viability probes for example, such as Hoechst or propidium iodide, you can define the modulus of cell-death straightforwardly. For more information on probe and labelling techniques to assess cell-death for live-cell imaging purposes we refer to our previous contribution in the Formatex Book series [8].
Moreover, when cells are transfected with FRET pairs (e.g., GFP – YFP), and light- and concordant fluorescence live cell imaging is performed, cell function, protein dynamics and/or intracellular signalling pathways can be easily studied (for reviews, see [9-10]). Scale bar, 50 µm.

With the above set-up, acceptable data can be recorded up-to one hour under artefact-free imaging conditions. However, the largest improvement came into effect when a dedicated live-cell imaging chamber was developed (Fig. 2C). In brief, a circular opening was drilled in the bottom of a 35-mm petri dish and a glass coverslip with a matching breaking index for the DIC objectives was glued over the opening with silicon. A matching lid based on plexiglass and a rubber ring was fabricated that contained openings for tubings. In this way, extensive evaporation of growth medium was avoided (i.e., osmotic stability) and the in- and output tubing facilitates the exchange of cell culture medium or the addition of agents–even cells–during imaging thereby reducing position-drift by touching the sample stage/holder. A low-debit/flow pump such as the ones used for chromatography as well as taking advantage of gravity-flow via a syringe can be employed for fluid exchange. A typical experimental outcome of the application of our home-built live-cell time-lapse light microscope is presented in Figure 3 and discussed in further detail in the corresponding figure legend. In brief, from these time-lapse experiments we could discern the reason behind the differences observed in our biochemical cytotoxicity assays. We noted that the killing ability of purified lymphocyte in freshly isolated cell preparations differed quite significantly. In some instances, a 3 to 5-fold higher killing ability was observed than was present in other routine cell preparations. Hence, one liver-associated NK cell could kill several target cells (i.e., 3 up to 5); whereas other isolations led to effector targeting efficiency of up to 1 and 2 cancer cells only. From the practical perspective, we concluded that our ‘single-position’ system could really benefit from a motorised microscope stage so we could track our moving effector cells over time and when they disappeared out of field of view. This was also our direct motivation to move towards a multi-position live-cell imaging system that allowed us to capture different subsets of samples or points of interest within one single time-lapse experiment (see, section 3).

The possibility of connecting the recording device (i.e., camera) directly to a computer instead of HDD time-lapse (video) recorder is worth mentioning. Frame-grabber video cards can be used for this purpose such as the ones provided by EPIX or Matrox Corona. This has the obvious advantage of data being directly available for image processing and/or analysis under standard image software programmes. The free shareware image program ImageJ [11] even has downloadable macro’s available allowing to record data under time-lapse mode [12].

