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Pavel Vesely
Academy of Sciences of the Czech Republic, pvy@img.cas.cz

Alan Boyde
University College London, United Kingdom

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VIDEO RATE CONFOCAL LASER SCANNING REFLECTION MICROSCOPY IN THE INVESTIGATION OF NORMAL AND NEOPLASTIC LIVING CELL DYNAMICS

Pavel Vesely1* and Alan Boyde2

1Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic, 2Department of Anatomy and Developmental Biology, University College London, London, UK

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Abstract

The introduction of video rate confocal laser scanning microscopes (VRCLSM) used in reflection mode with high magnification, high aperture objective lenses and with further magnification by a zoom facility allowed the first detailed observations of the activity of living cytoplasm and offered a new tool for investigation of the structural transition from the living state to the specimen fixed for electron microscopy (EM). We used a Noran Odyssey VRCLSM in reflection (backscattered) mode. A greater degree of oversampling and more comfortable viewing of the live or taped video image was achieved at zoom factor 5, giving a display monitor field width of 10 µm. A series of mesenchyme derived cell lines - from normal cells to sarcoma cells of different malignancy - was used to compare behaviour of the observed intracellular structures and results of fixation. We contrasted the dynamic behaviour of fine features in the cytoplasm of normal and neoplastic living cells and changes induced by various treatments. The tubulo-membranous 3D structure of cytoplasm in living cells is dynamic with motion observable at the new limits of resolution provided by VRCLSM. All organelles appear integrated into one functional compartment supporting the continuous 3D trafficking of small particles (vesicles). This integrated dynamic spatial network (IDSN) was found to be largest in neoplastic cells.

Key Words: reflection confocal microscopy, backscattered light imaging, high spatial and temporal resolution, functional integrity of cytoplasm, speed of cytoplasmic motility, fixation, neoplastic cell compartmentalization

Introduction

A fully confocal, unitary (laser origin) beam confocal laser scanning microscope (CSLM) for the reflection mode was developed by Draaijer and Houpt (1988). The later commercial development of the design principles involved - AOD (acousto-optic deflector) scanning on the line and galvanometer mirror scanning on the frames axes - led to the release of the Noran Odyssey instrument (with which we have worked). Very much higher scan rates are achieved in the multiple beam scanning confocal microscopes designed by Petran et al. (1968). These "tandem scanning" (the priority term for, and synonym for, confocal) microscopes gave excellent reflection images. However, their advantage in high speed scanning was lost in attempts at recording at high speed. Furthermore, although there are far fewer pixels in a TV scanning arrangement than in a mechanical scanning, Nipkow disc, confocal microscope, the temporal resolution at each pixel is maximal in the direct video scanning arrangement. Information is only written into a pixel at the time that the focussed laser beam is illuminating the corresponding point in the specimen. Because of these considerations, because video-tape recording is cheap and simple and reliable (if not free of problems!) and because it is simple to achieve a comfortable degree of empty magnification, the Odyssey type of video rate CLSM (VRCLSM) represented a major revolution in the evolution of light microscopical techniques for live cell biology.

VRCLSM with high magnification, high aperture objective lenses and with further magnification given by a zoom facility allowed the first detailed observations of activity of living cytoplasm. Rapid motion in fine granular 3D structure of the interior of a living cell, some of which apparently exceeded the frequency which had been previously possible to register at the time, was revealed in a variety of cell types: these included rat and chick bone derived cells (Boyde and Jones, 1992; Boyde et al., 1994) and rat tumour cell lines (Vesely et al., 1993; Vesely and Boyde (1994)).

In spite of the potential of the VRCLSM in the investigation of intracellular processes - and in correla-
tive microscopy as a tool for investigations of the structural transition from the living state to the fixed specimen for electron microscopy (EM) - many questions about the nature of the observed intracellular dynamic structures remained to be answered. The conditions for reliable imaging had therefore to be defined, a technique (or better an art) for performing real time experiments to be developed and managed, and comparative biological examination of various cells to be performed. The purpose of the present paper is to review our findings with this technique (to the date of the 14th Pfefferkorn Conference, August 1995), and to discuss the necessary future developments before this technique can become an ordinary tool of cell biology.

