# Proving Tumour Cells by Acute Nutritional/Energy Deprivation as a Survival Threat: A Task for Microscopy

HANA JANECKOVA[1], PAVEL VESELY[2] and RADIM CHMELIK[1]

[1]*Institute of Physical Engineering, Faculty of Mechanical Engineering, BUT, Brno, 616 69;*
[2]*Institute of Molecular Genetics, AS CR, Prague 4, 142 20, Czech Republic*

**Abstract.** *Malignant cells appear to possess a special aptitude for survival. We attempted to prove this in vitro by acute nutritional and energy deprivation as a survival threat. A phosphate-buffered saline (PBS) survival test in cell culture allowed static observations. These were supplemented by classic and quantitative phase-contrast time-lapse microscopy. From one normal and four neoplastic cell populations, no cells survived 77 hours of exposure to PBS. Only G3S2 derived from a human breast carcinoma survived 60 hours. Cells in sparse culture were more vulnerable than those in dense. Epithelial cells were more vigorous than mesenchymal cells. Cells of greater malignancy resisted longer. Evaluation in culture as detailed by digital holographic microscopy (DHM) revealed an increase in the compactness of the intracellular mass motility from normal to metastasizing mesenchymal cells, thus reaching the level of epithelial G3S2 cells. Studying the PBS survival test with DHM opens a new approach to investigations of the structural integrity of neoplastic cells.*

We anticipate that tumour cells are endowed with some unusual attributes empowering them to survive and expand locally and/or distantly *in vivo* under critical conditions. In order to uncover genomic profiles of these cells in action, we attempted to simulate the *in vivo* situation under *in vitro* conditions. The *in vitro* situation allows for a direct microscopic observation of living cells' behaviour. Thus any cell that is observed to behave suspiciously can be picked up and isolated for immediate genomic or proteomic profiling; moreover, the molecular analyses of an isolated single cell are becoming more and more efficient (1).

Following previous experience from a test using Hanks' balanced salt solution (HBSS) (2), designed to examine the course of cell attachment in 3D, a next step in that direction at the cell biology level was carried out. A phosphate-buffered saline (PBS) survival test was designed to prove attached growing cells. PBS rapidly deprives cells of nutrients and energy sources and elicits fast cell reactions manifested as a motile activity which frequently leads to cell death.

Classic (Zernike) phase-contrast time-lapse micrography was used to record the detailed reactions of cells to such a sudden change in the microenvironment. This was later followed by the application of quantitative phase-contrast imaging in a transmission digital holographic microscope (DHM). DHM allowed for characterization of cell behaviour by dynamic phase difference.

*Correspondence to:* Hana Janeckova, Brno University of Technology, Faculty of Mechanical Engineering, Institute of Physical Engineering Technicka 2896/2 Brno 616 69, Czech Republic. Tel: +420 602548625, +420 541142833, Fax: +420 541142842, e-mail: hanajaneckova@email.cz

*Key Words:* Tumour cell, nutritional deprivation, energy deprivation, cell survival, cell death, digital holographic microscopy, dynamic phase difference.

## Materials and Methods

*Cells*. LF cells are primary human subcutaneous fibroblasts grown already for one year in culture and used at the *in vitro* 30th passage (kindly provided by Dr. Eva Matouskova). LW13K2 cells are spontaneously transformed rat embryonic fibroblasts (3). A3 (full name A337/311RP) were obtained from a metastasis that arose in a rat injected with A297 cells derived from LW13K2 cells (4) and showed high incidence of metastases (5). RsK4 cells are double transformants of inbred Lewis rat embryo fibroblasts. Spontaneous *in vitro* transformation was followed by *in vitro* induced super-transformation by Rous sarcoma virus (6). EM-G3 cells are a new clonal cell line derived from a primary lesion of human infiltrating ductal breast carcinoma (7). G3S2 cells were cultured in nutritionally stringent conditions for one year longer than G3S1 cells that were derived from EM-G3 cells by pressing them to neoplastic progression using chronic nutritional stress combined with two treatments of 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C. G3S1 cells then showed much increased proteolytic activity in 2D FITC gelatin zymography (8) and lost dependence on special growth factors needed by EM-G3 cells.

*Culture techniques*. All cell populations used were grown attached to a solid surface. The maintenance of these cells in culture was with standard medium (Eagle's minimal essential medium with

10% calf serum) and transfer to new culture vessels was carried out by trypsinization.

