UNIVERSIDADE VILA VELHA-ES

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

ANÁLISE DE IMAGEM EMPREGADA NA AVALIAÇÃO DE VIABILIDADE CELULAR

LUCIANA SIMÕES GASPARINI

VILA VELHA

JUNHO/2016

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Dr. Dominik Lenz (UVV) - Orientador

Dedico esta Dissertação a minha família. Minha base, meu apoio e sem a qual nada seria possível. São vocês que me fazem ser quem eu sou.

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“A capacidade de sonhar sempre foi o grande segredo daqueles que mudaram o mundo. Os sonhos alimentam a alma e dão asas a inteligência. É no solo fértil da memória onde semeamos os sonhos que farão grande diferença em nossa existência. Os sonhadores mudaram a história da humanidade. Eles fizeram da derrota, o pódio para a vitória; das críticas, o palco, de onde receberam os aplausos. ”

(Augusto Cury)

SUMÁRIO

RESUMO

GASPARINI, Luciana Simões. MSc., Universidade Vila Velha-ES, Junho de 2016. **Análise de imagem empregada na avaliação de viabilidade celular**. Orientador: Prof. Dr. Dominik Lenz. Co-orientador: Prof. Dr. Márcio Fronza.

O presente estudo foi desenvolvido para avaliar a viabilidade celular *in vitro* utilizando análise de imagem. Linhagem de células de hepatoma hepa1c1c7 e fibloblastos L929 foram expostas a substâncias isoladas (camptotecina, alcalóides licorina, tazetina, albomaculina, 3-epimacronina, thisfaeridina e galantina) e a três extratos, sendo dois de espécies das algas *Padina* sp. e *Sargassum* sp. e um da espécie *Habrantus itaobinus* Ravenna em diferentes concentrações e corados com panótico. Imagens das células foram obtidas utilizando um microscópio invertido equipado com uma câmara digital. O ensaio colorimétrico de redução do sal tetrazólio (MTT) foi utilizado para avaliar a viabilidade celular. As imagens foram analisadas pelo software CellProfiler. Após análise, o software identificou e quantificou os objetos em cada imagem. Uma comparação entre o padrão-ouro MTT e a análise de imagens quantitativa foi realizada utilizando o teste de Bland-Altman. O método de análise de imagem apresentou resultados semelhantes para a identificação de células viáveis e pode colaborar como parte dos procedimentos de análise biológica. A metodologia apresentada é de baixo custo e reprodutiva.

Palavras-chave: CellProfiler, análise de imagem, viabilidade celular

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ABSTRACT

GASPARINI, Luciana Simões. MSc., Universidade Vila Velha-ES, Junho de 2016. **Image analysis employed in evaluation of cell viability**. Orientador: Prof. Dr. Dominik Lenz. Co-orientador: Prof. Dr. Márcio Fronza.

The present study was developed to evaluate *in vitro* cell viability using image analysis. Hepatoma hepa1c1c7 and fibroblasts (L929) cells were exposed to isolated substances (camptothecin, alkaloids licorine, tazetine, albomaculine, 3-epimacronine, thisphaeridine, galanthine) and three extracts, been two from species of algae *Padina* sp. and *Sargassum* sp. and one from *Habrantus itaobinus* Ravenna in different concentrations and stained with panotic. Images of the cells were obtained using an inverted microscope equipped with a digital camera. The methylthiazol-tetrazolium salt (MTT) colorimetric assay was used to evaluate cell viability. The images were analyzed by the CellProfiler software. After analysis, the software identified and quantified the objects in each image. A comparison between the gold standard MTT and the quantitative image analysis were performed using Bland-Altman analytical test. The method of image analysis presented similar results in the identification of viable cells and may assist part of biological analysis procedures. The presented methodology is inexpensive and reprodutible.

Keywords: CellProfiler, image analysis, cellular viability.

1.INTRODUCTION

Some experiments in the laboratory, are sometimes considered tedious and time consuming and are not quantitative (Lamprecht et al., 2007). The obtaining of images and processing with an image analysis software represents several advantages when compared to visual analysis (Lamprecht et al., 2007; Bray et al., 2015), including quick analysis, objectivity, beyond a quantitative and reproducable analysis (Rexhepaj et al., 2013; Krajewska et al., 2009; Lamprecht et al., 2007; Bray et al., 2015). The analysis may reproduce similar results, decreasing experimental error (Tozetti et al., 2014) and especially random error (Romanoff et al., 2014).

The image analysis employs software that identifies cells and analyzes each one of them. The CellProfiler is one free software and capable to automatically identify cells in digital images and count them, recording a wide spectrum of measurements to every one of them, like for example, cellular counting and complex morphologic analysis, as shape, staining and texture (Carpenter et al., 2006). An automated method that generates high reproducibility assays and that perform a cell selection and differentiation with lower cost, becomes promising to researches in biological area (Lamprecht et al., 2007).

