Algorithmic analysis of drug induced apoptosis and proteasome inhibition in cancer cells based on time-lapse microscopy images

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ABSTRACT

Time lapse microscopy imaging has revolutionized the study of dynamic events taking place inside living cells. When combined with automated image analysis it provides a powerful tool that enables spatiotemporal large-scale quantification at the level of complete cell population. However, existing image analysis methods are rather limited in analyzing such time lapse datasets. Here is presented a fully automated approach to analyze phase contrast and fluorescence microscopy images using CellProfiler. Cellprofiler is an open source modular package that contains advance algorithms for image analysis for extraction of quantitative information from biological images. In this study pipelines based on CellProfiler were designed and implemented for analyzing drug induced apoptosis and proteasome inhibition in cancer therapy. A method for automatic extraction of apoptotic cells using phase contrast images based on their intrinsic shape features is also proposed.

1. INTRODUCTION

Analyzing microscopic images and videos to extract useful information is becoming an increasingly important activity in many scientific laboratories [1]. Time-lapse microscopy imaging is broadly applied to record living cells over an extended period ranging from days to weeks. Recent advances in this field have made it possible to study different cell processes including apoptosis, cell division and cell migration [2]. This technique provides an insight into the nature of cellular functions that can be helpful in research areas like drug discovery, stem cell research, genomics and proteomics [3-4]. However, with the rapid increase in amount of data generated, our ability to interpret this information remains limited. Manual analysis of these massive data files takes weeks of tedious work, with the possibility of losing vital information stored in these images. Therefore, there is a great need for an automated and quantitative cell population monitoring system that facilitates the analysis of massive biological data. For this one has to develop advance image analysis algorithms for cell segmentation and cell tracking. These algorithms should be robust and simple having the ability to treat wide range of images.

In this study an image analysis system based on open source image analysis package CellProfiler (www.cellprofiler.org) from Broad institute (www.broadinstitute.org) is presented that can be used for quantification of images obtained through time lapse microscopy. CellProfiler is an efficient toolbox based on advance algorithms having the ability to identify and track thousands of cells over the duration of an experiment. Using subset of these algorithms organized into a tailor made processing pipeline, here a method for automatic extraction of apoptotic cells from phase contrast images using their intrinsic features is proposed. Apoptosis is an essential cellular homeostasis mechanism that is required for correct development and functioning of multi-cellular organisms. Malfunctioning of apoptosis represents a major causative factor in the development and progression of cancer. Therefore, much attention has been paid to apoptosis in the field of clinical medicine and oncology, focusing on cures for cancer. Thus, by quantifying effects of drug treatment we can better understand the biological mechanisms of cancer and cancer therapy, thereby accelerating drug discovery and development. For this purpose CellProfiler was used for quantifying the efficacy of apoptosis inducing drugs (Etoposide and KKF1) when cell lines were treated with them in combination with a caspase-3 inhibitor. In the last part of the study reported here, a comparative analysis was performed in which cell line was treated with different concentrations of the proteasome inhibitor MG132.

2. THEORETICAL BACKGROUND TO MAIN TOPICS AND METHODOLOGIES

This thesis spans a broad spectrum of different topics and methodologies, therefore, in this section brief theoretical background to the most important topics are presented together with suitable literature references

2.1 Apoptosis and cancer development

Apoptosis is a highly regulated process of programmed cell death present in multicellular organisms. It plays an important role during different stages of development and normal physiology [5]. Cells undergoing apoptosis can be characterised by cell shrinkage, plasma membrane blebbing, DNA fragmentation and chromatin condensation [6]. Inactivation of this process is central to the development of cancer. Besides enabling malignant transformations defects in apoptosis, cancer also may result in resistance to chemotherapies [7]. Therefore much research has been done to find a way to get around this resistance in order to improve the anti cancer therapies. It has provided the basis for novel targeted therapies that can induce death in cancer cells that include those targeting extrinsic as well as intrinsic pathways.

2.2 Proteasome Inhibition and cancer therapy

Apoptosis is controlled by multiple regulatory pathways and their proteins. Examples include p53, the nuclear factor kappa B, the phosphatidylinositol 3 kinase pathway, and the ubiquitin/proteasome pathway [8]. In ubiquitin/proteasome pathway, proteins are tagged by ubiquitin and presented to the proteasome, where the protein is digested and ubiquitin is recycled in the cell [9]. The proteasome system is important in the degradation of damaged or unneeded proteins and serves as an important regulator of cellular processes, therefore it plays a considerable role in tumorigenesis [10]. The proteasome is found in all eukaryotic cells, both normal and cancerous and is highly conserved from yeast to man. It is a multicatalytic enzyme with an important impact on many regulatory pathways.

The inhibition of protein degradation through the ubiquitin-proteasome pathway is a recently developed approach to cancer treatment that extends the range of cellular targets for chemotherapy [11]. Bortezomib was the first proteasome inhibitor that was approved by US FDA in 2003 for treatment of refractory multiple myeloma [12]. Other inhibitors include natural products such as lactacystin, peptide aldehydes such as MG132, ALLN, and MG115, which are in the preclinical stages.

2.3 Microscopy

2.3.1 Phase contrast microscopy

Phase contrast microscopy is an optical microscopy contrast-enhancing technique that is used to produce high-contrast images of transparent specimens, such as living cells [13]. It employs a mechanism that translates minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast. When light travels from one medium to another, it undergoes a change in amplitude and phase depending on the properties of that medium. These changes give rise to familiar absorption of light which gives rise to colors which is wavelength dependent. The human eye measures only the energy of light arriving on the retina, so changes in phase are not easily observed, yet often these changes in phase carry a large amount of information.

A phase contrast microscope does not require staining to view the slide and living cells can be examined in their natural state. This provides an insight into the dynamics of ongoing cellular processes which can be analyzed in high contrast with sharp clarity of minute specimen detail. This technique is widely applied in biological and medical research for example it is used in diagnosis of tumor cells and the growth, dynamics, and behavior of a wide variety of living cells in culture.

2.3.2 Fluorescence microscopy

A fluorescence microscope is used to study properties of organic or inorganic substances using the phenomena of fluorescence and phosphorescence [14]. The basic function of a fluorescence microscope is to illuminate the specimen that is labeled with a fluorescence dye with a specific band of wavelengths, and then to separate the much weaker emitted fluorescence from the excitation light. As a result part of specimen marked with fluorescent dye will light up against the dark background [15].

In recent years fluorescence microscopy has gained immense importance in fields of biology and medical research due to advancement in technology of microscopy and development of fluorescent molecular probes. By using these fluorescent probes we can characterize subcellular structures, location of signaling proteins and indicators of physiological states. Green fluorescence protein [16] has been frequently used as a reporter of expression. The presences of such fluorescent probes have triggered the development of fluorescence microscopy to visualize cells over time expressing specific proteins that have been fluorescently tagged. These probes can also be used in expressing the protein in small sets of specific cells. It allows to optically detect specific types of cells in vitro, or even in vivo.