Materials and Methods

Microscopy

We have used a Noran (Middleton, WI) Odyssey video rate laser scanning microscope (VRCLSM) with an argon ion laser 488nm line in the reflection mode, mainly with Nikon NA 1.4, 160 mm tubelength, oil-immersion objective lenses with nominal magnifications of 100X and a 60X, NA 1.2 water immersion lens. The first has been strongly preferred for the type of study considered here. To minimize spherical aberration, living cells were grown on coverslips so that no additional water layer might distort the image (Inoué, 1990).

Image recording and analysis

Video tape was used throughout the experiments to record all images at real time as well as the synchronous voice commentary on the actions and effects. Tapes were evaluated by thorough visual inspection and were also used for measuring the timing of activity and for the estimation of the frequency of dynamic phenomena.

Further magnification given by scanning a reduced area was regularly used in the majority of the present studies, usually to give a zoom factor of 5 corresponding to a display monitor field width of 10 μm.

We used two image analysing computers with the Odyssey: first, the Noran TN8502, a Unix based machine which permitted sequential frame capture and averaging, and secondly, the FENESTRA system of Kinetic Imaging Ltd (Liverpool, UK), which allowed sequential single field or frame capture, but could not Kalman average images in a sequence. Because rapid motion is blurred if the odd and even fields of one TV scan are interlaced, sets of 8 individual half frame (single “field”, half the interlaced TV frame) images were grabbed at video rate giving half the number of lines and a time interval between subsequent images of 1/25s (“FENESTRA”, Kinetic Imaging Ltd, Liverpool, UK).

For printing, the FENESTRA images were trans-
Reflection mode video rate CLSM
Amphipleura pellucida nearest neighbours in time. This deficiency can be partly video leads to an unfortunate downgrading of temporal drift over periods of several minutes. It permitted the rapid exchange of fluid media by pipetting at one end and blotting at the other.

Before examining a few cells in each sample at this, the highest useful magnification, we scanned each sample with the focus adjusted to the plane of contact of the cells with the substrate in order to examine whether the cells were in standard condition.

Results

Both temporal and spatial components of the final resolution have to be assessed against the level of noise and approached in different ways. Standard interlaced video leads to an unfortunate downgrading of temporal resolution in that adjacent lines in the image are not nearest neighbours in time. This deficiency can be partly compensated for by throwing away half the lines in the image so that adjacent data points in the frame direction may be spaced physically (by an empty line), but are as close as possible in time: they are only separated by 1/256 of 1/50th second in our case. Pixels in the line direction are only separated by 1/(512x512)sec. In either case, this excellent time resolution adds to the well known enhancement of XY, and particularly, Z, resolution, greatly to extend the possibilities of visualizing activity in living cytoplasm (whilst still respecting the Shannon-Kotelnikov theorem about the need for a 2 to 3 times higher sampling frequency than the frequency observed). Real temporal resolution currently can also be estimated visually from observing biological activity and its changes during experimental manipulation.

Spatial resolution can be measured using established procedures in light microscopy. To assess the zoom factor necessary to view the phenomena of rapid motion in comfort, we used mounted specimens of the diatom Amphipleura pellucida (purchased from Northern Biological Supplies, Ipswich, UK). Using the 100/1.4 lens, we found that the 0.25 µm transverse periodicity could be seen at zoom 2, and the 0.2 µm longitudinal striping at zoom 3. However, more comfortable viewing of the live or taped video image was achieved with a greater degree of oversampling at zoom factor 5, and this was the standard we adopted for examining intracytoplasmic motion: this gave a display monitor field width of 10 µm.

In the types of biological experiments which were undertaken, there are intrinsic and extrinsic sources of noise (Pawley, 1995). Intrinsic noise is exemplified by the mainly thermal, Brownian motion seen as shimmering mainly over the cellular compartments: this can be dealt with by appropriate setup of the experiments and examination of the effect of blocking particular types of motility and evaluating the residual activity. Extrinsic noise is due mainly to the electronic instability of the whole imaging system: it can be explored by using solid, motionless specimens (grids, fixed cells). Thus true cellular activity can be recognized in spite of the presence of background noise of thermal origin which constantly slightly blurs the entire image, and of an irregular flickering of the image caused by the imaging system. True motility is revealed by movements which (a) extend over distances larger than just a few pixels, and which (b) also proceed in oblique directions (and are therefore not related to TV scan problems) and/or (c) contrast with the relative stability of other parts of the cell, e.g., the cell edge. The cell edge can be used with advantage as a point of reference not only for relative motions in X,Y axes but also for controlling the stability of the focus in Z-axis. This very important aspect as the dynamic behaviour of intracellular structure could be to some extent simulated by slight focus wobbling.