It is software employed in experimental and biological analysis, assisting in assays and optimizing the time spend (Lamprech et al., 2007; Bray et al., 2015; Boatright et al., 2015). According to Kamentsky and collaborators (2011), the Cellprofiler possess funcionability and throughput, been also possible to be integrate to another open code software. Biological phenotypes can be measured automatically and quantitatively from a number of images.

The CellProfiler is a program easy to use by researchers that do not have great computer skills and very interesting to scientists that wish to implement an additional methodology based in image analysis (Jones et al., 2008). It is also necessary an algorithm, creating a sequence of configurations in order to process the image according to the criteria of the researcher standards for loading and analysis of the study's images (Carpenter, 2006). Cardoso and collaborators (2015) using image analysis evaluate fibroblasts and considered the method as easy, low cost, efficient, as well as Buzin and collaborators (2015) when employed image analysis to detect necrosis and apoptosis.

Abnormal or atypical cells possess great matter to medical and scientific community (Campaner et al., 2007). By that, evaluation and triage methods in artificial or natural substances that showed some biological or antitumoral activity present notorious relevance in studies (Mahavorasirikul et al., 2010; Ali et al., 2014).

This study evaluates plant extracts and isolated substances. Several vegetable species are constantly selected and analyzed to verify their phytochemical composition and potential for the Pharmaceutical scientific area (Mans et al., 2000). Studies about traditional medicine have been largely investigated to contribute the development of new drugs (Yuan et al., 2016). The alkaloids are a class in evidence in the scientific area, especially because a lot of them have been reported to be active against many existent pathologies (Perviz and Pervaiz, 2016; Lee et al., 2016; O'Rourke et al., 2016; Mabhiza et al., 2016) and some algae species have also awakened the scientific interest, due to several compounds with important properties (Zorofchian et al., 2014; De Jesus Raposo et al., 2015; Patra et al., 2015).

The Bland Altman statistical method is the used most suitably to compare two different methods (Bland-Altman, 1986) and in this study was used to evaluate the similarity between the results of the MTT assay and image analysis.

1. MATERIALS AND METHODS

2.1 Cell line and reagents

Hepatoma cancer cells, lineage Hepa-1c1c7 (ATCC® CRL-2026) and NCTC clone 929 [L cell, L-929, derivative of Strain L] fibroblast cells (ATCC® BCRJ 0188) were kept on culture medium Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich. USA) with 3.7g $NaHCO_3$, supplemented with 10% fetal bovine serum (FBS) (Cripion, Brazil) and 100 IU/ml penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, USA) and maintained at 37ºC on humidified atmosphere with 5% CO_2. Camptothecin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich® Chemical Co, St. Luis, MO, USA. Panotic staining was purchased from Instant-Prov, Newprov, Brazil. Isolated substances and extracts tested in this study were from the laboratory of natural products from the Federal University of Espírito Santo and University of Vila Velha. Other reagents and chemicals used were of analytical grade and were obtained from various commercial sources.

2.2 Cellular treatment for image analyses and MTT assay

Hepa-1c1c7 cells and L929 cells were seeded at a density of 7 x 10^4/mL in a 96-well plates and cultured for 24 h, in the presence of increasing concentrations of camptothecin (0.045 - 100 µM); *Padina* sp. algae methanolic extract (0.300 – 312.5 µg/mL); *Sargassum* sp. algae methanolic extract (0.300 – 312.5 µg/mL); lycorine (0.1 – 83 µM); tazettine (0.03 - 30 µM); *Habranthus itaobinus* AcOH extract (0.08 – 312.5 µg/mL); Albomaculine (0.75 - 46 µM); 3-epimacronine (3.03 – 19 µM); trisphaeridine (4.50 - 28 µM) and galanthine (0.88 – 56 µM). After 24 h incubation, medium was removed and the cells were stained either with panotic staining for Cell Profiler analyses or with MTT for the colorimetric viability assay as Mosmann, 1983.

2.3 Image Analysis Preparation

Medium was removed and 50 µL of Panotic dye (Instant-Prov, Newprov, Brazil) was added each for cellular staining. Then 10 photos were made each sample concentration using an inverted microscope LGD3 model, Eikonal Brazil. Cell analysis was performed using the CellProfiler (CP) software for cell parameters.

2.4 Analysis of images, mobile identification CellProfiler Software

For each concentration studied was used the same standard analysis - the pipeline (Table 1), only changed in identify Primary Objects item, minimum area and maximum area depending on the need for each concentration. After identifying with CellProfiler, the images were analyzed, quantified and saved.

The percent viability of each tested concentration of each substance was calculated using the control group as a base (live cells Standard 100%) and expressed as mean and standard deviation. The Cellprofiler identified in each image, the objects corresponding to the labeled cells and bound to the surface of the test plate according to figures 1 and 2. Upon identification and quantification, the sum total cells and percent viability calculated using as a basis the control group. These were carried out the calculation of mean and standard deviation values for each concentration.