Automated fluorescence microscopy and high performance computing have allowed the emergence of high content screening as a useful tool in the early stages of drug discovery [17]. Use of fluorescent proteins has provided novel insights into compound-induced responses in drug discovery. Since they are non-invasive, non-destructive and can be genetically-encoded, fluorescent proteins are attractive candidates for labeling drug target of interest based on live-cell analysis.
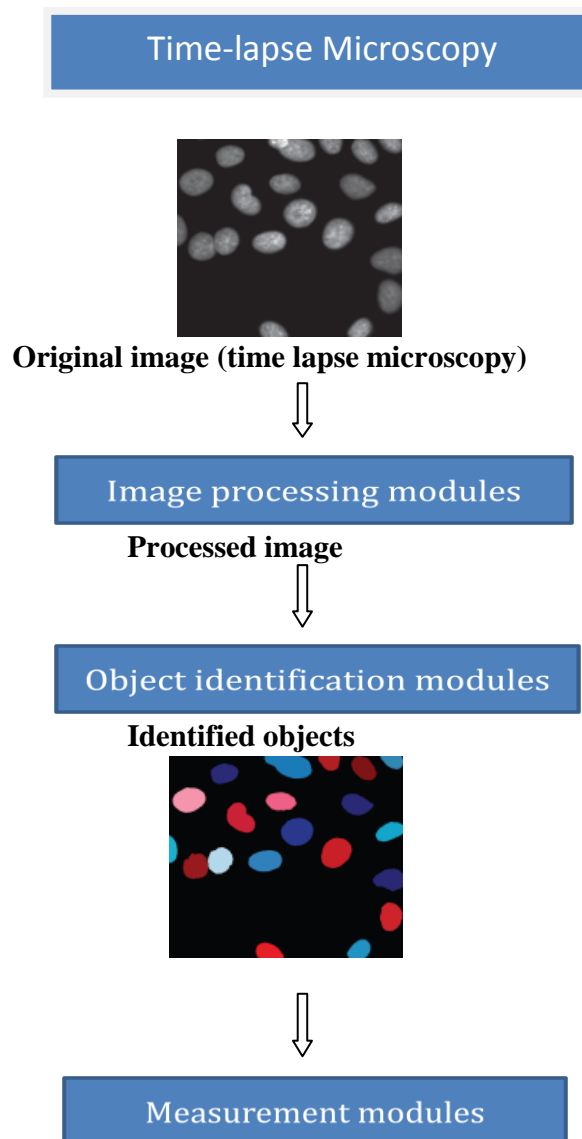
2.4 Automated Image Analysis System

Difficulty in handling and analyzing large amounts of image dataset generated has urged the need for a sophisticated image analysis system. Existing tools for image analysis, such as NIH ImageJ [18] and ImagProPlus [19] are limited in their functionality to analyze high-throughput image analysis data. Commercial software are also available, developed by companies like Cellomics, Molecular devices and GE healthcare. In addition to being limited in their scope, these software's are expensive and come along the hardware which makes it impractical to test several programs for a new project. Moreover, one cannot easily get information about the algorithms used and make any modification in them as they are not based on open source code. Therefore to process high throughput data there is a need for more transparent and cheaper software having the ability to correctly identify objects and analyze their size, texture, shape and intensity quantitatively. In order to extract meaningful measures from images one has to design and implement efficient algorithms, which are used for feature extraction, cell segmentation, pattern recognition and statistical modeling [20]. To bridge the current technological gaps, there are many ongoing activities aimed at developing new algorithms that are fast and accurate in identifying objects and extracting their features.
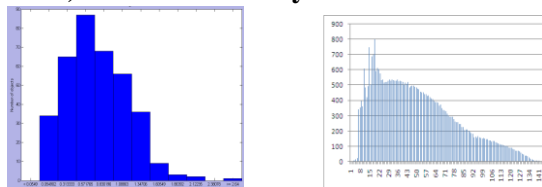
2.5 CellProfiler

CellProfiler (http://www.cellprofiler.org/) is automated open-source image analysis software that can analyze thousands of images obtained through image acquisition instruments. The software contains already developed methods that are applicable to diverse assays [21]. It produces rapid, quantitative, and accurate results. Since the software is open-source it allows researchers to design and contribute new methods and improve the existing ones. CellProfiler contains advanced algorithms for image analysis that can accurately identify cell clumps and non mammalian cell types. It has a user friendly graphical user interface (Figure 1) with a modular flexible design allowing analysis of new assays and phenotypes. A pipeline is constructed based on individual modules that are placed in a sequential order. Image is processed through each module and sent to the next module in line. CellProfiler contains published and tested algorithms for object identification [22-26]. For each identified cell it can measure a large number of features including size, shape, intensity, texture and location. These measurements can be viewed either by CellProfiler's built in viewing and plotting data tools or they can be exported directly to excel or database (MySql or Oracle). A system workflow for time lapse microscopy is presented in figure 2.
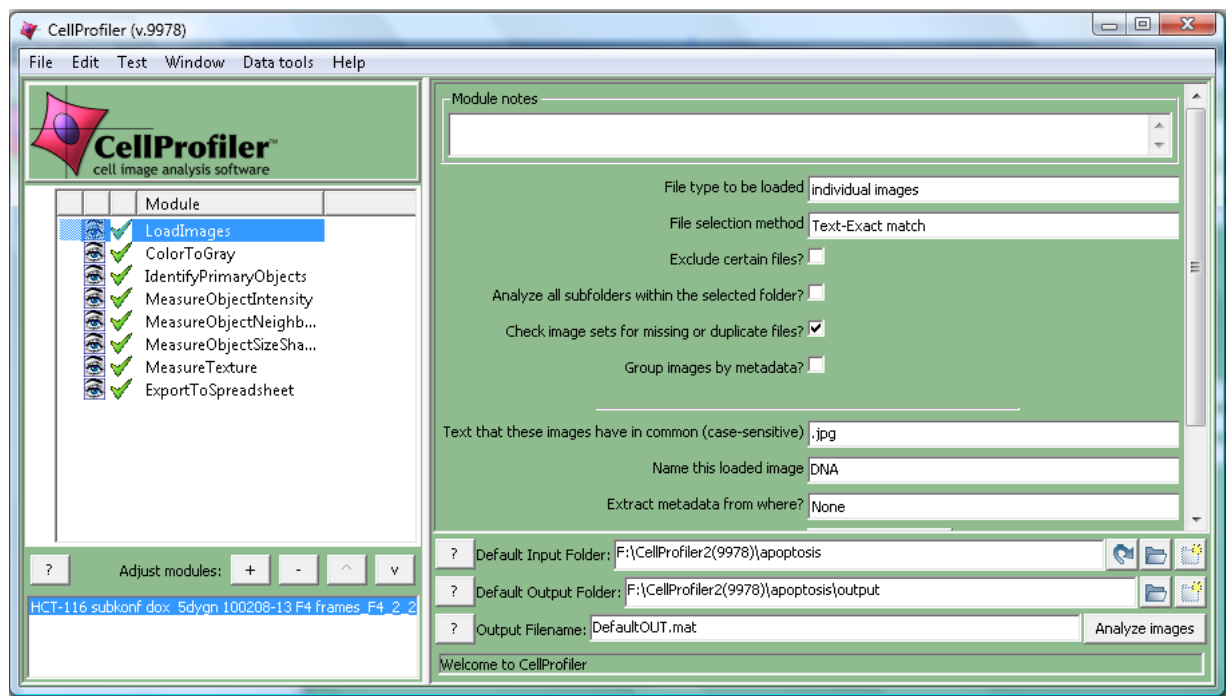
Figure 1*. Cell profiler interface*

Figure 2. *System workflow for time-lapse microscopy in which a set of cell profiler modules are combined into a pipeline used to identify and characterize cell population.*

2.6 Identification of Apoptotic Cells

CellProfiler is used to study the effects of drug induced cell death using time-lapse microscopic images. These Images studied were obtained from a live cell imaging system (IncuCyte from Essen Biosciences, http://essenbioscience.com/) offering time-lapse microscopy. Apoptotic cells are identified from phase contrast images using their special features that distinguish them from normal cells.