For microscopy observations the same medium was modified. It was without phenol red and only with one third of sodium bicarbonate while buffered to pH 7.4 with 20 mM of nonvolatile *N,N-bis*(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) buffer. For PBS challenge, standard PBS (NaCl 8 g/l, KCl 0.2 g/l, $KH_2PO_4$ 0.2 g/l, $Na_2HPO_4$ 1.15 g/l, pH 7.4) was used.

*PBS survival test*. Freshly prepared suspensions from trypsinized cells were seeded in NUNC 35 mm diameter culture dishes for determining the survival endurance, or into the bottom part of an observation chamber placed in such a dish for DHM examination. Two cell densities were used: $10^3$ cells/cm$^2$ for sparse and $10^4$ cells/cm$^2$ for semi-confluent coverage of the culture surface. Two days after seeding, the culture medium was thoroughly removed, cells were then washed twice with an excess of PBS, 2 ml of PBS were added and the cultures were incubated in a humidified $CO_2$ incubator at 37˚C, or if grown in observation chambers, immediately mounted for the DHM recording.

To determine the remaining viability of cells in culture, 2 ml of standard medium were added at desired time points without removing any PBS so as not to lose any potentially surviving cells floating in the PBS. The next day, an ordinary medium exchange followed and cell survival was scored by searching microscopically for cells recovering morphology and starting growth and multiplication.

*Classic phase-contrast time-lapse micrography*. A Nikon Diaphot 300 for computerised phase-contrast time-lapse observations was employed using a ×10 objective lens, UniBrain digital camera and software. Cells for PBS survival follow-up were placed in Falcon T25 flasks with filter screw caps to minimize drying out and prevent variation of pressure inside the flask.

*Observation chamber for DHM*. The observation chamber consisted of a round coverslip (22 mm diameter) glued with silicon adhesive to a stainless steel annular ring spacer (0.8 mm thick, inner diameter of 15 mm). This formed the bottom part of the observation chamber. The chamber was closed after filling with another coverslip (22 mm diameter) with a vacuum grease seal.

*DHM*. The DHM, which was built in our laboratory, used four ×10 objective lenses (two as condensers, two as objectives), digital camera Astropix 1.4 and software developed by us for the camera and for the image reconstruction. The light source was a halogen lamp spectrally restricted by an interference filter (FWHM=10 nm, maximum transmissivity at 650 nm). The DHM setup, principle of the hologram formation, and the image reconstruction have already been described (9).

We used the DHM in a time-lapse observation mode to calculate the quantitative changes of the cell dry mass distribution during the reaction to the PBS.

*DHM imaging modes: Intensity contrast* is formed by the image intensity similarly as in conventional light microscopy (bright field). *Quantitative phase contrast* is formed by the specimen-induced phase shift between the reference and the object waves (9). It visualizes the dry mass distribution in the specimen (10). *Dynamic phase difference* results from the subtraction of two quantitative phase-contrast images taken with a time interval. It visualizes the changes of the cell dry mass distribution within this deliberately chosen interval. Phase contours connect the points of the same phase-shift value and accentuate the shape of the cell.

Table I. *Cell survival after PBS challenge causing acute nutritional and energy stress*.

| | Cell density | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Sparse | | | | Semi – confluent | | | |
| PBS exposure (hours) | 4 | 24 | 36 | 60 | 24 | 36 | 60 | 77 |
| Cells | | | | | | | | |
| RsK4 | + | + | – | – | + | – | – | – |
| A3 | + | + | + | – | + | – | – | – |
| LW13K2 | + | – | – | – | + | – | – | – |
| G3S2 | NT | + | + | – | NT | + | + | – |
| EM-G3 | NT | + | + | – | + | + | – | – |

+ Cell survival and multiplication; – cell destruction; NT not tested.

## Results

*Biological framework*. The cells listed in Table I were examined in the PBS survival test in order to determine their endurance in this deprivation condition and also to define most interesting periods of time that deserved further detailed microscopical examination by DHM.

*PBS survival test*. The overall assessment of cell survival in PBS is summarized in Table I. It shows that in G3S2 cell populations, few cells were able to survive for up to 60 hours in PBS and their survival was dependent on the cell density. The individual isolated cells in a sparse culture survived for only 36 hours. An epithelial organization of the cell cytoskeleton appears to be more resistant towards this kind of stress. More malignant cells in pairs such as mesenchymal A3 *versus* the antecedent LW13K2 and epithelial G3S2 *versus* the antecedent EM-G3 cells emerged as being more robust.

