UNIVERSIDADE VILA VELHA - UVV

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

COMBINAÇÃO DE SOFTWARE LIVRE, MICROSCOPIA DE LUZ E CORANTES INESPECÍFICOS PARA ANÁLISE DE ALTO RENDIMENTO EM CONTAGEM DE CÉLULAS VIVAS E MORTAS

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Catalogação na publicação elaborada pela Biblioteca Central / UVV-ES

C268c Cardoso, Luciano Favalessa.

Combinação de software livre, microscopia de luz e corantes inespecíficos para análise de alto rendimento em contagem de céluas vivas e mortas / Luciano Favalessa Cardoso. – 2015.
58 f : il.

Orientador: Dominik Lenz.

Dissertação (mestrado em Ciências Farmacêuticas) – Universidade Vila Velha, 2015.
Inclui bibliografias.

1. Citologia. 2. Células mortas. 3. Sobrevivência celular. 4. Software gratuito. I. Lenz, Dominik. II. Universidade Vila Velha. III. Título.

CDD 611.0185

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Aprovada em: 06 de maio de 2015

Banca Examinadora

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Prof. Dr. Dominik Lenz (UVV - Orientador)

VILA VELHA

MAIO/2015

DEDICO ESTE TRABALHO A DEUS, que me suportou nos tempos de angústia, me deu palavras de conforto e sabedoria, impediu que eu desanimasse e me guardou do mal. Dedico também a meu pai, que me ensinou, me treinou e me apoiou até o fim.

AGRADECIMENTOS

Agradeço imensamente àqueles que colaboraram para que eu chegasse até aqui: minha esposa Dâmaris, meu filho Bruno, minha irmã Joseanne e todos os amigos e familiares que sempre estiveram por perto.

Agradeço a Fundação de Amparo a Pesquisa do Espírito Santo (FAPES) e a Universidade Vila Velha (UVV)

Agradeço a professora Bianca Prandi Campagnaro e a todo pessoal da UFES (em especial, a Bianca Rodrigues) por me “emprestar” o laboratório.

Também agradeço a meus mestres Dominik Lenz e Marcio Fronza (obrigado pela ajuda e pela paciência professor!).

Não poderia terminar esse agradecimento sem lembrar das melhores amigas que alguém pode ter: Cátia (Cacá) Vittorazzi e Luciana (Lú) Simões Gasparini (valeu pela mão, garotas!).

Porque a mim se apegou com amor, EU o livrarei; pô-lo-ei a salvo, porque conhece o meu nome.
Ele me invocará, e EU lhe responderei; na sua angústia EU estarei com ele, livrá-lo-ei e o glorificarei.
Saciá-lo-ei com longevidade e lhe mostrarei a minha salvação.

(Salmos 91: 14-16)

SUMMARY

ACRONYMS AND ABBREVIATIONS LIST

DMSO – Dimethyl sulfoxide

MTT - 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide

CP – CellProfiler®

CPA – CellProfiler® Analyst

DMEM - Dulbecco's Modified Eagle's Medium

FBS – Fetal Bovine Serum

IU – International Unit

mL - Milliliter

µg - Micrograms

g (rcf) - relative centrifugal force

DAMP – Damage-associated molecular pattern

HMGB1 – High-mobility group box 1 protein

HDGF – Hepatoma-derived growth factor protein

HSP – Heat Shock Protein

µM – Micromolar

µL - Microliter

h - Hour

CPT – Camptothecin

PI – Propidium Iodide

LIST OF TABLES AND FIGURES

CARDOSO, Luciano Favalessa, M.Sc., Universidade Vila Velha – ES, Maio de 2015. **Combinação de software livre, microscopia de luz e corantes inespecíficos para análises de alto rendimento em contagem de células vivas e mortas.** Orientador: Dominik Lenz.

RESUMO GERAL

Os estudos de diferenciação celular são importantes para identificar a viabilidade celular como para identificar o tipo de morte celular. Em ambos os casos a correta identificação das populações celulares é fundamental para diagnósticos e prognósticos em diversos ramos da pesquisa científica. A citometria é uma tecnologia disseminada desde os anos 60, porém o material utilizado, as máquinas necessárias para a execução das análises e o preparo dos técnicos ainda são de alto custo. O objetivo da presente pesquisa é utilizar microscopia de luz, corantes inespecíficos e software livre, com o mesmo nível de precisão de padrões tradicionalmente já utilizados, reduzindo custos e ampliando a difusão desse tipo de análise. Foram utilizadas imagens para as análises das amostras, criando banco de dados que podem ser reanalizáveis sempre que necessário. Além disso, essa metodologia, depois de estabelecida, diminui o viés humano por identificar menores quantidades de falsos-positivos e falsos-negativos.

Palavras-chave: Morte celular, Citometria, Contagem de alto rendimento, CellProfiler®, Viabilidade

CARDOSO, Luciano Favalessa, M.Sc., Universidade Vila Velha – ES, Maio de 2015. **Combination of free software, light microscopy and non-specific dyes for high throughput analysis in live and dead cell count.** Orientador: Dominik Lenz.

GENERAL ABSTRACT

The studies in cell differentiation are important to identify cell viability and to identify the type of cell death. in both cases the correct identification of cell populations is critical for diagnosis and prognosis in many fields of scientific research. Flow cytometry is a widespread technology since the 60'ies, but the material used, the machinery necessary to carry out the analysis and preparation of technicians are still expensive. The aim of this research is to use light microscopy, nonspecific dyes and free software, with the same accuracy level of standard methods that has been traditionally used, reducing costs and increasing the dissemination of this kind of analysis. Images were used for the analyzes of the samples, creating databases that can be re-analyzable whenever necessary. In addition, this methodology, once established, decreases the human bias by identifying minor amounts of false-positives and false-negatives.

Keywords: Cell Death, Cytometry, High-Throughput Count, CellProfiler®, Viability

1. INTRODUCTION AND THEORETICAL BACKGROUND

Definitions

The life and death may be defined in various ways. Biochemically, physiologically, culturally, artistically, philosophically and so on. "Viability" of a system can be defined in several ways, but none of them is a simple definition. Thinking about a "viable" organism, it is expected that this organism is alive and physiologically able to represent other individuals of that population from which it was originated. When thinking about "living organism", hooks up this idea to the definition of "life." Thus, as discussed by Hazel Davey, the definition of viability goes beyond what common sense meant by "life" and "death". As discussed by Davey, to questions about "what is death?", can be rationally answered that is the interruption of the physiological processes that medicine and biology understand of "life", i.e., absence of viability where it previously existed[1].