(Figures 5-8 on facing page)

Figure 5 A,B: Interior of K4 cell, showing part of the nucleus in the lower left and IDSN to the right, focussed 7 µm deep in the cell from the coverslip. Two images 1/25s apart. To detect the relative motion of features (only easily achieved by viewing the live dynamic image!), the images may be viewed as a stereo-pair, when features which have not moved will appear to lie in one plane: this will demonstrate the overall translocation of particles of 0.2 µm size of the IDSN. Picture width 10 µm, zoom factor 5, pinhole size 2.94 mm.

Figure 6 A,B: Interior of nucleus of K4 cells 3 µm deep in the cell from the surface of the coverslip showing differences in the image of the cell interior depending on the confocal detector pinhole size. The same field taken with pinhole 5.66 mm for (A) and 2.62 mm for (B). Picture width 10 µm, zoom factor 5.

Figure 7: Interior of a normal (rat bone derived) cell 1 µm deep to the coverslip. Nucleus to top right, to the left of which lies IDSN: 'communication' layer seen in bottom left corner. Picture width 10 µm, zoom factor 5, pinhole size 4.08 mm. (Cells kindly provided by Dr. Colin M. Gray)

Figure 8: Edge (top) and interior of a normal (rat bone derived) cell 0.5-1 µm deep to the coverslip. Thin microspikes along the edge of the cell (which could be used as an indicator of focus stability) and structures in the communication layer are seen. Picture width 10 µm, zoom factor 5, pinhole size 2.98 mm.
Reflection mode video rate CLSM

5A

6A

205
The way in which the image is created in the reflection mode of VRCLSM strongly depends on the level of focus inside the cell and particularly on the distance in the Z-axis from the surface of coverslip on which the observed cell is attached:

(a) At a level of up to 0.5 µm from the coverslip, the image is dominated by interference reflection contrast. It essentially shows the structure of the cell edge (Fig. 1) and maps the focal contacts and other regions (Fig. 2) in which the ventral plasma membrane is fairly close to the coverslip. For inspection of cell-to-substratum contacts, we used zoom 1 with the 100X objective, a physical pinhole size of about 3 mm and photomultiplier gain of 1000 arbitrary units.

(b) When the focus is moved 1-2 µm deep into the cell in thin (2-3 µm) normal cells, the image is composed from overlying processes: namely, simple reflection-scattering contrast from the intracellular cytoplasmic structures (Fig. 9) and interference between reflection from the surface of the coverslip and from the dorsal surface of the cell (the side away from the coverslip and facing the culture medium). The dynamics of this type of image indicate rather fast fluctuation of the cell dorsal surface seen as rapid changes in darker sinuous stripes in the center of the image (Fig. 10).

(c) If focussed between 2 and 6 µm into thicker, in our case usually neoplastic cells, and on increasing the PM gain to about 1400, the signal from the central parts of the cell is dominated by simple reflection and backscattering contrasts (backscattered light imaging) (Fig. 4). If the steep side slope of a thick neoplastic cell becomes a part of the image, then parallel contour lines produced by interference between strong reflection from the coverslip and from the side slope of the cell are seen and can be used as a measure of the height of the cell (λ/2 = 244 nm gives the difference in height between neighbouring black stripes) (Fig. 3).

Using the highest possible resolution (spatial and temporal) of light microscopy achieved so far in the VRCLSM, with the Nikon (Kingston, Surrey, UK) 100/1.4 lens and in the reflection mode, the known structures of the living cell are regularly seen, including all types of contacts between the cell and substratum, surface microvilli and filopodia and ruffles, and, inside the cell periphery, elongated mitochondria, stress fibres and various granules, and, inside the main body of the cell, nuclei with nucleoli. In addition to these structures, a much sharper image of the cell edge is obtained which clearly shows the difference in "hairiness" and cell-to-substrate contacts between neoplastic (Fig. 1) and normal (Fig. 2) cells.

Deep inside the cell, the spatial network of the cytoplasm (Fig. 4) is seen, the dynamics of which can be revealed if A and B images in this text are looked at (Figures 9-11 on facing page)

**Figure 9 A,B:** Cell periphery of normal (rat bone derived) cell 1 µm deep in the cell where reflection interference image from cell-to-substrate contacts is mixed with reflection scattering image of the intracellular cytoplasmic features. Viewing the image pair as if they were a stereoscopic pair reveals motile changes in 1/25s. Picture width 10 µm, zoom factor 5, pinhole size 2.98 mm.