Features of an apoptotic cells
The proposed method identifies apoptotic cells from observed images based on following features [27].
- The edges contain much higher grey level as compared to central area
- Their shape is quasi-circular form.
- Their edges do not overlap with neighbouring cells.

Using these features a pipeline can be designed using CellProfiler for accurate identification of apoptotic cells.

3. AIMS OF THE STUDY

The overarching goal of the project reported in this thesis was to develop, implement and validate pipelines of image processing algorithms, based on CellProfiler, that are able to extract information rich features of growing cell populations that can be used to quantify drug effects related to apoptosis and proteasome inhibition. The pipelines should be able to successfully extract the information from phase contrast as well as fluorescence microscopy.

4. MATERIALS AND METHODS

4.1 CellProfiler

Compiled versions of CellProfiler 1.0 and 2.0 were used for analysis of microscopic images. A brief description of methods and modules available and used for analysis are described below.

4.1.1 Color to gray
This module is used for the conversion of RGB (Red, Green, Blue) color images to grayscale. There are two options Combine or Split. If we select Combine All channels will be merged into one grayscale image and if we select Split each channel will be extracted into a separate grayscale image.

4.1.2 Enhance edges
This module takes greyscale image as an input and finds the edges of objects producing a binary image where the edges are white and the background is black.

In this study several algorithms have been employed in different attempts to enhance edges including the methods called Ratio, Sobel, Prewitt, Roberts, LoG and Canny. In the final analysis Ratio method was selected. This method works by applying two smoothing filters to the image (sum of squares and square of sums), and then takes the ratio of the two resulting images to determine the edges. Thickness of the edges depend upon the size of the filter, the larger the filter size, the thicker the edges will be. The recommended size of filter is 8 pixels, or roughly half the width of the objects we want to edge.

4.1.3 Identify primary object
This module is used for identification of primary objects (e.g. nuclei) in grayscale images that show bright objects on a dark background. It contains a modular three-step strategy for object identification.
- In step 1 it is determined whether an object is an individual nucleus or two or more clumped nuclei.
- In step 2 edges of objects are identified, using simple thresholding if the objects do not appear to touch. Otherwise if the object is actually two or more nuclei that touch each other, more advanced processing is taking place.
- In step 3 identified objects are either discarded or merged together based on user defined rules. For example, if the objects are at the border of the image and are incomplete they can be discarded, and objects that do not lie in specified size limits can either be discarded or merged with nearby larger ones.

4.1.4 Measure object size shape
This module extracts area and shape features of each identified object for example area, form factor, solidity and orientation.

4.1.5 Measure object intensity
This module extracts intensity features for each identified object based on one or more corresponding grayscale images.

4.1.6 Measure object neighbor

This module determines how many neighbors each identified object has. One can specify which objects should be considered neighbours by providing a distance threshold. If objects fall within this distance they are considered neighbors.

4.1.7 Classify object

This module classifies objects into a number of different bins according to the value of a measurement (e.g., by size, intensity, shape). It reports how many objects fall into each class as well as the percentage of objects that fall into each class.

4.1.8 Filter by object measurement

This module removes selected objects based on measurements produced by another module in pipeline. All objects that do not satisfy the specified parameters will be discarded.

4.1.9 Export to Spread Sheet

Measurements are converted to character-delimited text formats and saved to the hard drive in one or several files.

4.1.10 Export to Database

Measurements can be exported directly to a database or to a SQL-compatible format through this module.

4.1.11 Methods for thresholding the background

To distinguish between foreground and background pixels we need to set an intensity threshold. CellProfiler contains several methods including Otsu, Mixture of Gaussian, Background, Kapur and Ridler-Calvard that are based on efficient algorithms having the ability to automatically calculate an intensity threshold.

We used Background method in our analysis because the images that we processed had much of their area as background and this method works best for such images. It finds the mode of the histogram of the image, which is assumed to be image background, and selects a threshold at twice that value. This can be very helpful for images that vary in overall brightness but the objects of interest are always twice as bright as the background of the image.

4.1.12 Methods for cell clump segmentation

These methods were used for segmenting clumps into the appropriate number of objects of interest.

- *Intensity*
  This method takes each intensity peak as a separate object and works best for objects that have only one peak of brightness. The objects should have a smooth texture but can be of any shape, so they need not be round and uniform in size.

- *Shape*

  When objects are separated by definite indentations, this method works best for objects that are round in shape. Since declumping is based on shape the objects need not be brighter towards the interior as is required for the *Intensity* option. The image is converted to black and white (binary) and the shape determines whether clumped objects will be distinguished.

- *Laplacian of Gaussian (LoG)*

  This method performs a LoG transform on images containing objects that have an increasing intensity gradient toward their center. It thresholds the result and finds pixels that are both local maxima and above threshold.

4.2 Identifying apoptotic cells in phase contrast images

4.2.1 Cell culture and image acquisition
HCT-116 human cell lines were treated with the drug doxorubicin (apoptosis inducer). Plates with fixed cells were analyzed using the IncuCyte live cell imaging system from Essen Bioscience. Each image was obtained after duration of hour (Figure 3).

4.2.2 Image analysis
Images were analyzed using CellProfiler 1.0. A pipeline was setup based on modules shown in table 1.

Table 1 Pipeline for identification of apoptotic cells in phase contrast images

Module used	Parameters used
Load images	Image format (tif)
Color to gray	Combine (orig grey was selected)
Enhance edges	Edge finding method (Ratio)
Identify primary automatic	Size of object (15-40), thresholding method (background global), threshold correction factor (1.2), method to distinguish clumped object (shape), method to draw dividing lines between clumped objects (intensity).
Measure object intensity	Identified objects
Filter object	Category of measurement (intensity), Feature (mean intensity edge),Minimum and Maximum value required (0.6 - 1)
Measure object area shape	Filtered objects
Filter object	Category of measurement (AreaShape), Feature (form factor),Minimum and Maximum value required (0.8 - 1)
Measure neighbor	Filtered objects
Filter Objects	Category of measurement (neighbours), Feature (Percent Touching), Minimum and Maximum value required (0- 0)
Export to spreadsheet or database	Data can automatically be exported to spreadsheet or a database.

4.3 Identifying apoptotic cells in fluorescence images

4.3.1 Cell culture
Mv4-11(Nacute myeloid leukemia cell line) was used to study the effect of drugs in apoptosis induction.

4.3.2 Experiment
Six experiments were set up each with different combinations of apoptosis inducing drugs and caspase3 inhibitor Ac-DEVD-CHO (Table 4). DEVD-NucView™ 488 caspase-3 substrate was added in all wells. It consists of a highly negatively charged DEVD peptide that is attached to a DNA-binding dye to make the dye unable to bind to DNA and thus unable to produce fluorescence in the presence of DNA. The substrate rapidly crosses cell membrane to enter the cell cytoplasm, where it is cleaved by caspase-3 to release the high-affinity DNA dye. The released DNA dye migrates to the cell nucleus to stain the nucleus brightly green. Images were captured through IncuCyte from a single time point from each experiment i.e. after 8 hours.

4.3.3 Image analysis
Images were analyzed through cell profiler 2.0. The modules used in identification of fluorescent objects are shown in table 2.

Table 2 Modules and settings used for identification of apoptotic cells in fluorescence images

Modules	Parameters
Load Images	Movie format (.avi)
Color to gray	Split (orig green was selected)
Identify primary object	Size of object (3-30), thresholding method (Robust background), threshold correction factor (1.1), method to distinguish clumped object (intensity), method to draw dividing lines between clumped objects (intensity).
Measure object Shape Size	No adjustment required
Measure object intensity	No adjustment required

4.4 Proteasome inhibition

4.4.1 Cell line and image acquisition
The human melanoma cell line MelJuSo was used. These cell lines were treated with different concentrations of MG132 (0.1µM, 1µM, 10µM) and were incubated for three days. Microscopic images and videos were obtained through IncuCyte.