Thus, we can discern that, like Dubik and Wood commented[2], a nail cannot be considered dead, since never been alive. The Michaelis dictionary, in its online version, relates the word "death" only to beings from animals and plant kingdoms (mandatorily beings endowed with biochemical processes), thus considering that death is an interruption of the physiological processes, a cessation of these biochemical processes of which these beings are necessarily endowed.

In short, in order to consider a system as "dead", then we must consider that in a past time, this system was endowed with physiological and / or dynamic and constant biochemical processes that were interrupted for some reason.

Physiologically viable

A line can be traced going from life to death or going from death to life, based on some cellular features such as level of metabolism, the plasmatic membrane

structure, DNA and RNA level present within the cell, level of organelles degradation (especially, the mitochondria) and so on. In order to consider a cell as "viable", it must fill some necessary (and other essential) requirements[1].

Regarding the requirements, we can mention, for example, the level of metabolic activity. If we have a cell whose plasmatic membrane and organelles are intact, but where is not observed metabolic activity, it appears that we are facing a cell that does not fully meet the requirements for a claim that it is alive, though it can be found in torpor state, waiting for favorable conditions to resume its full metabolic activities. But when it comes to essential requirements, can be used as an example the plasmatic membrane and the integrity of the DNA. If there is a cell with severely damaged cellular membrane or degraded DNA, it is considered that this cell fulfills more requirements that link to cell death than requirements related to viable cells, since damages to the membrane allow to astray intracellular content necessary for the metabolism and DNA degradation preclude the manufacture of proteins necessary to maintain cellular viability.

In scientific experiments, cells, even those with a disorder or mutation such as cancer cells, need to be viable. Cell viability is an indispensable condition to proceed to any scientific experiment involving cell culture (in vitro), since the cells in viable conditions have identical features (or very close to it) to the cells installed in living organisms (*in vivo*).

Viability detection mechanisms

The importance of detection of cell viability is related to the necessity to evaluate the toxic effect of substances with pharmacological potential, or when it is necessary to determine the degree of degradation of preserved samples, or even when it is necessary to evaluate processes in which cells are subjected.

Due to the importance inherent to the subject, when referring to the viability of organisms (in particular, cells) involved in scientific experiments, the use of the viability detection methods is necessary. With technological advances, the

viability assessment techniques earn features that let you enhance the count, reduce the incidence of false-positive and optimize the time and costs required[1]. Some techniques to detect viability are more rudimentary and imprecise, as the use of dyes and manual counting in a Neubauer chamber, but easier and faster to execute. The state-of-the-art techniques involves flow cytometry and specific fluorescent markers, but are more costly and require advanced training to run.

Trypan blue dye exclusion

Among the methods used to determine cell viability, the trypan blue dye exclusion fits among the most practical, cheap and quick options[3]. It is an established technique and widely used in many laboratories around the world for providing highly reliable results. This is a dye which penetrates only into dead cells as the transmembrane transport metabolism of living cells prevents binding of this dye[4]. Technically, what happens is that the trypan blue mark only cells whose plasmatic membrane is permeabilized[5], feature that makes the cell unviable.

This is a dye used in light microscopy, so it is possible to assess the cell morphology, characteristic that more advanced techniques, such as flow cytometry, do not possess. Thus, it is possible, at the same time, to estimate the percentage of viability and to evaluate the morphology of the cells present in a given population.

The trypan blue does not classify the type of cell death, since we have two main types, necrosis and apoptosis. It only classifies the cell as viability. The cells may be non viable and have undergone necrosis, or may be non viable and have undergone apoptosis. Furthermore, apoptosis can be in different stages, which were classified as early stage or late stage. In manuscript 2 we will talk about necrosis and apoptosis.

2 MANUSCRIPT 1 - COMBINATION OF FREE SOFTWARE, LIGHT MICROSCOPY AND NON-SPECIFIC DYES FOR A HIGH-THROUGHPUT ANALYSIS IN LIVE/DEAD CELL COUNT

Combination of free software, light microscopy and non-specific dyes for a high-throughput analysis in live/dead cell count

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ABSTRACT

Background: Cellular viability studies are important in many different fields of cell biology research and are based on the estimation of live and dead cells from a specific cellular population. Recent progress in technology have brought important advances in the analysis of cellular viability mainly reducing the time of analysis and sample preparation; however the costs for equipment and reagents still a limiting factor in the feasibility to conduct those studies. The purpose of the present study was to use free software, non-specific dyes and light microscopy to establish a reproducible image analysis test and to compare the results with an established method. **Method:** Mouse fibroblast cells were exposed to different concentrations (ranged from 0.5% to 16%) of dimethylsulfoxide (DMSO) for 24 h and cellular viability estimated in the proposed technique (image analysis performed on free software) and in the methylthiazol-tetrazolium salt (MTT) assay. A Bland Altman analysis was applied to compare the methodologies. **Results:** Image analysis by CellProfiler®/ CellProfiler® Analyst was compared to MTT using Bland-Altman test and both methodologies were considered equivalents. Cellular viability decreased in a dose dependent manner with increasing doses of DMSO and both methodologies were capable to distinguish between live and dead cells, producing comparable results (with bias = 1.3550) evaluated by the Bland Altman analysis. **Conclusions:** The proposed image analysis can be used as a simple, rapid and low-cost technology for high throughput analysis of live-dead cell differentiation.

Keywords: CellProfiler, Image Analysis, Trypan Blue, Cell Identification, Cell Viability, Cytometry.

1. INTRODUCTION

Cellular viability studies are important in many different fields of cell biology research. It is based on the estimating the amount of live and dead cells from a total cell population. Cellular viability studies offer a huge potential for a broad array of research and methodology and are continuously growing through ongoing field efforts. Many studies in this field have proven substantial importance, e.g., cell death can act as biosensor of contaminated material; or as a prognostic factor in health care[1,2,3,4].

The importance of this kind of analysis can be verified using the world's largest scientific library: The PubMed library. There are more than 4000 peer reviewed publications related to "alive dead assay" keywords (may 2014). Most of these studies, probably all of them, used flow cytometry or laser scanning cytometry (LSC), techniques that has been used successfully since the 60s[5] and each cytometry technique has some special particularities[6,7]. Cytometry is used to identify and count cells of different origins mostly by antibodies or morphology[8].

Despite its importance, flow cytometry and laser scanning cytometry (LSC) are cost-intense techniques as they require equipment, reagents and qualified staff. The costs and complexity of cytometry limited the distribution of cytometric analyses[9]. The costs for flow cytometry nowadays still need to be considered[10]. However, if it's possible to obtain the results similar to cytometry using more accessible techniques, like light microscopy and non-fluorescent dyes. Light microscopy images furthermore have, the advantage that objects (i.e. cells) of interest and results of analyses can be morphologically verified.
This research aimed to combine light microscopy, non-specific dyes and free software for reproducible high throughput analysis of live/dead cell count [11], in order to substantially lower the costs for automated cellular diagnostics and to compare the results of the proposed image analysis technique with methylthiazol-tetrazolium salt (MTT) assay.