*Classic phase-contrast time-lapse micrography.* Classic phase-contrast microscopy was used to capture the course of events from the onset of the PBS treatment till the end marked by cell death. Selected features are presented in Figure 1, which illustrates the rapid cell response as early as after 3 min in PBS.

The upper three images of Figure 1A show the course of a rapid detachment of normal human fibroblasts followed by a retraction of cells toward a remaining higher density group of cells. The bottom image documents the resulting necrotic cell death after 18 hours in PBS.

In Figure 1B, the upper three images show the course of a rapid detachment of rat sarcoma A3 cells and their on-spot motile activity. The bottom image shows oncotic cell death after 24 hours in PBS.

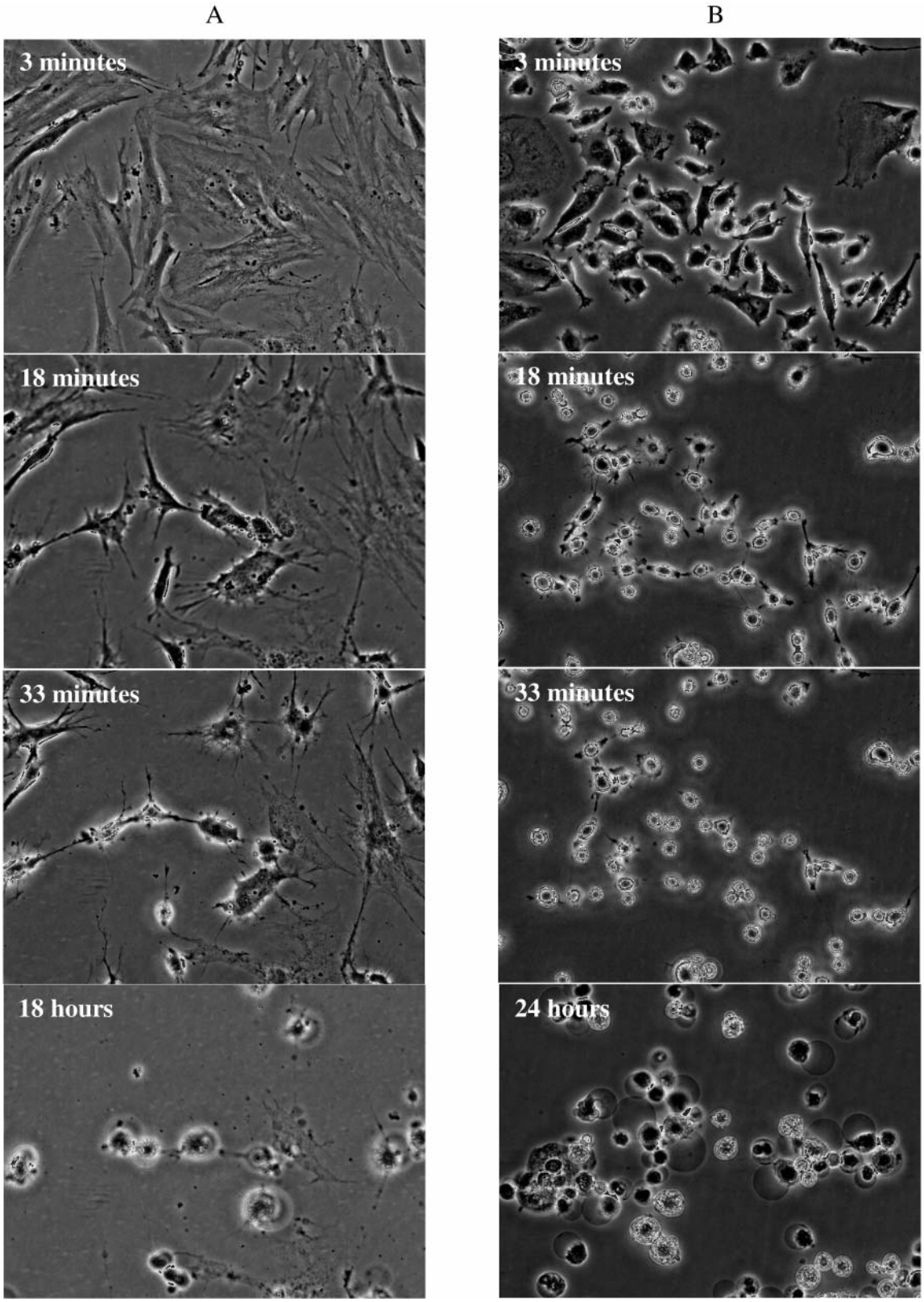


Figure 1. *A, Human subcutaneous fibroblasts LF. The duration of the exposure to PBS is stated in each image. The bottom image shows necrotic cell death after 18 hours in PBS. B, Rat sarcoma A3 cells. The bottom image shows oncotic cell death after 24 hours in PBS. Image width is 350 μm.*