3. Advanced multi-position live-cell imaging microscopes

Current microscopy solutions for live-cell imaging differ significantly from their historical predecessors. Besides their contemporary appearance they are fully built-in platforms in which all components like camera, shutters, piezo Z-positioner and optical illumination are fully integrated and optimised for light efficiency and acquisition speed (Fig. 4). Dedicated image-capture speed board(s) synchronise the electronic control units (ECU) of the microscope thereby directly reducing the acquisition idle times to a minimum. This allows for recording of dynamic processes at unprecedented speeds. Dedicated illumination hardware components such as special filter wheels or monochromator technology that are computer-controlled allow users to dial fast into the required excitation wavelength thereby permitting to perform multi-color live cell experiments. Superior systems also let users to record differential interference contrast images simultaneously. Due to optical and recording speed improvements the samples are exposed to minimal amounts of ‘toxic’ light rays during imaging (see also, section 4). Especially, when combined with the most sensitive cameras on the market in conjunction with optical dimming technology, light-induced stress to the samples is almost negligible, ensuring that time-lapse experiments can go over hours even up to days. The minimal optical exposure (i.e., dimming technology) is in particular useful when dealing with different excitation beam lines in one experiment. Finally, fast and accurate piezo-driven sample scanning stages permit the reliable recording of different spots of interests (i.e., cells) within one single time-lapse experimental set-up thereby inherently benefiting the statistical value of the observations. This all together is in essence the major difference between basic single-point vs. total solution live-cell imaging platforms. In section 4, we further elaborate on some important points to consider that directly contribute to the outcomes of a successful live-cell imaging observation.
Two examples of typical configurations of commercial available ‘advanced multiposition live-cell imaging’ solutions. As one can notice in a glimpse the designs between suppliers differ significantly and are mainly based on company philosophies. For example, what about the enclosed humidity-controlled tissue-culture cabinet (white arrows) under A that seems to like floating in the air while in B the cabinet rests on the anti-vibration table. One says that it is more accessible under design A whereas B has the vibration stability advantage. In addition, under B all auxiliaries units are clearly separated from the other hardware and electron control units, again benefiting stability issues. Also solutions to deal with thermal equilibrium of the instrument are significantly different. In Fig. 4A a dedicated local heating strategy is the preferred solution while the system in Fig. 4B opts for a combined local stage and overall circulation of hot air within the enclosed cabinet. Between both systems a significantly different performance in thermal stability is observed and this is also known as thermal drift. In general, overall heating is far more superior, when imaging over prolonged periods of time (i.e., 4 hours and more). Thermal fluctuations are a significant source of live cell image-related artefacts during data acquisition and mainly effect the Z-dimension information. Solutions for out-of focus images, whether caused by thermal drift or not, can be compensated to a certain extent. Finally, when it comes to optical performance, a high-resolution low-noise bottom mount camera (white arrow under B) is the preferred solution if the budget allows. Legend: (A) Carbon-dioxide supply (black arrow); fully computer-controlled inverted microscope (1); CCD camera (2); computer and electronic control units (3-4); temperature control (5). (B) CCD camera (white arrow); temperature control (black arrow). For full comparison, compare also Figure 2 with Figure 4.

Before we go over to a practical application of an advanced multidimensional live-cell imaging experiment it is worth mentioning that the raw image data collected on a typical live-cell microscope under fluorescence imaging mode significantly differ from the data collected with more advanced laser optical technologies such as confocal or multiphoton microscopy. However, this is the price to pay for the speed we gained and the minimal light exposure we work with under dedicated live-cell imaging conditions. It is well known that confocal images are inherently superior to live cell image data because of the efficient rejection of out-of-focus fluorescent signals. However, the prolonged time and intense laser light needed for confocal imaging makes this optical technology highly unsuitable for long-term live cell data capturing. However, this can be partially compensated for by applying deblur or deconvolution algorithms to the raw image data stack [13]. A practical example of the outcome before and after applying non-blind deconvolution of the stacks can be seen under Figure 5. In this example, permanent eGFP-transfected CC531 colon carcinoma cells were followed over a time course of 15 minutes using the multidimensional live-cell imaging microscope depicted under Fig. 4B.
Fig. 5. Live-cell time-lapse series over a time span of 15 min using an advanced multidimensional live-cell imaging workstation equipped with a dedicated ‘Live Cell Imaging Objective’ (PL APO 63×/1.3 N.A. Glyc Corr at 37°C) (see, Fig. 4B). Enhanced green fluorescent protein (eGFP)-expressing rat adenoma carcinoma cells were cultured for 24 hours before live cell imaging commenced [14]. (A-C) The upper panel shows the unprocessed data that were collected from the live cell imaging microscope while the images under a-c show the corresponding data after non-blind deconvolution via the Leica DEBLUR Software. Note the overall obvious difference in image quality between the top vs. lower panel. Crisp subcellular details such as small intracytoplasmic vesicles become readily visible (large white arrow). Furthermore, cytoplasmic extensions at the rim of cell can be observed and which appear as sheets of membranes waiving through its immediate surroundings (small white arrow). Scale bar, 20 µm.

The multidimensional live-cell microscope system that we used to study the effect of anti-cancer drugs on cultured colon carcinoma cells in a time-lapse manner was essentially composed of: an inverted microscope (Olympus Live CellR IX81) equipped with a dedicated 40× differential interference contrast objective (N.A. of 0.95); a multi-function all-in-one illumination system (Olympus MT20); a tissue cabinet connected to carbon dioxide supply and temperature incubator controller units (Solent Scientific); and a low noise and thermal stable CCD camera (Hamamatsu Orca-ER). The Olympus live CellR software was used as interface for the image acquisition, the control of the shutters, lenses and sample positioning (Fig. 4A).