4.4.2 Image analysis
Images obtained are analyzed through cell profiler 2.0. A list of modules used for detection of cells emitting fluorescence signal are showed in table 3.

Table 3 Modules and settings used for analysis of proteasome inhibition

Modules	Parameters
Load Images	Movie format (.avi)
Color to gray	Split (original green was selected)
Identify primary object	Size of object (3-30), thresholding method (Robust background), threshold correction factor (1.1), method to distinguish clumped object (intensity), method to draw dividing lines between clumped objects (intensity).
Measure object Shape Size	No adjustment required
Measure object intensity	No adjustment required

5. RESULTS

5.1 Identifying apoptotic cells in phase contrast images

Apoptotic cells were identified from cell lines treated with doxorubicin using image analysis software CellProfiler. A pipeline (Table 1) was constructed based on modules that used apoptotic cell specific characteristics (circular Shape, thick edges, and non-overlapping) to distinguish them from normal cells. Figure 3a represents a phase contrast image that was processed through this pipeline. Identify Prim Automatic module was used that identified all the objects within this image as shown in figure 3b. A number of thresholding methods were applied on this image (see material and methods) but Background Global was the one that gave the best result. Intensity was calculated for each identified object using module 'Measure Object Intensity' and a filter was setup based on this measurement. Since apoptotic cells have a thick contour, the objects having high intensity level at edges were taken as apoptotic. While filtering the objects, limits for 'Mean intensity edge' were set between 0.6 and 1. After applying this filter we obtained cells having thick contour. These objects were again filtered based on their shapes. As we know that apoptotic cells are circular in shape, therefore a second filter was setup using shape as a measure for filtering cells. Shape was calculated for each identified object using 'Measure Object Area Shape'. After applying this filter we obtained cells that were circular with thick edges (figure 3d).

A third characteristic feature of apoptotic cells is that they are non-overlapping. This feature was measured through module 'Measure Object Neighbors'. It gave the number of neighbors of each object and the percentage of contact between two adjacent objects. A third filter was setup and only those objects were selected that had zero percent touching with neighboring cells using feature 'Percent Touching'. This final filtering yielded cells that were quasi circular shaped, had thick contours and were non-overlapping, three characterisitic features of apoptotic cells (Figure 3e).

5.2 Identifying apoptotic cells in fluorescence images

5.2.1 Measurement of Apoptosis in an AML cell line

An AML cell line was treated with six different combinations of apoptosis inducing drugs and caspase-3 inhibitor Ac-DEVD-CHO. During apoptosis caspase-3 was produced that cleaved DEVD-NucView™ 488 caspase-3 substrate. The fluorescence signal emitted as a result of this cleavage was detected through CellProfiler. Results of the analysis are shown in Table 4.

Table 4 Six treatments analyzed with respect to induction of apoptosis using fluorescence microscopy

| Experiment	Drugs	No of cells showing fluorescence	% of cells showing fluorescence
1	Etoposide	1637	46
2	Etoposide + Caspase inhibitor	2	0.05
3	KKF1	1230	35
4	KKF1 + Caspase inhibitor	10	0.28
5	No drugs	90	2.5
6	No drugs + Caspase Inhibitor	1	0.02
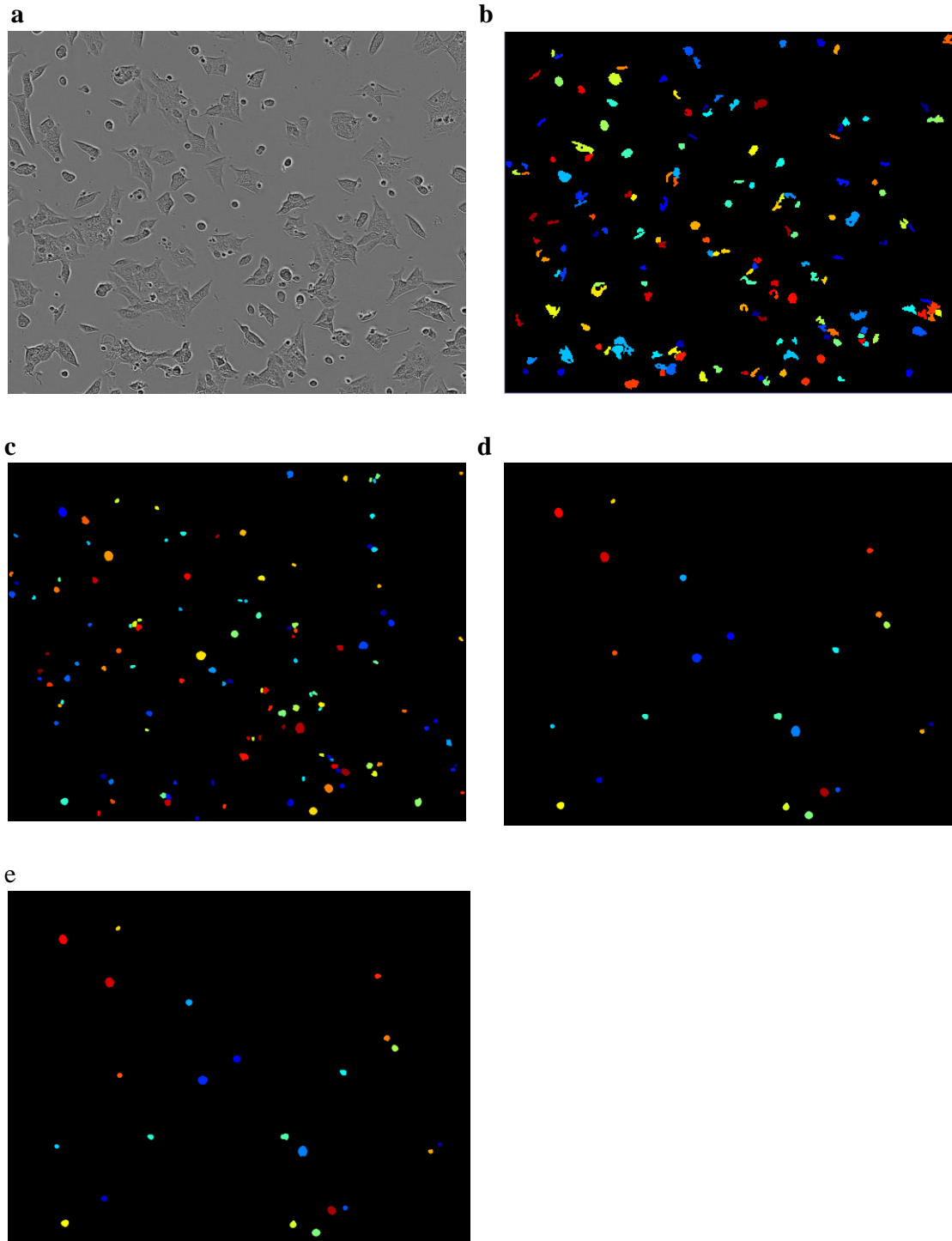
*Fig 3. (**a**) Phase contrast image showing cell line treated with doxorubicin. (**b**)Figure representing total number of identified cells in the frame. Different colors are just to distinguish between objects. (**c**) Cells after applying a filter based on edge intensity. (**d**) Cells obtained after applying a filter based on shape. (e) Image representing non-overlapping cells.*
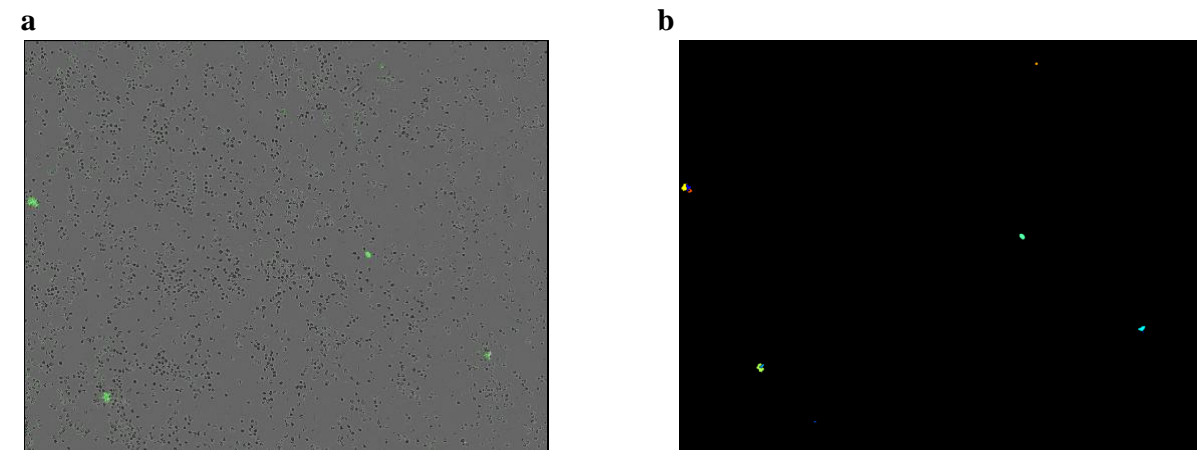
5.2.1.1 Cell line treated with KKF1