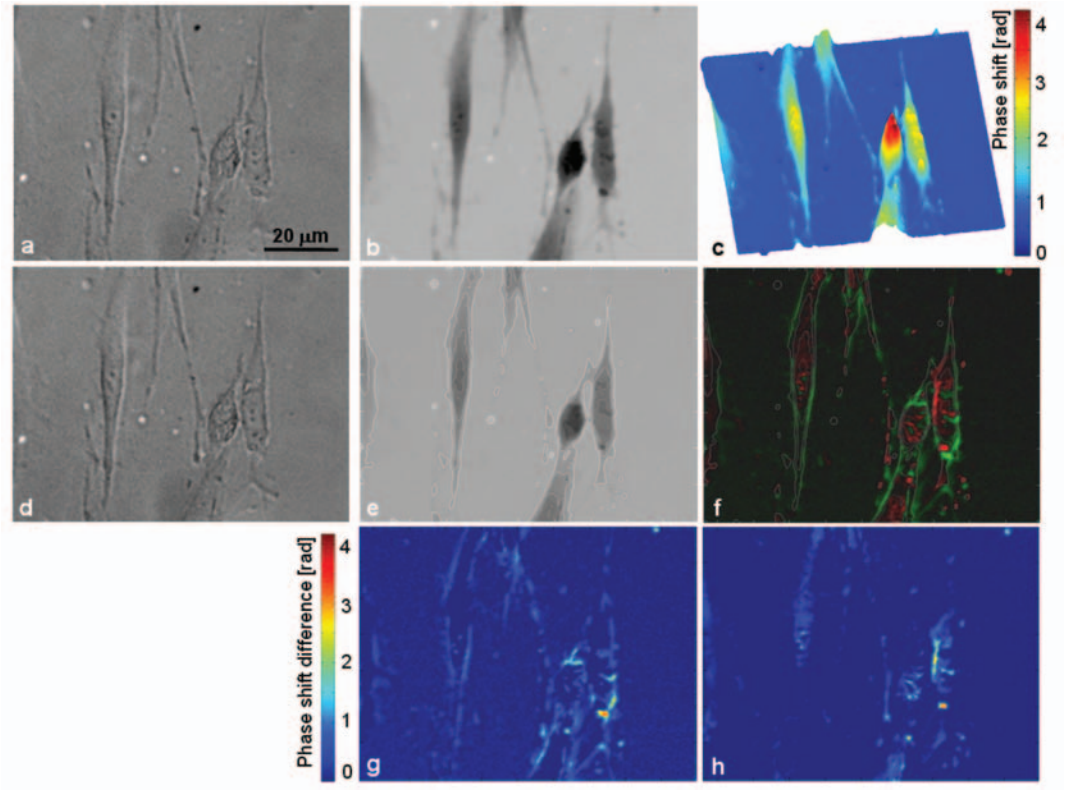


Figure 2. *Sparse LF cells in the 5th (a-c) and in the 13th minute of the reaction to PBS (d-h). Figure size 94 μm ×70 μm.*