2. MATERIALS AND METHODS

2.1 Cell Culture

Swiss 3T3 albino mouse fibroblasts (ATCC CCL-92 - Cell Line Service, Rio de Janeiro, Brazil) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Cripion, Brazil), 100 IU/mL penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, USA), at 37ºC in a containing 5% CO_2 humidified atmosphere.

2.2 Chemicals and reagents

Trypan Blue and 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt was purchased from Sigma - Aldrich Company (Sigma-Aldrich, USA). Dimethylsulfoxide (DMSO) was purchased from Neon Comercial (São Paulo - Brazil). Sodium chloride, potassium chloride, sodium phosphate dibasic and potassium phosphate monobasic was purchased from Cromoline Quimica Fina (são Paulo, Brazil). All other agents were analytical of grade.

2.3 Methylthiazol-Tetrazolium Salt (MTT) Assay

The *in vitro* cytotoxic activity was evaluated in the colorimetric MTT assay as previously described in literature[12] with minor modifications. Briefly, 3T3 fibroblasts cells were plated in 24-well flat-bottomed tissue culture plates with 4 x 10^4 cells/mL. After overnight incubation at 37 ºC (5% CO_2 and 95% air) cells were exposed to different doses of dimethylsulfoxide (DMSO) ranged from 0.5% to 16.0% and incubated for additional 24h. Vehicle control medium was used as negative control. After incubation, 100 µL of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (2.5 mg/mL in PBS Medium (1:4)) was added per well, and the plate was incubated for 2 hours. The excess of MTT was aspirated and the formazan crystals formed were dissolved with 100 µL of DMSO and the absorbance of purple formazan was measured at 595 nm using a microplate reader (Molecular Devises, Spectra Max 190, USA). Experiments were carried out at least in duplicate.

2.4 Image analysis preparation

The same conditions previously described in the MTT experiments was used. Briefly, 3T3 fibroblasts cells were plated in 24-well tissue culture plate, exposure with DMSO in the same concentrations and after 24h incubation the medium, with dead cells from each respective well were

collected in microtubes and the remaining adherent cells were trypsinized and placed to the same microtube. These tubes were centrifuged at 300 G for 5 minutes. Then, supernatant was discarded and the cells were resuspended in 100µL trypan blue dye. Microscope slides were prepared and cellular images were made using camera Samsung SDC-415ND, coupled to Leica microscope model DMLS, and connected to a computer, where the images were processed and stored. Cellular analysis was performed using the free software CellProfiler® (CP). CellProfiler® Analyst (CPA) was used for statistical analysis and cellular diagnostics.

2.5 Cell Identification by CellProfiler®

To identify live and dead cells, it is necessary to run an algorithm in CP. This is an important step, once the correct algorithm is linked to a correct identification of sample cells. Different algorithm will return an incorrect identification by the software, and this will cripple the analysis. This step comprehends to load all images in the software, identify all cells present in these images, turn all cells identified into a measurable object and then set measurable values to these objects. These values (e.g. form or light intensity) were used by CPA to create a profile of each cell population. The define algorithm used are showed in table 1.

Table 1: The established algorithm used in the CellProfiler program to perform the analysis.

LoadImages	Load all images to be analyzed
ColorToGray	Turn all color images to black and white
ApplyTreshold > Below treshold > Otsu Global	Enhance cell contours
ImageMath > Invert	Invert light intensities
IdentifyPrimaryObjects > RobustBackground Adaptive	Turn all identified images to objects, which can be measured
MeasureObjectIntensity	Measure the intensities of all identified objects
MeasureObjectSizeShape	Measure the form of all identified objects
FilterObjects > AreaShape > FormFactor	Filter objects by a determinado parameter
MeasureObjectIntensity	Measure the intensities of all filtered objects
MeasureObjectSizeShape	Measure the form of all filtered objects

2.6 Cellprofiler® Analysis

Cellular image analysis was performed with CP. First, each image was converted to grayscale. Thereafter, intensities were converted. The algorithm used for segmentation was Robust Background Adaptive. 15 and 45 pixels were chosen for minimum and maximum areas respectively (Figure 1), i.e. objects smaller than 15 and greater than 45 pixels were not identified.

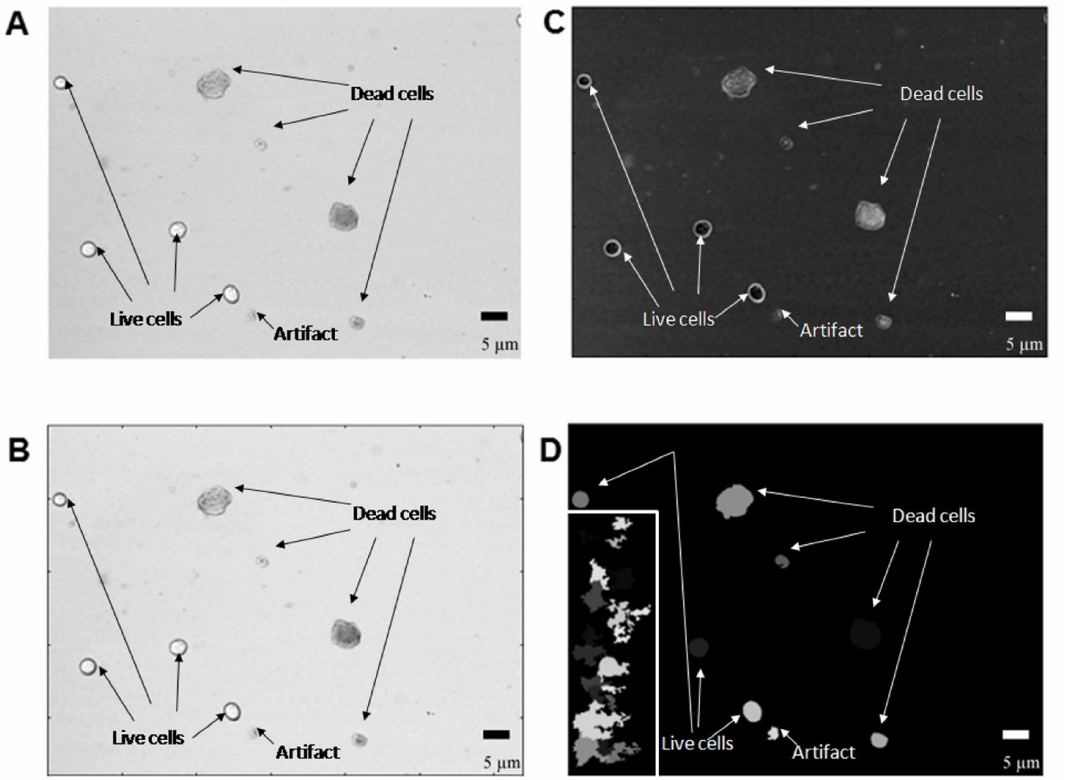

Figure 1: Cell identification in CellProfiler. The original image (1A) was turned to grayscale (1B) and the light signal was inverted to highlight the cells (1C). Then, the cells identified were transformed to measurable objects (1D). The gated objects are non cellular artifacts.