When cells were treated with KKF1 (A novel kinase inhibitor), it made them undergo apoptosis. Caspase-3 was produced that cleaved DEVD-NucView™ 488 caspase-3 substrate. After cleavage the released DNA dye entered the nucleus and stained it bright green (Fig 4a). Image obtained was processed through CellProfiler that correctly identified cells emitting fluorescence (Fig 4b).

In another experiment cells were treated with KKF1 in combination with a caspase inhibitor Ac-DEVD-CHO (Fig 5). When these images were analyzed the results showed a decrease in number of cells showing fluorescence signal (Table 4). Caspase-3 was inhibited by the addition of this caspase-3 inhibitor.

a   b
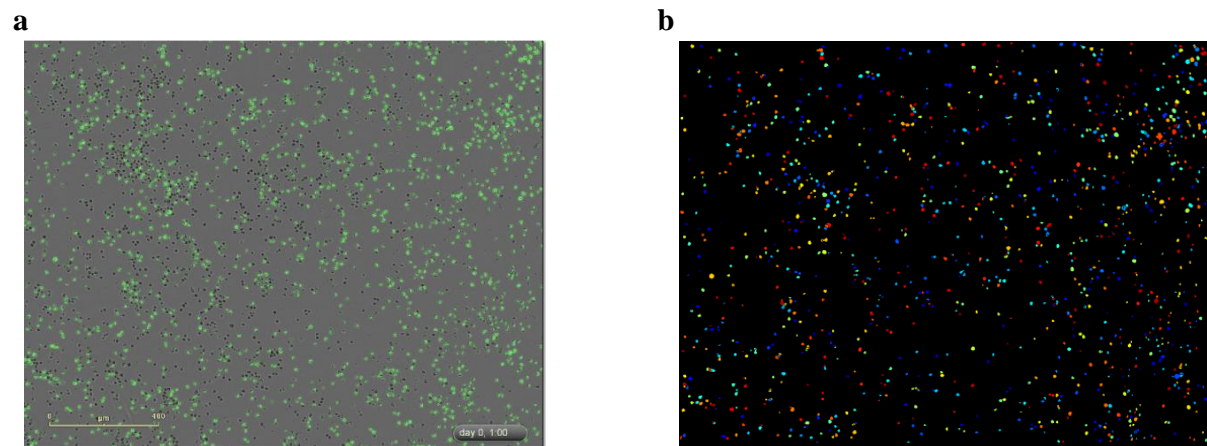
Figure 4. Cell line treated with KKF1. (a) Microscopic image of cell line treated with KKF1 when processed through CellProfiler based pipeline identified objects emitting fluorescence signal as shown in figure b.
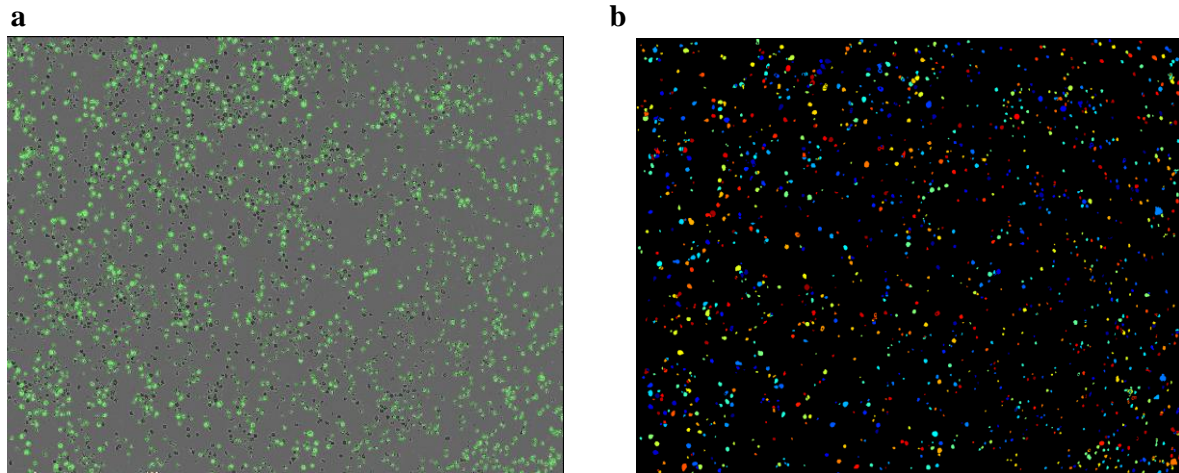
a   b

Figure 5. Cell line treated with KKF1 +caspase inhibitor. (a) Image of Cell line treated with KKF1 + caspase inhibitor Ac-DEVD-CHO when processed through CellProfiler identified the objects emitting fluorescence as shown in figure b.
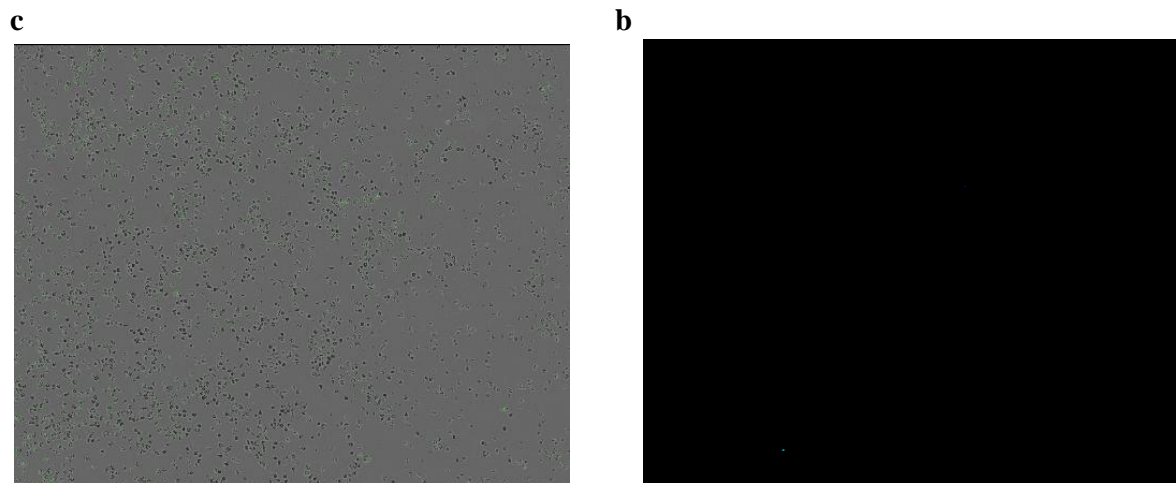
5.2.1.2 Cell line treated with Etoposide