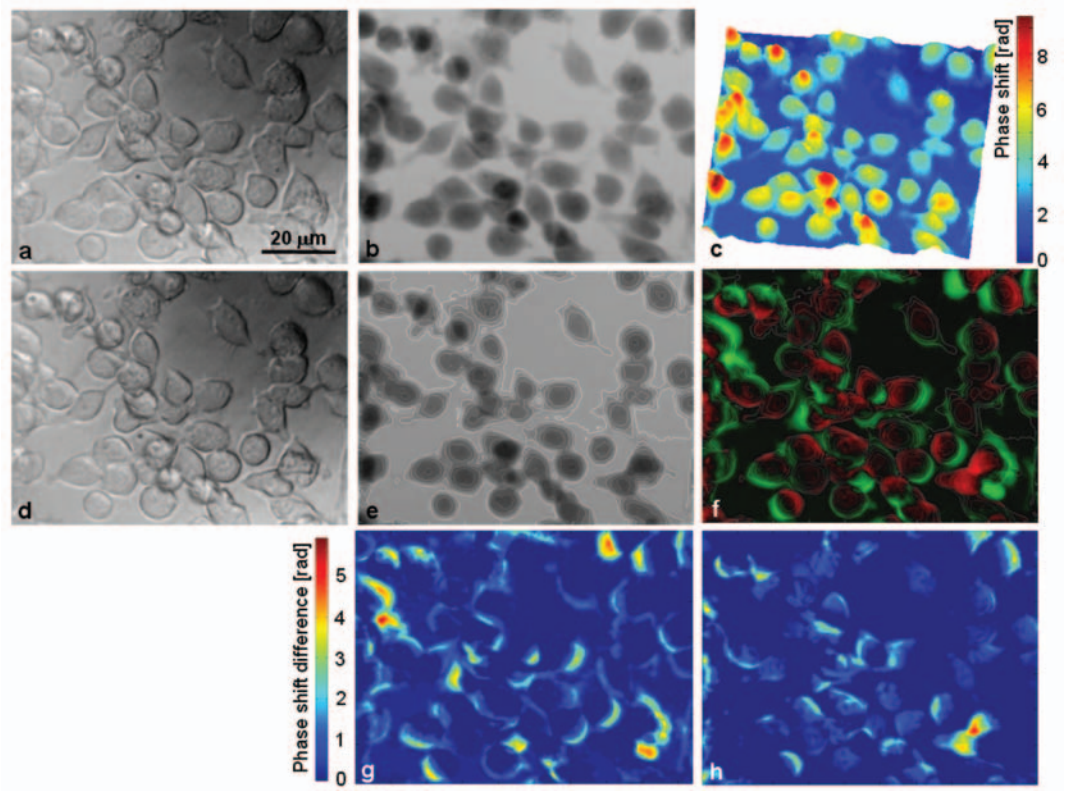


Figure 3. *Dense LW13K2 cells in the 5th (a-c) and in the 8th minute of the reaction to PBS (d-h). Figure size 94 μm ×70 μm.*

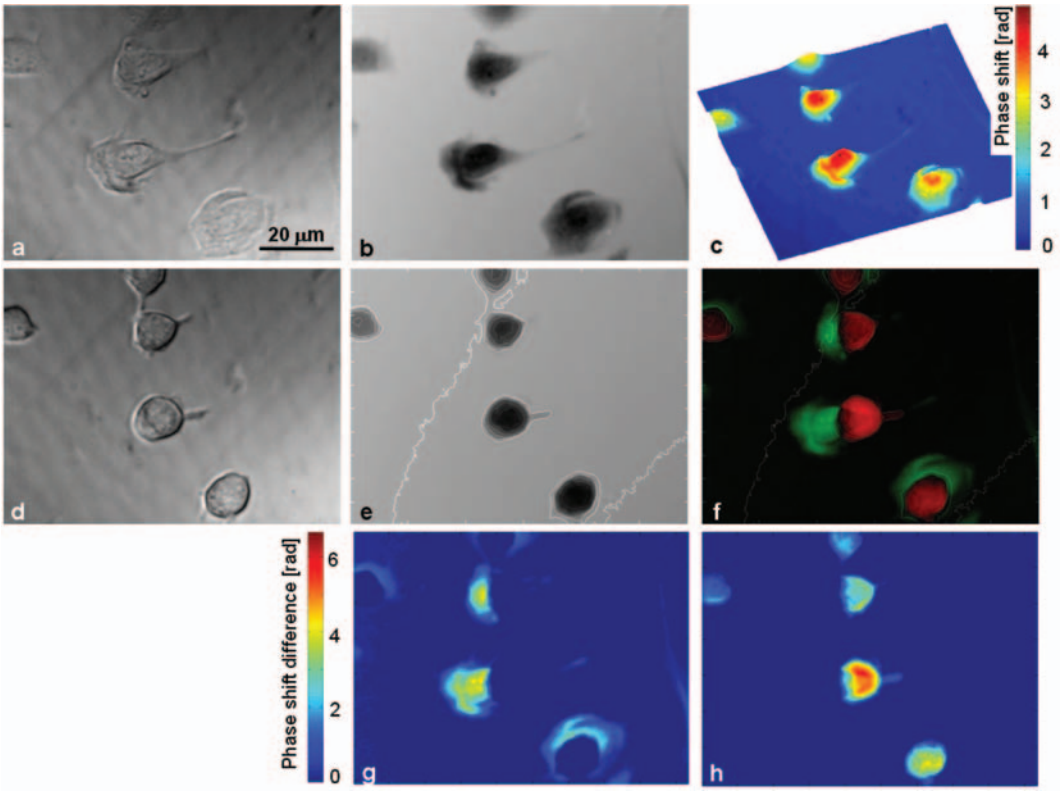


Figure 4. *Sparse A3 cells in the 5th (a-c) and in the 22nd minute of the reaction to PBS (d-h). Figure size 94 µm ×70 µm.*