With the growing awareness that the actin cytoskeleton is involved in practically all aspects of cell behaviour, but also in cancer and other human diseases, it has become increasingly important to identify new agents with well-defined actin-binding properties that preferentially affect certain aspects of actin filament organisation and dynamics in a reversible manner [15]. Such agents are not only necessary as research tools to better understand how the different actin structures are assembled, organised, and function in cells, but also may be potentially useful as therapeutic agents in the treatment of diseases such as colorectal cancer [16]. Our team uses time-lapse imaging on a routine basis to assess the effectiveness of novel candidate anti-actin compounds and/or therapeutic nanomaterials with strong anti-malignant properties on organoid-like cell cultures. These time-lapse studies are conducted in conjunction with biochemical assays and subsequent correlative electron microscopy studies. A typical practical result of our current studies is presented in Figure 6. The observations are discussed in detail in the corresponding figure legend. This approach has been shown to be extremely helpful in determining the kinetics and bioavailability of the drugs and/or drug-complexes and to understand how they affect ultrastucture and function of the cell in a dynamic way. This important information can directly tailor the anti-cancer compounds before moving onto more complex and ethically more sensitive animal studies.
4. Notes and tips for successful live-cell imaging

The most common issues and problems that one may encounter during live-cell time-lapse imaging are discussed here irrespective when you operate a home-built or commercial microscope system. In addition, practical hints and suggestions are provided.

Vibration is the greatest source of image noise in live-cell time-lapse imaging. This can be easily determined when short time-lapse sequences of a given sample are recorded. An ideal way to determine environmentally- or instrumentally-derived vibration is to image a petri dish that contains latex beads with a size in the order of a few microns suspended in growth medium; i.e. after the beads settle (~5 min) they should remain fixed in their position during image acquisition. Therefore, to obtain high quality optical data, the microscope must be maintained in a vibration-free environment. Special vibration isolation tables are commercially available and isolate the live-cell imaging microscope from the ground-floor laboratory. In addition, it is advised to place auxiliary controller units on a table separate from the imaging system. Moreover, acoustic waves or air-conditioning currents can cause vibrations of the stage and can be minimised by using a plexiglass box around the microscope. Occasionally, the mechanical components of the sample holder and stage can give rise to vibrations. Therefore, attention should be paid to make the combination of sample holder and stage as rigid as possible to avoid mechanical vibration. In this case, one or two drops of corn oil between the edges of the sample and the stage of the microscope can solve the vibration problems. Tape can be a quick band-aid solution.

Thermal stability of the microscopes’ hardware components is essential to obtain image data free of thermal drift. As discussed before, thermal drift becomes apparent when the image repeatedly goes out of focus. Cheap basic local heating devices give rise to sinusoidal temperature pulses (sometimes up to ± 2°C) and cause the sample stage and/or sample chamber to expand or shrink during recording. Imaging latex beads as outlined before to assess thermal stability is crucial before analysing your samples. A good practice is to allow complete temperature equilibration of the entire system. Warming up the stage and sample holder at least two hours before imaging is recommended. In addition, the entire enclosed tissue culture cabinet built around the instrument should be set to 37 ± 0.5°C by using the hot-air circulating heating device (if available) (Fig. 4B). By doing so, temperature fluctuations during imaging are significantly reduced. For the same reason, the auxiliary equipment, the laser and the electronic control units (ECU) have to be switched on beforehand as well (i.e., to avoid ECU-derived thermal noise). Ideally, the instrument should be placed in a temperature controlled room to avoid drift problems. Noteworthy, the temperature of the room where our home-built instrument was installed was continuously set at 30°C for the same reason. Finally, temperature drift can also result in spherical aberrations by altering the refractive index of the optical components of the cell chamber. However, changes in refractive index of the immersion medium are the most likely cause. The use of dedicated live-cell objectives that allow correction at relevant temperatures are therefore recommended. Top-end solutions allow to compensate for temperature during imaging via a heater-sensor jacket attached to the objective.