A large number of cells emitted the fluorescence signal when they were treated with Etoposide (Figure 6a). In Etoposide treated cell line there were a higher percentage of cells (46%) showing fluorescence as compared to cell line treated with KKF1 (35%) but there wasn't much difference when intensities of the fluorescence signals were compared (Figure 8).

When caspase-3 inhibitor was added along with Etoposide (Figure 7), the percentage of cells expressing fluorescence was decreased to 0.05% (Table 4).

a   b

Figure 6. Cell line treated with Etoposide. (a) *Microscopic image of cell line treated with KKF1 when processed through CellProfiler based pipeline identified objects emitting fluorescence signal as shown in figure* **b.**
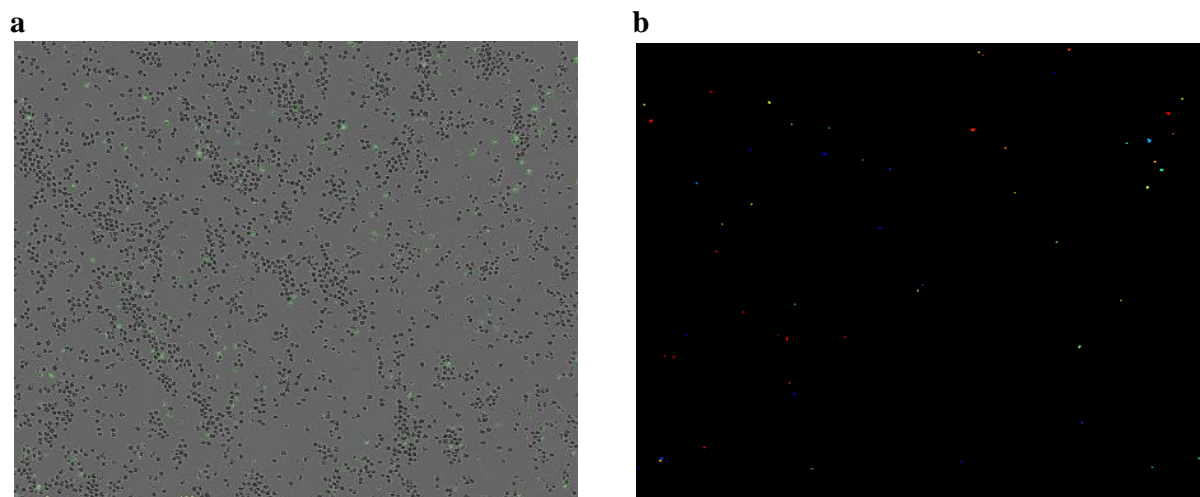
c   b

Figure 7. Cell line treated with Etoposide + Caspase inhibitor. (a) *Microscopic Image of Cell line treated with Etoposide + caspase inhibitor Ac-DEVD-CHO when processed through CellProfiler identified the objects emitting fluorescence as shown in figure* **b.**
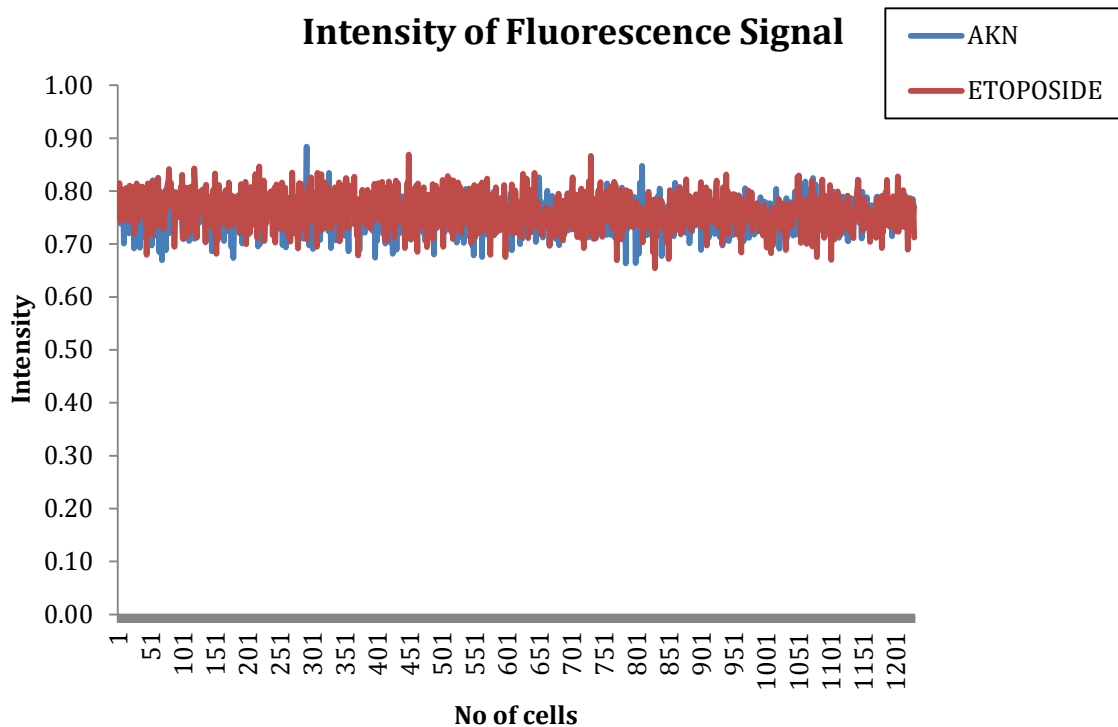
Figure 8 *. Graph representing comparison between intenities of fluorescence signal emitted from cells treated with KKF1 and Etoposide.*