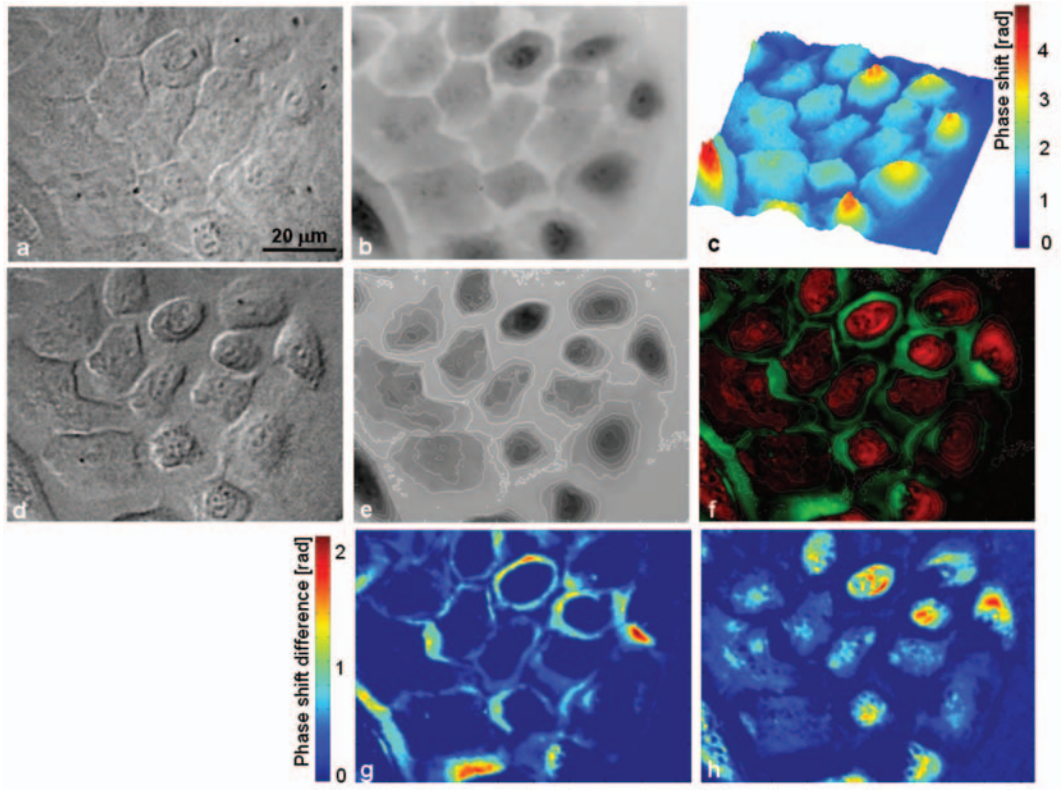


Figure 5. *Dense G3S2 cells in the 5th (a-c) and in the 10th minute of the reaction to PBS (d-h). Figure size 94 µm ×70 µm.*

*DHM imaging results*. Four sets of images were acquired, each characterizing one type of cell. For each type there are 3 images in the first row (a-c) which depict the specimen at the beginning of the reaction, usually after 4 minutes from the moment of the last addition of PBS marking the start of the PBS challenge. These images are a) the intensity contrast, b) the quantitative phase-contrast, and c) a 3D visualization of the quantitative phase-contrast, which is pseudo-coloured for a pertinent expression of the spatial dry mass distribution. The colour scale corresponds to the phase-shift values in radians.

The other two rows (d-h) characterize the specimen at certain time points during the PBS-evoked reaction that were selected after visual examination of the time-lapse recording, which showed interesting behavioural traits. Images in the second row are d) the intensity contrast, e) the quantitative phase-contrast, and f) the dynamic phase difference within the given period of time. In this image, the green colour marks a decrease and the red colour an increase of the dry mass of each cell from the beginning of time-lapse imaging to the chosen time point. The lightness of the colours is proportional to the dry mass differences. There are phase contours added to both the phase-contrast image and the dynamic phase difference image for a better orientation.

In the third row, the decreases (g) and increases (h) of cell dry mass are depicted separately and these are pseudo-coloured with a colour-scale corresponding to the values of the change for a quantitative evaluation of the dry mass motility.

