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The role of time-lapse fluorescent microscopy in the characterization of toxic effects in cell populations cultivated *in vitro*

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ABSTRACT

Responses of cell populations *in vitro* to toxic substances are very dynamic and exceed hours or even days. Toxicologists realise that cell-based dynamic assays can acquire important additional information about toxic responses of individual cell within a treated population. In the past, this type of cellular dynamics could have been monitored only with the help of time-lapse microcinematography. In spite of several advantages, the time-lapse approach has been used relatively infrequently in the routine *in vitro* cytotoxicity assessment. The main reasons were demanding time and work schedules, problems with quantification of visual information, and lack of mechanistic data. Recently, the situation has changed dramatically. The progress in digital imaging technology coincides with enormous development in the field of fluorescence microscopy. With the help of specific fluorochromes or fluorescent proteins we can now analyse practically all sub-cellular and cellular events.

Some producers developed large-scale high-throughput systems for live cell imaging. Nevertheless, there has been significant progress in small-scale approach as well. New versions of motorised microscopes are fulfilling principal demands for *in vitro* assessment of toxic effects: ease of performance, high-throughput of data, quantitative cell-based analysis, simultaneous assessment of several parameters, and control of the environment conditions for cultivation of cells. In this paper, we present our experience with two of these systems designed for long-lasting observation of living cells in phase contrast and fluorescence. Our aim is to draw attention to the suitability of small-scale cell-based assays for determination of cytotoxicity *in vitro*. We believe that these methods could be considered as a further step towards the replacement of animal testing.

1. Introduction

Continuous changes are one of the most fundamental characteristics of all living systems and as human beings are intensely visual creatures, there is a natural tendency to record and present all dynamic phenomena in the visual format. At the beginning of the 20th century our technological progress reached the level which allowed the construction and use of first cameras, thus recording and subsequently presenting motion as a sequence of still pictures – movies. Simultaneously with the first cinematographic experiments, some scientists tried to use this technique for recording of the dynamic behavioural patterns at the cellular level. The pioneer of such microcinematography was the French scientist Jean Comandon (1877–1970). In 1909, he was probably the first scientist in history who used a darkfield microscope connected to a camera to record movements of some bacteria. Over the next 50 years he produced numerous excellent scientific and educational movies and became the true classic in this field of science. For more details about the history of scientific microcinematography see Landecker, 2006.

In the middle of the last century, many biologists began experiments which employed cells cultivated *in vitro* to study many phenomena including toxicity assessment. Some of these scientists realized that cells *in vitro* models are quite suitable for observing processes which take place in living objects. Thus they connected their microscopes to 16 mm cameras and were able to produce movies for research purposes. This classical technique involved repeated imaging of cells in culture at defined points of time and was originally used mainly in the analysis of cell division and cell motility.

Dynamics at the cellular level can be recorded and analysed on very different scales of time, ranging from microseconds to days. The microsecond scale is important in studying the interactions of macromolecules, the activation of genes, spatial and temporal regulation of some signals such as calcium bursts, etc. For this purpose we need a very fast recording system employing high-speed and sensitive cameras along with automated software support. These advanced fluorescence microscopy techniques are reviewed

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elsewhere (Goldman and Spector, 2005; Day and Schaufele, 2005; Anonymus, 2007) and are not covered in this article.

On the other hand, there are cellular processes that last for days. Prime example of these processes might be the responses of cell populations to toxic substances. To record dynamics of this type we have to use time-lapse microscopy. Original time-lapse systems employed classical cameras and they were often developed by individual researchers rather than being commercially available. The turning point here is the 1980's when specific time-lapse video recorders became commercially available and time-lapse video microscopy has become a standard research methodology. In the last decade, there has been an enormous progress in digital technologies that brought together and integrated various hardware and software platforms. One consequence of this technological outburst is the integration of various time-lapse recording modes into imaging systems, where a time-lapse mode represents the standard imaging possibility with relatively easy employment and subsequent analysis.

The progress in digital imaging technology has coincided with significant developments in the field of fluorescence microscopy too. With the help of specific fluorochromes or fluorescent proteins we can analyse practically all sub-cellular and cellular events. For review see Lippincott-Schwartz and Patterson (2003) and Pepperkok and Ellenberg (2006). Live-cell imaging has thus become an important analytical tool in cell biology, and we have to think about rational applications of this technique in the field of *in vitro* toxicology. In this article, we will focus on the analysis of visual information acquired from living cell populations, where the major sources of this information in the form of individual pictures are fluorescence signals.