5.2.1.3 Cell lines treated with no drug

As a control a cell line that was not treated with any drug was employed. The results show that 2.5% of cells emit the fluorescence signal (Figure 9). When the cell line was only treated with caspase-3 inhibitor Ac-DEVD-CHO (Figure 10) this percentage dropped to 0.02%

a   b

Figure 9. Cell line without any drug (control). (a) *Microscopic image of cell line without any drugs when processed through CellProfiler based pipeline identified objects emitting fluorescence signal as shown in figure **b.***
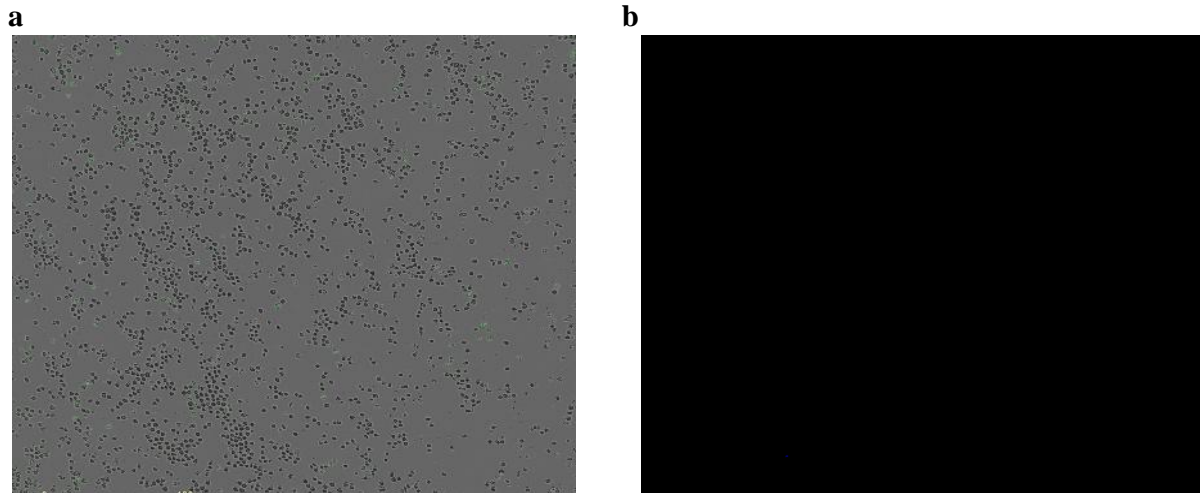
Figure 10. Cell line treated with caspase inhibitor. (a) Microscopic image of cell line treated with caspase inhibitor Ac-DEVD-CHO when processed through CellProfiler based pipeline identified objects emitting fluorescence signal as shown in figure b.

5.3 Proteasome inhibition

Mg132

The human melanoma cell line MelJuSo was treated with three different concentrations of proteasome inhibitor MG132. It was evident from the results that an increase in concentration of MG132 resulted in an increase in number of cells showing proteasome inhibition over time (Figure 11). Cell line that was treated with 0.1 µM of MG132 showed the least number of cells undergoing inhibition, whereas the cell line treated with 10 µM of MG132 showed the highest number of cells showing inhibition. At a particular time point the percentage of cells showing fluorescence is shown in figure 12.
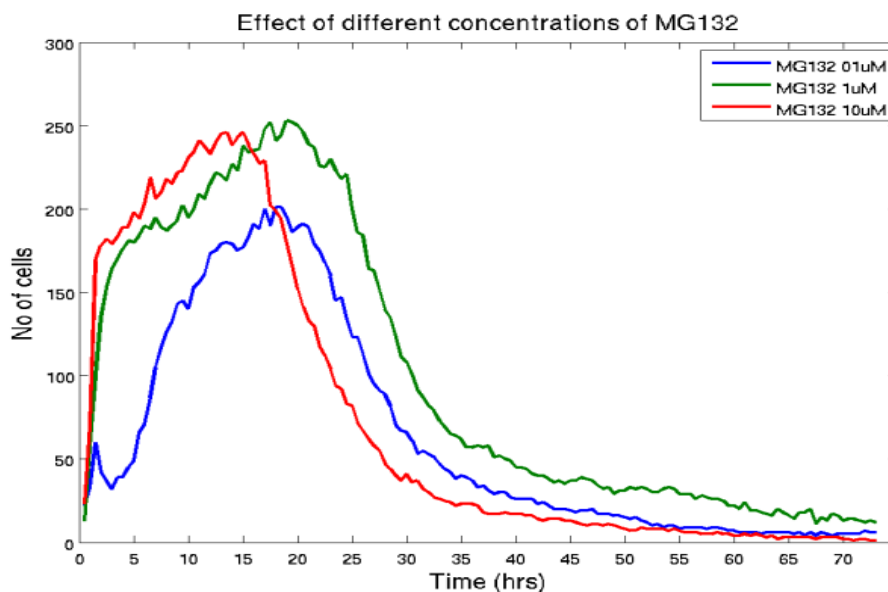

Figure 11. Graph representing number of cells showing fluorescence signal over time when treated with different concentrations of MG132.

20
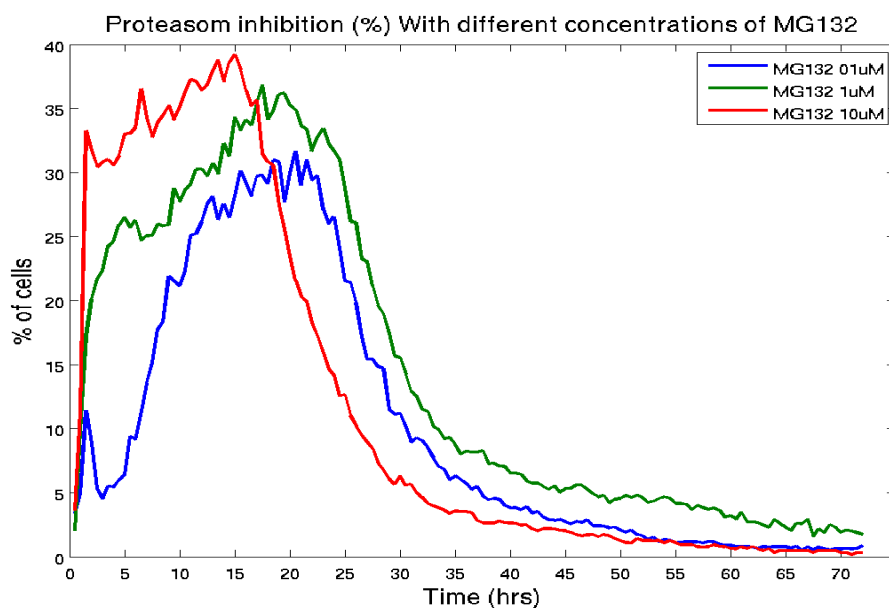
Figure 12. *Graph representing percentage of cells showing fluorescence signal when treated with different concentrations of MG132.*

There was a slight difference between intensity of fluorescnence signal emitted, when cells were treated with different concentrations of MG132 (Figure 13). Cells treated with 10 μM MG132 showed highest fluorescence signal intensity during early hours of experiment but after 5-6 hours cell lines treated with 1 μM MG132 showed highest intensity. As the experiment proceeded the intensity level became similar in all three cell lines.
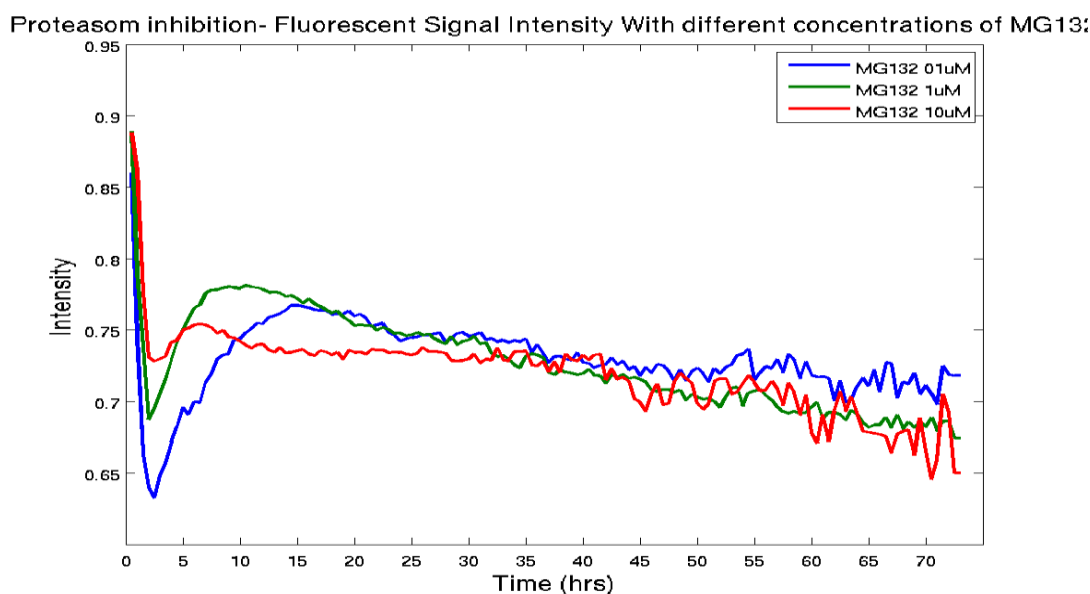
Figure 13. *Graph representing intensity of fluorescence signal emitted over time when cells are treated with different concentrations of MG132.*

Form factor represents the shape of identified objects. By calculating this feature we can determine the changes in shape over time in response to different drug doses. Figure 14 represents a comparison in change of form when cells were treated with different concentrations of MG132.