To eliminate the interference of artifacts an algorithm was used to filter this objects based on roundness (form factor). Objects with a low, user-defined form factor value were excluded. After the exclusion of the artifacts, the remaining objects were analyzed and live/dead cells were classified and counted using CPA (Figure 2).

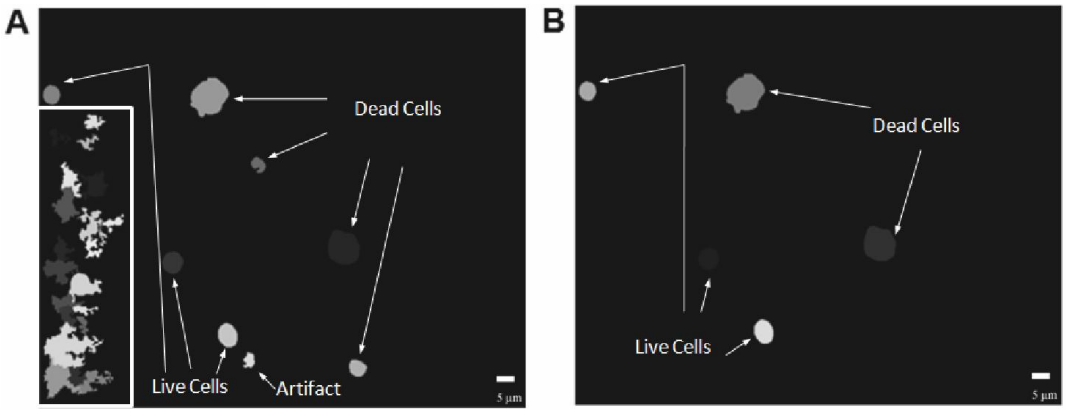

Figure 2: To exclude the non-cellular objects (called artifacts), an algorithm was created. This algorithm takes into consideration the cell and the artifact size. The figure 2A shows the image with the artifacts (gated) and 2B shows the same image without it.

2.7 Statistical Processing

To evaluate the concordance between the methods used for the present study, a Bland-Altman test was applied. This test assesses the correlation between the means of two different methodologies, or

in the case of this study, the correlation between the results of MTT assay and the results of image analysis.

3. RESULTS AND DISCUSSION

Fibroblasts were exposed to different doses of DMSO and live cells present was evaluated by cellular image analysis using CP and by the standard gold MTT assay. The percentage of live cells quantified demonstrated that both methodologies were able to estimate the cellular viability in a similar manner. e.g 8.0% of DMSO produce 22.10% (±0.03%) and 29.39% (±0.83%) of viability in the MTT assay and in proposed image analysis, respectively (Table 2).

Table 2: Identification of cells in CellProfiler. The algorithm used to perform the cell identification in the sample.

DMSO Concentration (%)	MTT assay (%)	Image Analysis (%)
0.5	93.87 ± 0.95	93.60 ± 0.42
1.0	89.90 ± 0.14	74.47 ± 0.11
2.0	88.30 ± 0.57	84.30 ± 0.33
4.0	56.60 ± 1.13	79.07 ± 0.06
8.0	22.10 ± 0.03	29.39 ± 0.83
16.0	4.04 ± 0.13	2.00 ± 0.25

To assess the viability, another commonly used technique a Neubauer chamber count. Although being a quick and low-cost technique, it is not reproducible and does not allow to assess the morphological features with the accuracy of image analysis. Moreover, these tests depend on the skill of the technician and there may be a bias in the results if the same sample is analyzed by two different technicians.

After staining with trypan blue, the cells showed distinct colors. Dead cells are stained with trypan blue and became dark blue, while unstained cells are alive and remain colorless.

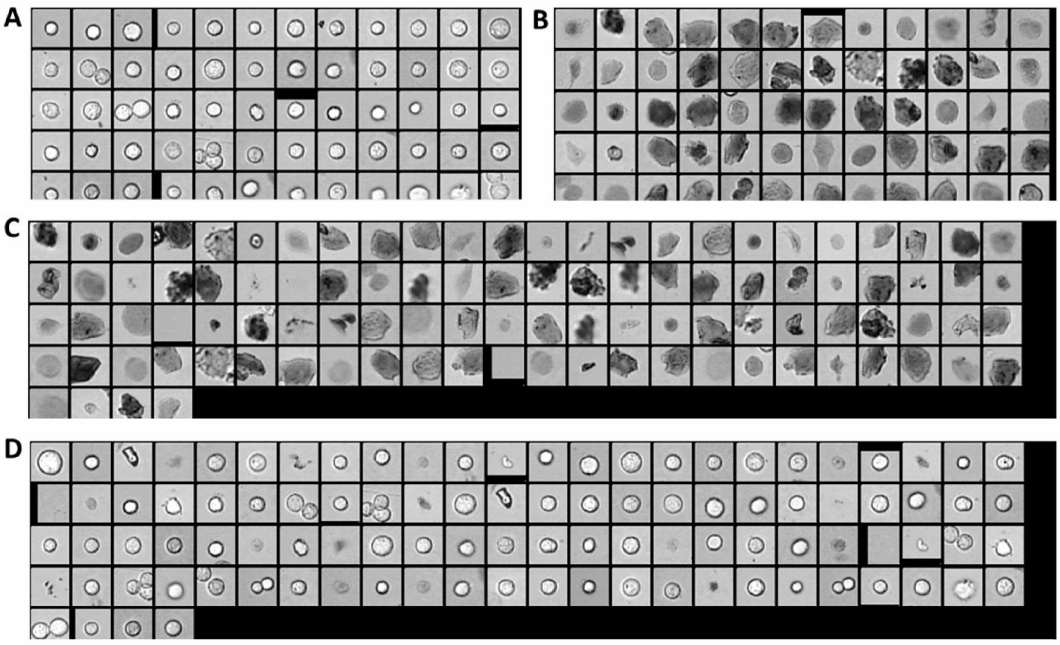

Figure 3: Cell Profiller analysis of live and dead cells. Software training for A) Live cells, and B) for dead cells. C. Software training when was requested to return 100 live cells. D) Software training when was requested to return 100 dead cells.