Figure 2 depicts normal sparse fibroblasts in the 5th (a-c) and in the 13th (d-h) minute of the PBS challenge. LF cells manifested detachment starting in longest lamellae and around the borders of the cell peripheral regions (see Figure 2 f - green colour near the borders of the cell). They showed massive disintegrated intracellular dry mass motility, which is pictured with red/green spots inside of the cell (Figure 2 f).

There are dense LW13K2 cells in Figure 3 in the 5th (a-c) and in the 8th (d-h) minute of their reaction. Their detachment is mostly well proportioned (uniformity of red and green colour in Figure 3 f) and polarized (one part of the cell attached to the surface and the other retracting), however, with motionless centre. The cell centre shows no dynamic disintegration.

Figure 4 depicts the state of the reaction of sparse A3 cells to PBS in the 5th (a-c) and 22nd (d-h) minute. The detachment was strongly polarized and intracellular mass motion compact until they formed a round shape in which they resisted the condition for a long time, showing thin protrusions engaged in fast locomotive activity.

The reaction of dense G3S2 cells is depicted in Figure 5. In Figure 5 a-c, cells in the 5th minute of the reaction are shown. The first act was the smooth disconnection of the cell-to-cell adhesions. Cells in the 10th minute of their reaction to PBS are shown in Figure 5 d-h. Cell retraction can be seen to be unpolarized, regularly around the whole cell border and some unevenness in the cell mass within the otherwise compactness of the intracellular mass motility can be seen (see Figure 5 h for non-uniformity of central cell mass).

## Discussion

Proving tumour cells by acute nutritional and energy deprivation as a survival threat using PBS as a tool revealed a vast variation of survival potential within rather uniform cell populations. The resistance of individual cells to a sudden sharp change in the microenvironment depends on intrinsic factors and their 'social situation' *in vitro*. The origin of the cell, be it mesenchymal or epithelial, belongs among these intrinsic factors, as well as a degree of malignancy. This is what our experiments demonstrate at least. The 'social situation' is according to the density of cells in the layer covering the culture substratum and mainly reflects the multitude of cell-to-cell contacts. The greater sensitivity of single cells and retraction of cells in groups towards an imaginary centre seem to indicate an emerging danger of anoikis *i.e.* cell death from lack of such contacts in this condition. However, the sweeping misery brought about by PBS challenge apparently compelled cells to abandon all efforts aimed at programmed cell death, apoptosis that is energy dependent. Perhaps just because of a lack of time. Although not all cell fates were screened by classic phase-contrast time-lapse micrography in every detail, apoptosis was not observed and the most sensitive normal fibroblasts died by necrosis, while hardened A3 cells (11) by oncosis. This, together with some surprisingly long enduring resistance to PBS influence and in the end survival, indicates that the signal initiating apoptosis is an early signal and one which could be missed irreversibly.

Transmission digital holographic microscopy was used because quantitative phase-contrast imagery of a cell is generally a picture of dry mass distribution. Various modes of DHM imaging were examined and finally the dynamic phase difference mode appeared to be the most explicative. This means that one printable image facilitates grasping of the course of the whole time series of images. This mode follows the intention to visualize patterns of intracellular motility by the subtraction of digital images from scanning acoustic microscopy (12) and is an improvement on the method proposed by Dunn and Zicha who calculated cell margin areas of protrusion and refraction as dry mass changes though with low temporal resolution (13). Due to the temporal resolution provided by DHM and the digitizing technique, interesting patterns of motility over the whole cell area during the course of PBS treatment were revealed along the developmental line of malignancy *i.e.* from normal fibroblasts (LF in Figure 2) over tumorigenic fibroblasts

(LW13K2 in Figure 3) to metastasizing fibroblasts (A3 in Figure 4). Normal fibroblasts showed signs of internal disintegration. Tumorigenic fibroblasts produced mainly polarized activity, while metastasizing fibroblasts showed compact translocation of their cell mass. The motility pattern of G3S2 cells (Figure 5), which were most robust in the survival test, resembled those of the A3 cells, with a minor difference in the appearance of a lower density patch within the accumulated cell mass.

It seems to be a general rule that polarized (mesenchymal) cells (with one axis longer than the other) show a slower or faster polarized retraction, however, non-polarized (epithelial) cells demonstrate an unpolarized retraction. Thus the reaction follows the cell morphotype.