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The results show that as experiment proceeds cells begin to change their shape and at the end of experiment all the cells had more or less circular shape which holds true with features of apoptosis.
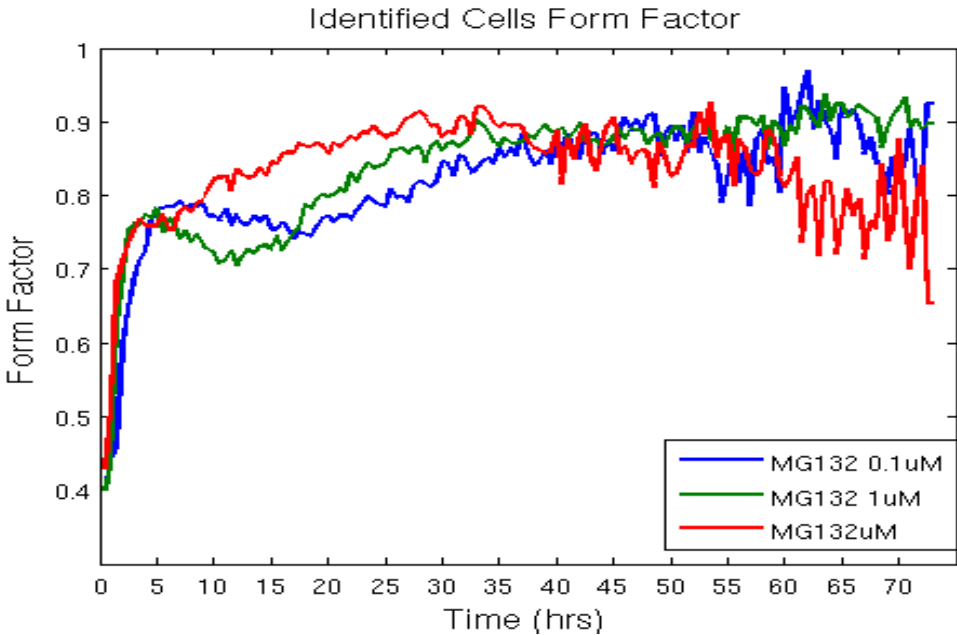
Figure 14. *Graph representing form factor of cells over time when treated with different concentrations of MG132.*

6. DISCUSSION

Merging time-lapse cell imaging with image analysis provides an efficient means of quantitative characterization of dynamic cell behavior [28]. This characterization provides insights into the potential mechanisms of action of compounds that can be of immense importance in studies of anticancer drugs. Since high-content screening of this kind generates data on multiple parameters in single cells as well as in populations of cells [29], we need highly sophisticated image analysis methods for quantification of drug induced effects. In this work we present the development and application of an algorithmic analysis of drug induced apoptosis and proteasome inhibition in cancer cells based on time-lapse microscopy images. Apoptosis and proteasome inhibition are important players in cancer drug therapy [4, 30].

For this purpose we used automated image analysis software CellProfiler [21] which contains advanced algorithms for image analysis that can accurately identify cells and extract user defined cellular features. To evaluate and demonstrate the potential of these algorithms we performed an analysis on data obtained by treating different cell lines with anti cancerous drugs to study apoptosis and proteasome inhibition.

In the first experiment we identified apoptotic cells from a phase contrast image (Figure 3a). These images were obtained by treating HCT-116 human cell lines with doxorubicin, an apoptosis inducing drug. Since apoptotic cells have certain morphological characteristics [27] they can be distinguished from normal cells. We used CellProfiler for their identification and made a pipeline based on different modules (Table 1). CellProfiler used a modular three step strategy for object identification. By selecting different options for each of these three steps we analyzed a variety of different types of objects.

In the second evaluation which was based on fluorescence imaging, CellProfiler was used to quantify apoptosis in an AML cell line that was treated with apoptosis inducing drugs and caspase-3 inhibitor. This comparative analysis was conducted to check the efficacy of a novel kinase inhibitor KKF1 in inducing apoptosis. Etoposide was used as a positive control for apoptosis induction since it is known to induce apoptosis through DNA damage induced p53 activation [31].Six experiments were set up and an image was acquired from a single time point from each experiment. The results (Table 4) showed that cell lines that were treated with Etoposide showed a higher number of apoptotic cell (46%) as compared to cell line treated with KKF1 (35%). It shows that Etoposide is more efficient in inducing apoptosis. Through quantification of these results, efficiency of KKF1 in inducing apoptosis and ability of caspase-3 inhibitor in blocking caspase-3 was confirmed.

In third analysis, the task was to determine how different concentrations of the drug MG132 affect its ability in inducing proteasome inhibition. MG132 induces a caspase dependant apoptotic cell death [32] and is used in cancer therapy. The human melanoma cell line MelJuSo was treated with three different concentrations of MG132. The results showed that cell line treated with 10μM MG132 had highest percentage of cells with proteasome inhibition detected through a fluorescence signal (Figure 12). Cell line treated with 0.1uM MG132 showed the smallest number of cells with proteasome inhibition. Moreover cell line treated with 10 μM MG132 inhibited proteasome more rapidly as compared to the other two doses (Figure 11). It shows that efficacy of MG132 is directly proportional to its concentration. The intensity of the fluorescence signal was calculated as a function of time for all three doses (Figure 13). The signal intensity was strong

during the early hours of the experiment but became weak over time. This effect was consistent for all three doses. At the end of experiment there was higher fluorescence intensity in cell line treated with 0.1 μM but it is unclear if the difference observed is significant enough to reflect a true difference.

7. CONCLUSION

In conclusion, live cell imaging, when combined with automated image analysis allows fast quantification and characterization of cell behaviour. It has become a powerful tool in medical cell biology and requires novel algorithms based on dynamical system analysis, image processing, and statistical modelling. In our work we evaluated the potential use of an automated image analysis system, CellProfiler, for quantification of *in vitro* anti cancer drug treatments. CellProfiler is capable of identifying thousands of individual cells and offers a modular and flexible structure that allows design of tailor-made analysis of new assays and phenotypes. By using this software in the work presented here a method for automatic extraction of apoptotic cells from phase contrast microscopy images based on apoptotic cells shape features is proposed. Furthermore this software was employed for quantifying and evaluating time and dose dependant induction of apoptosis and proteasome inhibition after treating cell lines with different drugs. In conclusion, the CellProfiler is not only useful for the particular applications studied here, due to its great flexibility and performance it has an outstanding potential to become a key player in the future of quantitative live cell imaging.

8. ACKNOWLEDGEMENT

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