**Figure 10 A,B:** Cell periphery of normal (rat bone derived) cell 2 µm deep in the cells shows complicated image which arises from superimposition of the reflection cum backscattering image of the internal cytoplasm over the reflection interference image: the latter is produced by interference of the light reflected from the coverslip and the dorsal surface of the cells (facing the medium side). Rapid changes in 1/25s of the interference pattern seen as darker sinuous stripes in the center of the image indicate rather fast fluctuation of the cell dorsal surface. Picture width 10 µm, zoom factor 5, pinhole size 2.98 mm.

**Figure 11 A,B:** Nucleus of normal (rat bone derived) cell shows very fine structures. 3D viewing reveals motile changes accomplished in 1/25s. Picture width 13 µm, zoom factor 4, pinhole size 2.98 mm.
the internal structure of the normal cell nucleus (Fig. 11) is even more surprising; it shows structures for which identification will depend on dynamic analysis.

The rate of motile exchanges in the cell internal network is rather stable and robust, but there appear to be some differences between the types of cell examined. Its frequency can be estimated to be within a range which extends from a distinguishable 5-10 Hz to over 25 Hz. The measurements are difficult as the background noise slightly blurs the entire image. 3D trafficking of particles means that particles move in all directions. A proportion of them therefore pass the plane of focus and their coming and going out of this plane thereby contributes falsely to the noise level.

The speed of these intracellular motions does not change quickly after (a) instantaneous exchanges of full medium; (b) change to serum-free medium; or (c) phosphate buffered saline (PBS); or (d) a slight drop of pH to 6.5; or (e) moderate temperature changes; or even (f) during the first 10-20 seconds after treatment with distilled water (hypotonic shock). This behaviour indicates a functional stability of this type of motility which appears not to be easily influenced by the presence, or the short absence, of growth factors or by rapid changes of microenvironmental conditions. Only changing to ice-cold full medium slowed down the overall rate of motion after some minutes. This remarkable stability, together with the fact that the range of speeds seen in the IDSN fills the gap in the spectrum of observable speeds in the cell, raises the question as to what extent the regulation of speeds of exchanges and trafficking within the IDSN can be of importance in the epigenetic regulation of cellular processes.

There is also some indication of the mechanical robustness of the IDSN which may be related to internal tensions: it takes more than 10 seconds for hypotonic treatment to start to show the swelling and bursting in the IDSN which leads to its disruption.

Several modes of 3D motile activity in the IDSN were observed. Overall motility spans the entire cell interior, and this may make it difficult to detect the nucleus. In primary rat fibroblasts incubated for 5 hours in serum free Eagle's MEM pH 6.5 prior to examination, the motile activities in the nucleus and cytoplasm were found to be asynchronous. In some cases, "particles" appeared to move across the nuclear membrane in one or more regions, whilst they were apparently blocked in others. Within the cytoplasm, some areas showed higher rates of motility compared to their surroundings, reminiscent of the motility domains (of "nucleating" type) visualized in motile cells by the differencing mode in scanning acoustic microscopy (Vesely et al., 1994). Other than the variations in motile activity noted within the nucleus of some cells, images of rather stable nucleoli surrounded by very active motion in the peri-nucleolar area were seen.

The course of fixation with 2.5% glutaraldehyde in PBS and/or in 96% ethanol was studied in order to differentiate between active intracellular motility and thermal motion and also to examine the usefulness of this approach for correlative microscopy in the control of specimen preparation for electron microscopy. There are differences in the effects of these two fixatives. Glutaraldehyde stabilises structures within approximately 5 seconds and they appear unchanged as the consequence. There is a slight variation in the time (4-6 seconds) needed for full immobilization of the cell: it depends on cell thickness even in a monolayer culture. Ethanol fixation is even more rapid: it fixed within 3-5 seconds, but induced changes seen as released tension and partial restructuring. The granularity of structure was preserved, but differed slightly from the living state.