2. Time-lapse fluorescence microscopy in toxicology

Soon after the establishment of toxicology *in vitro* as a science, many toxicologists have realized that to describe properly the toxic effects *in vitro* we need information about the dose–response relationship. Moreover, it has been suggested that beside this basic characteristics it might be advantageous to acquire information on:

(a) the dynamic aspects of the toxic response in a treated cell population;
(b) the heterogeneity of the toxic response within a given cell population;
(c) the recovery or demise of the treated cell population.

In experiments focused on discovering the mechanisms of action of toxic substances, observations can be made over relatively short periods of time (minutes). On the other hand, when we intend to use living cells to study basic cellular responses to toxic damage or stress, it is necessary that the observation period is extended over many hours or even days to cover several cell cycles. To accomplish this purpose, non-invasive (non-destructive) methods for toxicity assessment are needed.

For many years the only method fulfilling the criteria for non-invasive, long-term recording of individual cell behaviour has been time-lapse microcinematography, time-lapse video microscopy or, more recently, computer-enhanced video microscopy. All those approaches essentially rely on two main techniques for visualisation of living cells *in vitro* – phase contrast or differential interference contrast (DIC). Using this visualisation system, it was possible to record and analyse

- the number and quality of cell divisions including any aberrations;
- the characterisation of cell cycle duration;
- G0–G1 transition;
- cell morphology dynamic;
- cell adhesion and motility;
- the types of cell death;
- the heterogeneity in the response to toxic stimuli.

In spite of a broad extent of measured parameters, the time-lapse approach has been used relatively infrequently in routine *in vitro* cytotoxicity assessment. The main reasons were demanding time and work schedules, problems with quantifying visual information, and a lack of mechanistic data. Regardless of the aforementioned difficulties, there were some laboratories, which traditionally specialized in this methodological approach and with help of some more or less originally developed technical devices they were able to successfully address selected questions in this toxicological field. For our contribution see Cervinka (1992) and Cervinka and Drobnik (1984).

The situation has changed dramatically in recent times, and the new technological possibilities such as software-based calibration and measurement of changes in visual signals help to overcome many of the above-mentioned limitations.

3. Methodological requirements for the visualisation and long-term observation of living cells *in vitro*

Before any relevant time-lapse, live-cell imaging experiments are performed, several essential technical and biological requirements need to be fulfilled. In every experiment, we have to guarantee optimal conditions for non-destructive visualisation of a treated cell population and for recording their behaviour and optimal conditions for the cells on the microscopic stage.

For optimal conditions to visualize living cells, we need a suitable visualisation system; e.g., phase contrast, DIC, fluorescence, all with the possibility of automatically changing mode of observation. Motorised (robotic) inverted microscopes or scanning devices are, therefore, needed. Another essential part of the whole system is the module where image recording and storage takes place; e.g., camera, control unit (exposure, intervals) and other peripheries. During observation and recording, we have to ensure the stability of the optical conditions, the stability of the focus plane, vibration free status of the mechanical parts and the stability of the lighting.

To maintain cells functionally normal during the recording process we have to control the culture environment. Obviously, the physical, chemical and biological properties of the cultivation–observation chamber are of utmost importance. The cultivation chamber should ensure the stability of the cultivation condition, as well as enable the exchange of cultivation media during observation. Another essential factor is precise temperature control. Properties of the gaseous environment (CO_2 concentration, oxygen concentration, and humidity) should also be regulated. Composition of the cultivation medium, pH, osmolarity and optical properties are important too. Finally, we have to ensure the proper lighting conditions, in particular when it comes to the interaction of cells with fluorophores.

3.1. Cultivation chambers

Live-cell imaging cultivation chambers have always been discussed in the history of time-lapse microcinematography. Cultivation–observation chambers are important because they have to ensure optimal culture condition for cells and at the same time they should have excellent optical conditions for observation. Nowadays, there are several commercially available chambers and in the literature you can find numerous examples of different homemade modifications. Many investigators have developed and fabricated customized chambers for living cell observation (for details see http://www.microscopyu.com/articles/livecellimaging/culturechambers.html).