The obtained cellular images displayed light blue color background and therefore it was not necessary to stain the live cells. The different colors between live and dead cell proportionated by trypan blue stained associated with the different light intensities emitted by each color, allows the CPA to differentiate between live/dead cells (figure 1). To be able to identify live and dead cells, at first it was necessary to define specific parameters in the software helping it to recognize which cells were alive and which were dead, in order to quantify the size of each population. Before training, artifacts were excluded (Figure 2). Then, after the machine learning process, the software itself identified and counted live and dead cells of the sample. Subsequently the user ordered the software to show 100 live and dead cells, respectively. Using false positive and false negative events of each of the populations, sensitivity and specificity were calculated figure 3).

Being an alternative methodology, image analysis assessed by CP/CPA was compared to the established MTT assay (as seen in Figure 4). To compare two methods, the correlation coefficient cannot be used because it does not evaluate how the methods correlate, but how the results of each method are associated to each other. Therefore, the Bland-Altman test which correlates the means of two independent tests is generally used to compare methodologies [13]. The means values of the recorded results in both methodology should be close to zero to be consider equivalent the tested methodologies. The image analysis offered the advantage allowing morphological verification and documentation of the cells. The CP/CPA free software allows automated and reproductive high-throughput image analysis. In the proposed methodology, these characteristics associated with the low cost of the dyes used are more advantageous when compared to traditional methods of analysis.

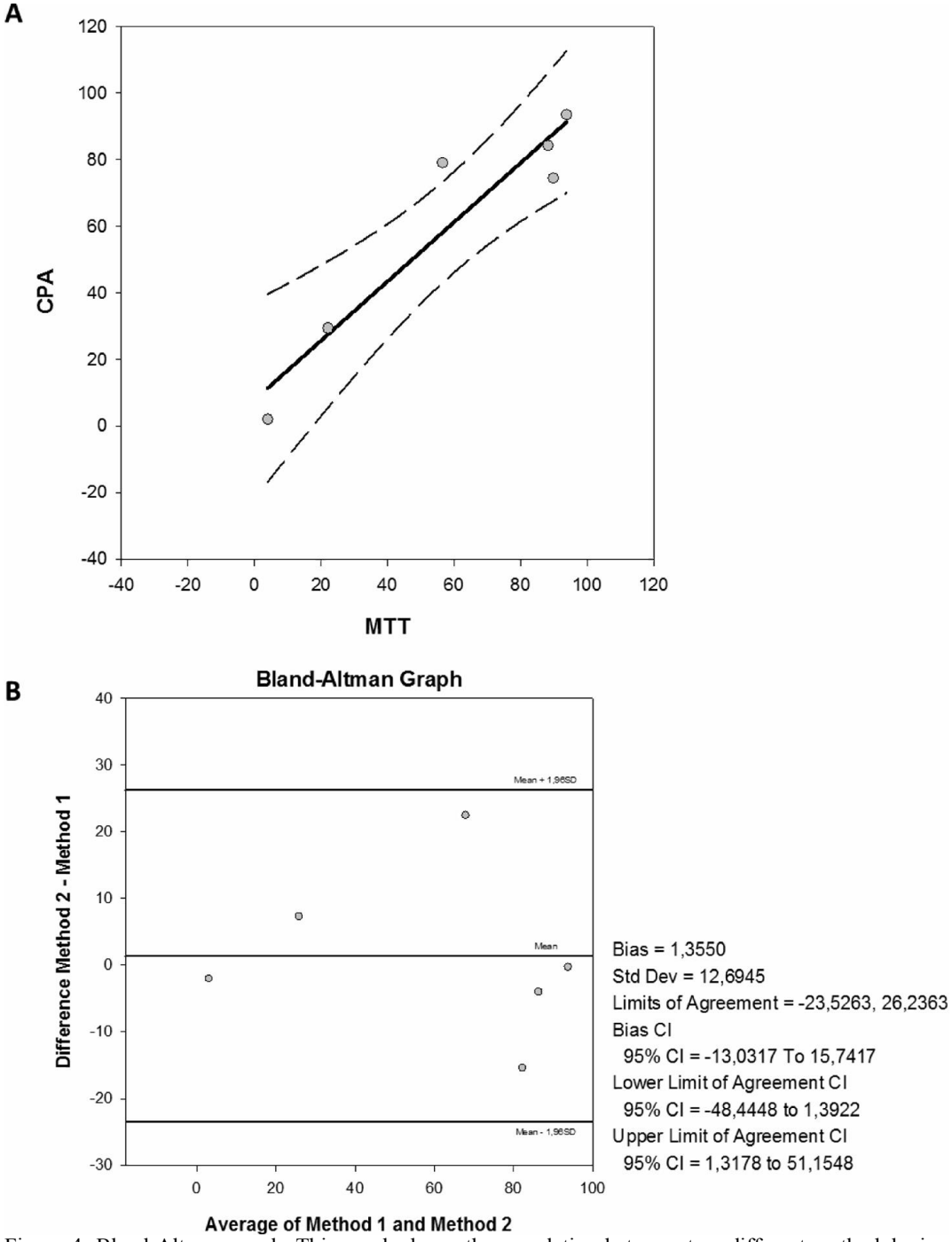

Figure 4: Bland-Altman graph. This graph shows the correlation between two different methodologies. The mean, close to zero, indicates that both methodologies are equivalent.

4. CONCLUSIONS

Our results and the statistical analysis allowed us to conclude that the image analysis by CellProfiler and CellProfiler Analyst is a simple, rapid and a low cost technique to explore cellular viability. Moreover, this technology, when compared to high-cost techniques like flow cytometry, allows the visualization and morphological verification of the cells. In comparison to low-costs techniques, like

Neubauer chamber, image analysis has the advantage to be reproductive, free of human bias, with high efficiency.

5. CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

6. ACKNOWLEDGEMENTS

The authors wish to thank the Fundação de Amparo a Pesquisa e Inovação do Espírito Santo (FAPES) for the financial support.

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3 MANUSCRIPT 2 – IS IT POSSIBLE TO USE JUST THE CELL MORPHOLOGY AND LIGHT MICROSCOPY TO DIFFERENTIATE APOPTOSIS AND NECROSIS?

Is it possible to use just the cell morphology and light microscopy to differentiate apoptosis and necrosis?