Glutaraldehyde did not stop all motility, but the remaining motility (bound Brownian motion) was stopped by additional ethanol treatment. It may be that glutaraldehyde crosslinking is less potent in fixing cell fluid compartments, whereas ethanol may possess greater potential to coagulate proteins from solution and, in this way, change everything and even embed loose structures left oscillating on internal surfaces after glutaraldehyde fixation. When hypotonic treatment with water had preceded ethanol fixation by several seconds, then the expansion of the internal network was slightly delayed behind the overall swelling of the cell, which again indicates how resistant the internal network is. After fixation with both fixatives, the optical contrast of the fine 'granular' structure characteristic of cytoplasm is immediately increased, possibly by rapid sequestration of water from proteinaceous structures.

The IDSN varies in size relative to the cell body, being largest in the epithelial-like, fully transformed, rat RSV (Rous Sarcoma Virus) sarcoma K4 cells, where it almost fills the cell body (Fig. 5). It is smallest, confined to the center of the cell, in primary fibroblasts, and almost missing in some rat calvarium bone-derived cells (Fig. 7). The IDSN in K4 cells attached to a solid substratum in vitro, where it is most developed, appears to extend up to the "dorsal" side (i.e., that side remote from the objective lens, facing the medium) of the cell membrane. It does not extend into either the cell periphery or into cellular processes. Its extent varies with cell type and with the time in culture after trypsinization, which may in turn be related to the position in the cell cycle.

From observations by optical sectioning and from the effects of fixation with glutaraldehyde and ethanol, it can be deduced that there is a very narrow fluid compartment between the "ventral" side (facing the
substratum) of the cell membrane and the core of the
IDSN. This represents a communicating layer (Fig. 8),
approximately 1 µm thick, in which both centripetal and
centrifugal trafficking and clustering of small particles
can be observed, sometimes along, or in, what appear to
be preformed channels. Moving particles were some­
times seen to arise from above or below the current
focus level, apparently through small openings, but to
continue to move horizontally.

The overall image of the internal structural and
dynamic organization of the cell creates a novel view of
compartmentalization along the height (Z) axis. The (< 1
µm) contacting layer of cell membrane and rather stable
structures close to the underlying substratum give rise to
the known reflection interference image of cell-to­
substratum contacts. Above the contacting layer (further
from the objective lens) lies an approximately 1 µm
thick layer (Fig. 8), apparently engaged in every kind of
particulate transport, which spans almost from one edge
of the cell to the other and also occupies the cell center
underneath the IDSN. This communication layer does
not extend into the peripheral rim of expanding lamellar
cytoplasm. Next, in normal cells, only the nucleus with
almost negligible IDSN in its vicinity can be found (Fig.
7). Lastly, parts of the top ("dorsal") surface of the cell
are seen at the same level as prominent nucleoli. In
neoplastic cells, an IDSN (Fig. 5) of up to several
micrometers in thickness is found. The IDSN appears to
integrate the cytoskeleton and all perinuclear cellular
organelles from the nuclear membrane, through endo­
plasmic reticulum, cis Golgi network, Golgi stacks, trans
Golgi network, through to the "dorsal" cell membrane
into one dynamic transport compartment.

Discussion

The intracellular spatial network apparently support­
ing 3D movement of small particles may be described as
an integrated or integrating dynamic spatial network
(IDSN) because this is how it is observed in video rate
reflected/backscattered imaging mode. From a functional
viewpoint, it seems interesting that the IDSN is much
more developed in sarcoma cells than in normal mesen­
chymal cells. Spatial periodicity in the IDSN was
estimated to be well below 0.5 µm: measurement with
two dimensional Fast Fourier Transform (using Noran
TN8502 software) in a T15 cell returned major
periodicities between 0.1337 and 0.168 µm⁻¹.

The static morphological image of the IDSN is
reminiscent of the networks in extracted cells shown by
Bell and Safiejko-Mroczka (personal communication), of
those of cytoplasmic filaments seen in stereoscopic high
voltage electron micrographs of whole cell mounts (Ris,
1985), and/or of actin meshworks of the types demon­
strated in cultured cells (Small, 1981) and in the termi­
nal web of intestinal epithelial cells (Hirokawa and
Heuser, 1981). A perinuclear structure of a similar type
was also imaged in high pressure frozen and freeze­
fractured leukemia cells by Malecki (1991). Finally, a
new video-rate confocal scanning laser microscope (built
for imaging human skin) using 100x objective lens
showed a perinuclear granularity of a type similar to the
IDSN in still images of epidermal cells (Rajadhyaksha et
al., 1995). It seems that the IDSN can also support
endocytotic function as well as exocytotic export of
proteolytic enzymes (Krepela et al., 1989): the dorsal
surface of the K4 cell is very actively engaged in
micropinocytosis (Vesely, 1972). The IDSN is rather
stable in respect of mild temperature changes or a short
absence of growth factors contained in full tissue culture
medium, and apparently enables 3D trafficking of small
"particles" (<0.2 µm) over distances that cannot
currently be assessed.