Similarity in compactness of patterns of cell internal mass motility between G3S2 and A3 cells revealed by DHM seems to fit well with the established knowledge of the epithelial–mesenchymal transition (14) of advanced carcinomas to a sarcomatous morphotype.

From these results, it is possible to conclude that introduction of DHM into cell biology *in vitro* will contribute to the simultaneous characterization of cells during an actual observation. As it is non-invasive and intravital it should have a significant impact on investigations of the basis of individual, cell properties (15) in contrast to average properties of the cell population.

## Acknowledgements

This project was supported by GACR (project no. 202/08/0590), by application research programmes of the Ministry of Education of the Czech Republic (project no. MSM0021630508) and project no. AV0Z50520514 of the Academy of Science of the Czech Republic. This work was also supported by the Faculty of Mechanical Engineering BUT (project no. BD1373002).

## References

1 Templer RH and Ces O: New frontiers in single-cell analysis. J R Soc Interface *5*: 111-112, 2008.
2 Vesely P, Blase C, Matouskova E and Bereiter-Hahn J: Arising podosomal structures are associated with neoplastic cell morphological phenotype induced by the microenvironment. Anticancer Res *26(2A)*: 967-972, 2006.
3 Vesely P and Weiss R A: Cell locomotion and contact inhibition of normal and neoplastic rat cells. Int J Cancer *11*: 64-76, 1973.
4 Vesely P, Chaloupkova A, Urbanec P, Urbancova H, Bohac L, Krchnakova E, Franc F, Sprincl L, Vousden K, Moss R and Dilly N: Patterns of *in vitro* behaviour characterizing cells of spontaneously metastasizing K2M rat sarcoma. Folia Biol (Praque) *33*: 307-324, 1987.
5 Cavanna T, Pokorna E, Vesely P, Gray C and Zicha D: Evidence for protein 4.1B acting as a metastasis suppressor. Cell Sci *120*: 606-616, 2007.
6 Vesely P, Donner L, Cinatl J and Sovova V: Interaction of Rous sarcoma virus with rat embryo fibroblasts of inbred Lewis strain *in vitro*. Folia Biol (Praque) *14*: 457-465, 1968.
7 Brozova M, Kleibl Z, Netikova I, Sevcik J, Scholzova E, Brezinova J, Chaloupkova A, Vesely P, Dundr P, Zadinova M, Krasna L and Matouskova E: Establishment, growth and *in vivo* differentiation of a new clonal human cell line, EM-G3, derived from breast cancer progenitors. Breast Cancer Res Treat *103(2)*: 247-257, 2007.
8 Tolde O, Rosel D, Brabek J and Vesely P: Myzitiras and Vthela morphotypes as new signatures of neoplastic progression? *In*: Abstracts of the 8th International Conference of Anticancer Research, 17-22 October, 2008, Kos, Greece. Anticancer Res *28*, 3529, 2008.
9 Janeckova H, Kolman P, Vesely P and Chmelik R: Digital holographic microscope with low-spatial and temporal coherence of illumination. *In*: Optical and Digital Image Processing. Proc. of SPIE 2008, *7000 (70002E),* 1-8, 2008.
10 Barer R: Interference microscopy and mass determination. Nature *169(4296)*: 366-367, 1952.
11 Rosel D, Brabek J, Tolde O, Mierke CT, Zitterbart DP, Raupach C, Bicanova K, Kollmannsberger P, Pankova D, Vesely P, Folk P and Fabry B: Up-regulation of Rho/ROCK signaling in sarcoma cells drives invasion and increased generation of protrusive forces. Mol Cancer Res *6(9)*: 1410-1420, 2008.
12 Vesely P, Luers H, Riehle M and Bereiter-Hahn J: Subtraction scanning acoustic microscopy reveals motility domains in cells *in vitro*. Cell Motil Cytoskeleton *29(3)*: 231-240, 1994.
13 Dunn GA and Zicha D: Dynamics of fibroblast spreading. J Cell Sci *108*: 1239-1249, 1995.
14 Guarino M, Rubino B and Ballabio G: The role of epithelial-mesenchymal transition in cancer pathology. Pathology *39(3)*: 305-318, 2007.
15 Martini J, Hellmich W, Greif D, Becker A, Merkle T, Ros R, Ros A, Toensing K and Anselmetti D: Systems nanobiology: from quantitative single molecule biophysics to microfluidic-based single cell analysis. Subcell Biochem *43*: 301-321, 2007.

*Received December 11, 2008*
*Revised March 19, 2009*
*Accepted April 3, 2009*