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ABSTRACT

Background: Necrosis and apoptosis are important and dramatically different cell death mechanisms. Since they are clearly different mechanisms, its morphological characteristics are also different. In this work, the morphological aspect will be assessed by image analysis and, therefore, there will be a biochemical aid in the course of the analysis. **Method**: Mouse fibroblast cells were exposed to different concentrations (ranged from 1 to 4 μM) of campthothecin (CPT) for 24 h and morphological parameters of necrosis and apoptosis were assessed by image analysis through a computer software. **Results**: The software was trained to recognize apoptotic and necrotic cells just by cell morphology but this analysis did not reach satisfactory quality. **Conclusion**: Although it is still not completely possible to differentiate necrosis and apoptosis using cell morphology, this analysis has reached a certain degree of accuracy, which shows that further improvements can positively contribute in order to build analysis that reliably classify these two cell populations.

Keywords: Cellular Classification, Free Software, Cell Identification, CellProfiler®, Cytometry.

1. INTRODUTION

Necrosis and apoptosis are the two main mechanisms of cell death. They have initiation, process and conclusion distinct and dramatically opposite. Apoptosis is a programmed cell death mediated by caspases and which process depends on the active energy consumption. Occurs under physiological conditions during and even after the development as a way to maintain the cell populations present in the tissues. Furthermore, apoptosis is a silent mechanism of cell death, non-aggressive to the neighboring cells and that does not mobilize the immune system by not releasing aggression signaling molecules (Damage-associated molecular pattern - DAMP's)[1,2, 3]. In contrast, necrosis is a chaotic cell death, which occurs independent of energy consumption. It is an aggressive process to neighboring cells by releasing molecules called "alarmins" which are molecules that mobilize the immune system against agressions, such as an alarm. The alarmins are found only in the intracellular environment and, during necrosis, cells have their membranes disrupted[4] which allows these alarmins end up in the extracellular medium, and then identified by immune cells which readily initiate defense response. Among the main alarmins, we can mention the High mobility group box 1 (HMGB1), uric acid, the S100 protein family, hepatoma-derived growth factor (HDGF) which, despite its name, is a protein expressed by neurons, the heat shock proteins (HSP), among others. As noted above, these molecules bind to specific receptors that identify them and give beginning to the immune response[2].

Since they are clearly different mechanisms, its morphological characteristics are also different [5]. In necrosis, there is a cell swelling and vacuolation of the cytoplasm, followed by a disruption of the plasmatic membrane and intracellular content leakage, may also be leakage of intranuclear content, so the circulating DNA outside the nucleus is considered an alarmin[6]. On the other hand, in apoptosis, the structure of cell plasmatic membrane and apoptotic bodies remains intact. Instead of swell, increasing in size, apoptotic cell shrinks, reducing their size, and there is little or no vacuolation formed in the cytosol.

An important feature of apoptosis is the externalization of a phospholipid present on the inner face of the plasmatic membrane: the phosphatidylserine. This phospholipid acts as a marker of apoptotic cells, since only in this condition it is exposed on the external surface of the cell membrane. Before chromatin condensation that occurs during apoptosis, DNA encodes proteins that act in the externalization of phosphatidylserine. This stimulates macrophages engulf cell and its apoptotic bodies. Since this condition only occurs in apoptotic cells and is absent in necrotic cells, are commercially available specific fluorescent markers to phosphatidylserine.

Cell differentiation: Apoptosis x Necrosis

The studies about differentiation of cell death types have played an important role in many areas of cellular biology: In microbial infections, where some pathogens, like *M. tuberculosis* for example, are able to control or manipulate the cell death modes to promote their proliferation[1]; in inflammation, when some proteins are released (like HMGB1 protein in necrosis) or activated (like caspases cascade, in

apoptosis)[7]; This studies are also important in many researches of tumor cells[8,9] and in many researches about pharmacology [10,11].

The cell differentiation can occur in several ways: Biochemical, physiological and morphological[12]. In the present work, the morphological aspect will be analyzed and, therefore, there will be a biochemical aid in the course of the analysis. The cells will be labeled with fluorochromes in order to show which ones are apoptotic and which ones are necrotic, so we can configure the software to help identify them only by morphology. It is noteworthy that, once informed to the software which cells are necrotic and which ones are apoptotic, it should be able to sort the cells without the aid of fluorochromes.

2. MATERIALS AND METHODS

2.1 Cell Culture

Swiss 3T3 albino mouse fibroblasts (ATCC CCL-92 - Cell Line Service, Rio de Janeiro, Brazil) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Cripion, Brazil), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich, USA) at 37ºC in a container of 5% CO_2 humidified atmosphere.

2.2 Image analysis preparation

3T3 fibroblasts cells were plated and incubated in 6-well tissue culture plate for 24h. After this incubation, cells were exposed to camptothecin (CPT) at 1µM, 2 µM and 4 µM. After 24h of incubation, the medium was carefully removed and 500 µL of a binding buffer (from apoptosis detection kit, Sigma-Aldrich, USA) was added in each well. After 10 minutes, the cells were incubated with 5 µL of Annexin V-FITC and 10µL of Propidium iodide (both from apoptosis detection kit, Sigma-Aldrich, USA). Microscope slides were prepared, and cellular images were made using a Nikon camera model Ds-Ri1 coupled to a Nikon–microscope model Eclipse Ti and connected to a computer, where the images were processed and stored. Cellular analysis was performed using the free software CellProfiler® (CP). CellProfiler® Analyst (CPA) was used for statistical analysis and cellular diagnostics.

2.3 Cell Identification by CellProfiler®

Like identification of live and dead cells, here, it is necessary to run an algorithm in CP. Similary to that case, the step here includes loading all images in the software, identifying all cells present in these images, turning all cells identified into a measurable object, and then setting measurable values to these objects. These values (e.g. form or light intensity) were used by CPA to create a profile of each cell population. The established algorithm used is shown in Table 1.

Table 1: The established algorithm used in the CellProfiler program to perform the analysis.

LoadImages	**Load all images to be analyzed**
ColorToGray	Turn all color images to black and white
CorrectIlluminationCalculate	calculates an illumination function that is used to correct uneven illumination/lighting/shading or to reduce uneven background in images
ApplyTreshold	sets pixel intensities below or above a certain threshold to zero
ImageMath > Invert	Invert light intensities
IdentifyPrimaryObjects > MoG Adaptive	Turn all identified images to objects, which can be measured
MeasureObjectSizeShape	Measure the form of all identified objects
FilterObjects > AreaShape > FormFactor	Filter objects by a determined parameter
MeasureTexture	measures the degree and nature of textures within objects
MeasureObjectIntensity	measures several intensity features for identified objects
MeasureImageQuality	Measures features that indicate image quality
MeasureGranularity	outputs spectra of size measurements of the textures in the image

2.4 Cellprofiler® Analysis

Cellular image analysis was performed with CP. First, each image was converted to grayscale. Thereafter, this gray image was enhanced and a treshold was applied. Then, intensities were inverted. The algorithm used for segmentation was MoG Adaptive. 60 and 100 pixels were chosen for minimum and maximum areas respectively (Figure 1A, to 1F), i.e. objects smaller than 60 or greater than 100 pixels were not identified.