IDSN also seems to confer mechanical stability,
which in turn may have functional impact on important
growth properties such as anchorage dependent growth
(Stoker et al., 1968). The network may provide the cell
with a mechanical self-sustaining structure for intracellu­
lar 3D transport. Thus it may free the cell from the
dependence on attachment and consequent stretching that
is apparently needed for the communication layer to
resume function, which in turn seems to be vital for the
growth of normal cells. Elucidation of a possible
biomechanical role for the IDSN may enhance under­
standing of the differences between normal and neoplas­
tic cells. The methodology employed here may then
contribute in the rapid evaluation of the malignant state
of living neoplastic cells in biopsies.

Fixation with glutaraldehyde stabilizes the IDSN
without changes and fast enough to allow experiments in
correlative microscopy to develop further. Particularly
it should be of interest to try to compare in the EM the
visualization of the IDSN in various reactive stages of
the cell or phases of the cell cycle as seen by the
reflection mode of VRLSCM.

There is a kinetic difference between fixation with
glutaraldehyde and ethanol. It is possible that ethanol
disrupts some linkages in the trabecular structure of the
IDSN causing its rearrangement by the residual forces in
the net. Ethanol apparently possesses a greater potential
to coagulate proteins from solution, stabilizing pendulous
structures still moving on internal surfaces after glutaral­
dehyde fixation.

Exploratory experiments comparing video rate single
frame presentation (and grabbing) and averaging of 2, 4,
8, and 16 frames indicated that averaging of 2 frames
decreases both noise and the time resolution. Averaging
4 and more frames, the loss of time resolution sharply
increases.

Spherical aberration due to the use of the oil immersion objective in water is hardly a problem at the very short ranges, past glass (up to 10 µm deep), which we employed. To obtain images less influenced by this possibility, we also examined some samples using water immersion objectives, but found no difference in the way the IDSN was imaged and in the intracellular dynamics.

The requirements for correlative examination of the living cell by the reflection mode of VRCLSM followed by fixation and analysis of the observed structure in the EM can be summarized into several steps of accomplishment. The basic setup is composed of a VRCLSM based on an upright or an inverted microscope (less suitable for water immersion lenses) with reflection mode and a suitable lens, video recorder (date and time, including seconds), an open through-flush chamber of the blotting type and 37°C heating of a plastic "tent" incorporating the microscope. Image acquisition should consist of non-interlaced image grabbing and/or digital videodisc. Accommodation of cells and experimental manipulations can be further improved by introducing a closed through-flush chamber, temperature control of the microscope stage and the lens and finally by computer controlled operations. A substantial drawback in the design of contemporary light microscope stands is in the rather sloppy control of specimen positioning in X, Y and especially Z. Anything cheaper than feedback control (Lani, 1993) is not really suitable for the job. Another necessity is presentation of the contextual information about the present state of experimental manipulations with the cell under observation, or in another part of the same cell. An ordinary inverted microscope arranged to allow observation of the specimen at lower magnification from the opposite direction would be the easiest solution. Employing a system for bidirectional viewing (Maly and Vesely, 1979) should allow for much more sophisticated approach than that described here.

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Discussion with Reviewers

M. Malecki: Phototoxicity is a major plague in light microscopy of living cells. What was the energy delivered to the cells in your experiment? Did you evaluate the viability of the cells?

Authors: It was not our aim to preserve cells, except in the very short term. We did not evaluate the viability of the cells. The patterns of microscopic movement which we observed could be seen when we first found a field and immediately, as could be checked from the videotape record - from which we may conclude that they were not induced by the process of observation. There was no change on changing the zoom factor, whilst any radiation damage would be expected to square with doubling the magnification. We are not surprised by this evident lack of a phototoxic effect, because only a tiny fraction of the incident radiation is absorbed in the specimen: that part which is reflected and backscattered (1 in 10^6 according to M. Petran, personal communication, 1970) possibly being of no consequence because it is reflected.

From measurements made with a laser power meter, the maximum power given in the 488 nm line with the system we used was 570 W at the objective lens.