It becomes clear that environmental issues can play a major role in the ultimate performance of live-cell microscopy. Vibration and temperature issues are the two most important potential sources of errors to consider. Furthermore, the dedicated live-cell imaging room should also be equipped with light-darkening curtains to avoid unnatural background light, especially caused by induction-lamps, which give rise to noise-rich data with poor contrast. This can be easily tested with the light switch on or off and/or curtains are closed.
Fig. 6. Live cell imaging data (A-D) and corresponding correlative scanning electron micrographs (E-F) of caco-2 colorectal cancer cells during and after treatment with 50 nM swinholide A for 1 hr. Briefly, caco-2 cells were grown on 35-mm glass bottom petri dishes and labelled at the start of the live-cell imaging experiment with the membrane-permeable nucleic acid dye Hoechst 33342. After time-lapse imaging for 10 minutes, the actin-disrupting drug swinholide A was added. Live cell imaging was performed using the Olympus Live Cell R as outlined in Figure 4A and data were recorded every 5 minutes over a time span of 1 hr. At the end of the experiment, cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer enriched with 0.1 M sucrose. Next, samples were postfixed with osmium tetroxide, dehydrated in graded ethanol solutions and dried over hexamethyldisilazane as described previously [17]. Samples were coated with 7.5 nm platinum and imaged with a field emission-scanning electron microscope (Zeiss Ultraplus, Germany) at 3-5 kV. Cross-correlative image information as presented under E-F was obtained as previously outlined in the 2007 Formatex Book series [2]. (A-D) Cells responded within 5 min of anti-actin drug administration (B). Signs of cytoplasmic retraction (arrows) were evident and progressed as a function of time (B-D). Scale bar, 50 µm. As described before, colorectal cancer cells proceed into the final stages of
apoptosis when exposed to similar concentrations of swinholide A for more than 2 hrs [16]. (E-F) Scanning electron microscopy (SEM) and correlative methodologies on the same cells after 1 hr treatment revealed that the numerous dot-like cytoplasmic structures as observed at the optical level (B-D) are clearly associated with the cell membrane: i.e., the worm-like membrane protrusions as observed by SEM seem to be made up by those dot-like vesicles. We postulate that these micro-membrane structures are the pre-apoptotic morphological signs preceding the typical larger membrane blebbing structures as observed in the final stages of apoptosis (for comparative reasons, see Fig. 2B). It is conceivable to suggest that the loss of the actin cytoskeleton causes the indirect disorganisation of the normal membrane architecture and gives rise to micro-membrane blebbing. This directly illustrates the importance of extending the live-cell experimental data with information at a higher resolution scale (see also Fig. 1). Note, the dotted line boxes under D and E correspond with the image following. Scale bar E, 10 µm; Scale bar F, 2 µm.

As mentioned previously, light-induced cell stress also known as phototoxicity is a common recurrent problem during imaging. To prevent light-induced damage to the cells, a broad-spectrum green interference filter should be placed in the light path of the microscope. This filter blocks the light with a wavelength below 510 nm which is extremely toxic for cells and therefore prevents a decrease in cell viability. There is nothing worse to ‘cook’ cells under the light or laser beam. But this mainly applies to the home-built systems since commercial solutions have accounted for these problems (see, Fig. 2B). In addition, special growth medium for live cell imaging that is devoid of phenol-based indicators should be used as well for live-cell imaging. Standard cell culture growth medium contain these indicators as a quick visible marker for pH assessment during cell culture (see also below). However, phenols are well-known photon absorbers and hence contribute directly to the light-induced phototoxicity. Furthermore, also the type of objectives used contribute to photodamage to the cells. High numerical aperture (NA) lenses improves the final optical resolution that can be achieved according to Abbe’s and Rayleigh’s optical laws. High NA objectives also increase the brightness of an image at a given magnification, and because less light is needed to obtain low-noise high-resolved images, cells are less likely to suffer from light-induced stress. Finally, after considering the (i) filters, (ii) the chemical composition of the growth medium and (iii) the NA of the objectives; there is not much more you can do to limit photodamage. Addition of anti-free radical reagents to minimise free-radical mediated damage may help to some extent. Sodium ascorbate at 10 mM is particular an effective scavenger of free-radicals released in part by prolonged light exposure. Worth mentioning is that an alternative method was introduced recently to reduce phototoxicity and photobleaching during fluorescence live-cell imaging, also known as ‘controlled light exposure microscopy’ [18]. This controlled light exposure microscopy method is based on reduced excitation in confocal imaging of non-informative background and bright background pixels via a feedback system. Only dim foreground pixels are excited for the full pixel dwell time. This method of exposure is commercially available in the new Nikon confocal microscopes and reduces phototoxicity and photobleaching by a factor 2-10×.