To eliminate the interference of artifacts, an algorithm was used to filter these objects based on roundness (form factor). Objects with an user-defined form factor value were excluded. After the exclusion of the artifacts, the remaining objects were analyzed and apoptotic/necrotic cells were classified and counted using CPA (Figure 1G).

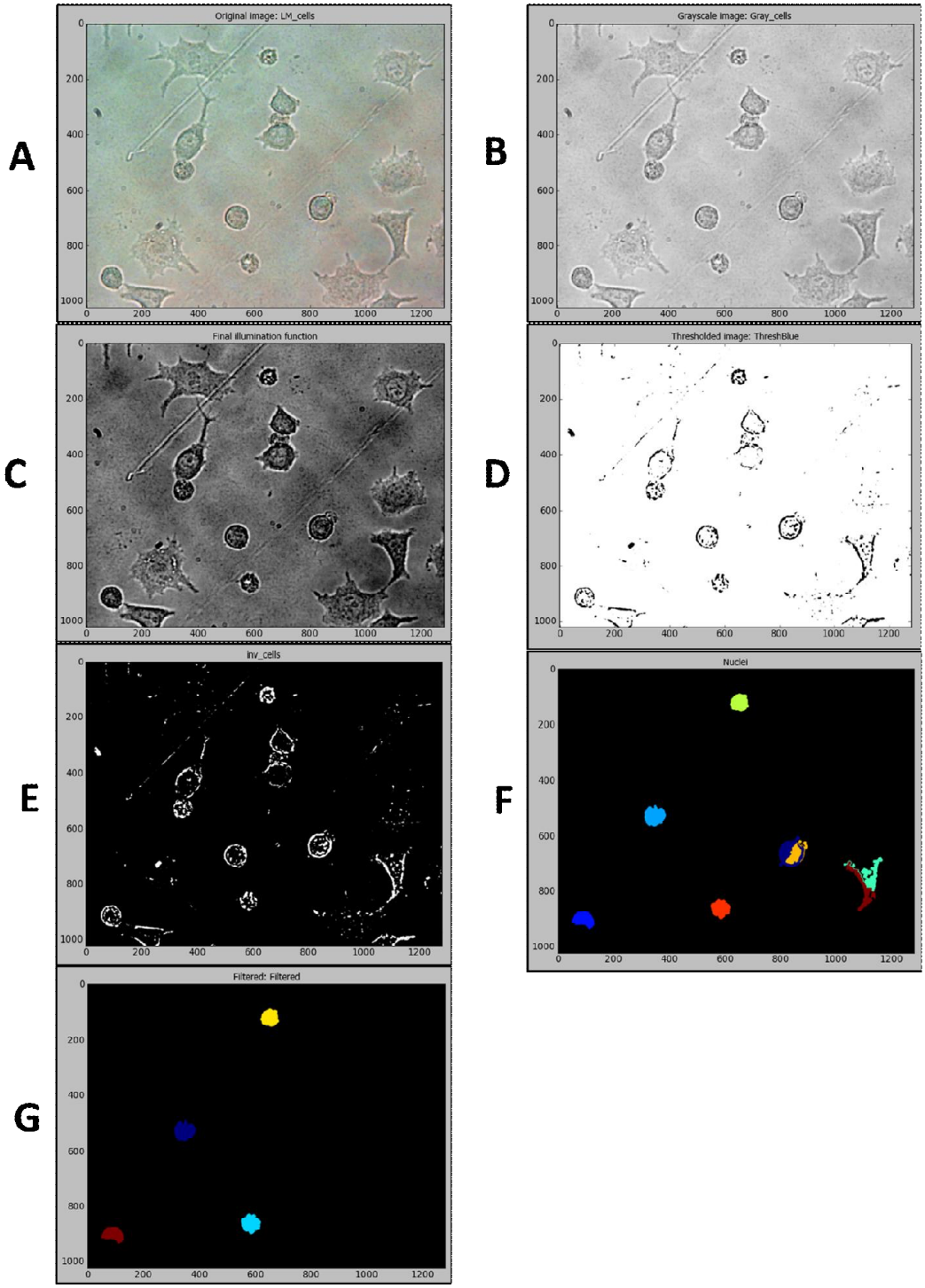

Figure 1 showing the cell identification by CP. 1A shows the original image of the cells captured by light microscopy; 1B shows the image 1A converted to black and white (necessary for the analysis); 1C shows the image 1B enhanced; 1D shows the image 1C after a threshold application, to highlight the cell borders; 1E shows the inverted light signal of image 1D; 1F shows the transformation of images into mesurable objects; 1G shows the image 1F after artifacts filtration.

3. RESULTS AND DISCUSSION

The methodology chosen to classify the necrotic and apoptotic cells was image analysis and counting by a free software called CellProfiler®. The identification of the cells is performed in this software and the classification in a software called CellProfiler® Analyst. These two softwares will be responsible for identifying and classifying cells based on commands provided by a trained technician. Once designed algorithm, other image analysis with the same cell population can be done without the need to rewrite the algorithm.

This technique allows researchers to keep track of the analysis, which will be subject to re-analyzes future at any time. This technique also allows the analysis of many cells at once, if there are sufficient images, without losses arising from the bias inherent to the observer, acting as a confirmation of the diagnosis of this.

Fibroblasts were exposed to different doses of camptothecin and the cells present was evaluated by cellular image analysis using CP. To highlight which cells were necrotic and which ones were apoptotic, fluorescent labels were used, but during the analysis, the fluorescence intensity was disregarded by the CPA, which used only the information related to morphology. It is possible to keep the fluorescence images to help researchers to determine which cells are marked for annexin V (apoptotic cells) and which are marked to Propidium Iodide (PI, necrotic cells) without the software use this information to classify populations. Thus, the researcher can load just the morphological parameters for the software, using only these parameters in the analysis.

In order to trace a morphological profile of the cell populations to classify, were informed to CPA more than 150 cell of each population (necrotic cells and apoptotic cells), labeled with annexin V and propidium iodide. Then, after the machine learning process, the software itself identified and counted necrotic and apoptotic cells of the sample. Subsequently the user ordered the software to show 100 necrotic and apoptotic cells, respectively. Using false positive and false negative events of each of the populations, sensitivity and specificity were calculated asking CPA to return 100 cells of each population in the sample. Figure 2 show the machine learning with the cells in fluorescence mode, in overlap mode and in black and white mode (showing just the morphology, as the software see).

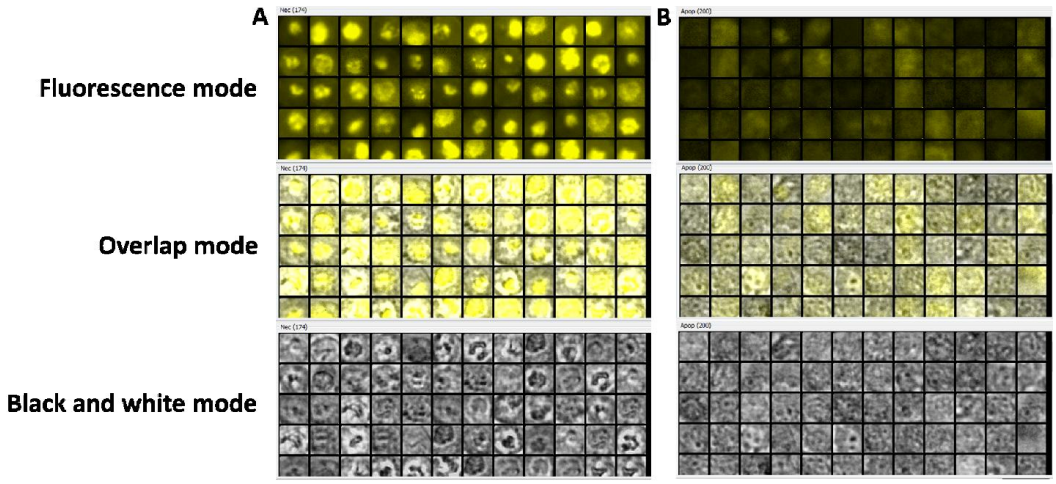

Figure 2. Demonstration of the machine learning by CPA. With the help of fluorescence, has been informed to the software which cells are necrotic (column A) and which are apoptotic (column B).

When asked CPA to return 100 necrotic cells, there were many false-positive, showing that the software had difficulty to classify correctly this population. The same happened when asked CPA to return 100 apoptotic cells. As we can see in figure 3, in a black and white mode (using only morphology), it is really hard to differentiate the cells.

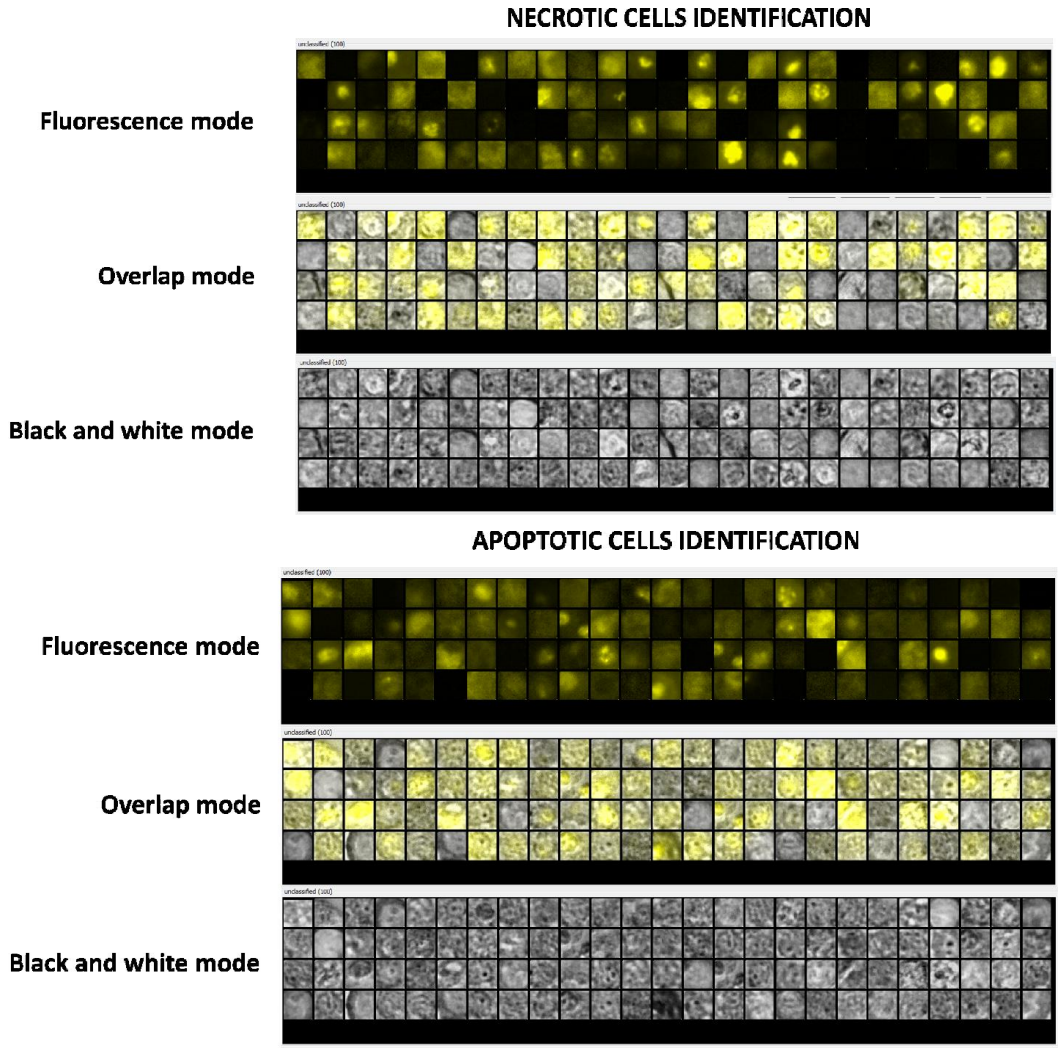

Figure 3 shows the cell identification by CPA. Observing the first line (fluorescence mode) can be noted the presence of much false-positive identification. This fact occurs because CPA uses only morphology for identification. in this kind of light microscopy analysis, in contrast to cell viability analysis, use only morphological criteria to identify necrosis and apoptosis seems to generate a low quality analysis

Despite difficult, it was possible to achieve a certain degree of accuracy, since the CPA recognizes, if not all, many cells from each researched population.

4. CONCLUSIONS

Although it is still not completely possible to differentiate necrosis and apoptosis using cell morphology, this analysis has reached a certain degree of accuracy, which shows that further improvements can positively contribute in order to build analysis that reliably classify these two cell populations.

5. CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

6. ACKNOWLEDGEMENTS

The authors wish to thank the Fundação de Amparo a Pesquisa e Inovação do Espírito Santo (FAPES) for the financial support.

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Bond spacing	18% of width
Fixed length	14.4 pt (0.500cm, 0.2in)
Bold width	2.0 pt (0.071cm, 0.0278in)
Line width	0.6 pt (0.021cm, 0.0084in)
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Font	Times New Roman
Size	8 pt
Under the Preference Choose:	
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