Directly related to the above, a good standard practice is that at the end of each live-cell imaging experiment, the overall viability of the sample should be determined. A cheap and non-toxic way of viability assessment is the use of the vital dye Trypan Blue. In brief, add 0.3 ml of 0.4% Trypan Blue Stain (Gibco BRL, Cat. No. 15250-061) into the 35-mm petri dish with the aid of a pipette and mix gently. Recalculate the volumes when smaller liquid cell chambers are used. The typical amount of growth medium in the 35-mm petri dish is 1.7 ml. Allow to stand 5 min at 37°C before counting. Observe under the inverted microscope. Non-viable cells stain blue and viable cells exclude the stain. Propidium iodide and Hoechst are also good candidate viability makers but only work when the system is equipped with fluorescence excitation. These fluorescent dyes also have the advantage of providing information about the type of cell death: i.e., apoptosis, necrosis or secondary necrosis [8]. But be aware that prolonged exposure (i.e., > 30 min) of cells to these dyes can cause cell death by themselves.

Finally, the pH of the cell culture medium during time-lapse imaging should be maintained in the physiological range of pH 7.4. Commercial systems have a continuous flow of CO₂ percolating in the humidified cell culture cabinet attached to the microscope so that a final concentration of ~5% CO₂ is achieved (Fig. 4A). When this capability is not available growth medium can be further enriched with HEPES (Gibco BRL, Cat. No. 32430-027). However, when HEPES is used in combination with exogenous gas, it is important that the HEPES concentration must be more than double for adequate buffering; i.e. 2% CO₂ ~ 10 mM HEPES vs. 5% CO₂ ~ 50 mM HEPES. Importantly, concentrations higher than 25 mM are toxic for cells and HEPES should be added in addition to, not in place of, sodium bicarbonate. Viability assessment is recommended when HEPES concentrations are used that are higher than the normal recommended working concentrations. It is also worth mentioning, that HEPES has a limited pH buffer capacity in function of time and usual restricts live-cell imaging experiments to two hours.

5. Concluding remarks

From this topical paper on multidimensional live-cell imaging microscopy it becomes clear that capturing high-quality live-cell light / fluorescence data requires significant capital investment as well as technical and scientific expertise in running such an imaging platform. For the occasional live cell microscopist, relatively inexpensive home-made designs...
can be built and result in satisfying data. However, the operator discovers often quite quickly the limitations of a basic design and immediately seeks the top-end advanced multidimensional live-cell imaging microscopes. This was definitely the experience of the authors of this paper.

In general, more sophisticated imaging tools offer a scope for greater insights and understanding, yet there is also an increased risk of researchers applying these instruments incorrectly. Understandably, top-flight researchers are attracted to high-end instruments based on the latest vogue in biological research, but they often make the fundamental mistake of not “learning the alphabet of live-cell imaging” first. With this in mind, one golden advice, before you start the ‘rediscovery’-journey, is to look around in your institution or nearby research centres that have established credentials in live-cell imaging and associated sample-preparation protocols [19].

For the nearby future, we can expect significant advances in two areas that will directly advance live-cell imaging: i.e., the availability of new probes that yield high spatial-resolution signals [20-21] and the development of new standardised sample preparation methods that allow producing accurate-, artefact free- and reproducible cross-combined data at high spatial and temporal resolution [22-23]. For example, companies heavily invest nowadays in solutions for sample stages and accompanying rapid-sample transfer systems that allow precise relocation of the sample between live cell- and electron microscopy platforms. Recent cross-combined studies of tissues, cells and microorganisms have illustrated the extraordinary possibilities for, and value of, visualising fine structures across different length and time scales [1]. This correlative imaging approach allows accurate relocation of the same regions first studied at the dynamic 4-D light optical level for subsequent molecular studies at the static 3-D electron microscopy level.

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