The substances which showed cytotoxicity, the IC_{50} was calculated through the Table Curve software (software AISN Inc. Purdue University).

2.5 Cellular viability - MTT Assay

The cytotoxic activity *in vitro* was evaluated by the colorimetric MTT assay, as described previously with added small modifications (Mosmann, 1983). The Hepa-1c1c7 cells and L929 cells were plated in 96-well flat bottom plates at a concentration of 0.7 x 10^5 cells / mL. After overnight incubation the cells were exposed to different concentrations of the substances and extracts has described above. After 24 h incubation, 100 µL of MTT (5 mg/mL solution) was added to each well and the formazan crystals were dissolved with dimethyl sulfoxide (DMSO). The optical density was measured at 595 nm, using a microplate reader (Molecular Devises, Spectra Max 190, USA). The experiments were carried out at least in triplicate.

2.6 Statistical Analysis

To evaluate the correlation between the methods used in this study a Bland-Altman analysis was applied with software SigmaPlot (Systat Software, San Jose, CA). A Bland Altman result to be acceptable, must present a normal distribution of data and a good correlation between the measures related between the two methods (Bland and Altman, 1999). The average close to zero, indicates that the two methods have low difference between them.

2. RESULTS

Cell viability was assessed by image analysis using free CellProfiler software and compared with the colorimetric MTT assay, used as a reference standard. The results are shown in Table 2. Among all tested samples tested, 3-epimacronine, albomaculine, galanthine and trisphaeridine and extracts of *Padina* sp. and *Sargassum* sp. showed no cytotoxicity at the highest concentration analyzed. The substance tazettine showed cytotoxicity only against hepa-h1c1c7 cell line. Lycorine and *Habrantus itaobinus* Ravenna extract exhibited cytotoxic effects in both cell lines, as well as camptothecin. For substances that exhibit cytotoxicity IC50 were calculated as shown in Table 3.

Bland Atman test is shown in Figure 3. The data presented normal distribution is confirmed by the histogram and the mean difference between the two methods is close to zero, shown by the bias of the value 3.0263. Moreover, 95% of the values is between established concordance limits, -35.6465 and 41.6991. The error lies within the limits of the confidence intervals and these values is presented with a narrow difference. The R^2 was added to show the correlation between the data.

3. DISCUSSION

Study on cytotoxicity IC50 are scarcer in relation a isolated substances presented this study. Luo and collaborators (2012) evaluate the citotoxicity of alkaloids 3-epimacronine, tazettine, trisphaeridine and lycorine extracted from a plant belonging to Amaryllidaceae family. Of these, only lycorine presented cytotoxicity and proved to be selective to tumoral cells when compared to non-tumoral cells. On the other hand, in the present study the lycorine presented cytotoxicity for cell line hepa-h1c1c7, well as for cell line L929 and tazettine showed cytotoxicity only in lineage hepa-1c1c7 with IC50 10.40 µM. No data were found in the literature that could be compared. The other alkaloids tested also showed no cytotoxicity, according to the literature, except lycorine.

Li et al (2014) showed an IC50 of 0.56 µM for camptothecin in a study with cancer line of human liver and Piao et al (2016) found IC50 0.7 µM for the same lineage. Fronza and colleagues (2011) found 0.4 µM in the pancreatic cancer cell line cells. Our study found IC50 0.47 µM in Hepa-1c1c7 and 0.7 µM in L929 cells. The values are close to the literature, and for the cancer strain showed up below the surveyed data.

The lycorine is a substance that has demonstrated interest of the scientific class (Wang et al, 2012; Guo et al, 2015; Shen et al, 2014). Lamoral-Theys and collaborators in 2009 found IC50 for lycorine in cancer cells between 4.3 and 8.5 µM and Liu et al (2004) close to 1 µM in HL60 and non-tumor cells, showing more pronounced inhibition of cell growth than in non-tumor cells. This study showed IC50 2.88 µM in Hepa-1c1c7 and 0.62 µM in L929.

Comparing the methodologies, MTT and image analysis, the study presented significant and acceptable results in the Bland-Altman test. The data show a normal distribution, as shown in Figure 3. The exposure of the histogram data confirm the normality, and the figure shows no distortion or very long tails (Giavarina, 2015). As Altman and Bland (1983) recommend that 95% of the figure points are within the difference of means in its standard deviation, and the closer to zero for the difference of means, greater your agreement. In this study we can see the difference between the averages with a value of 3.0263, being very close to zero, according to the recommendations of the authors. According Giovarina (2015), the confidence interval (CI) checks how precise are the estimates of the data and allow us to estimate a

possible sampling error. The larger the sample size, the narrower will be CI and greater reliability that the data correspond to the true values.

The data presented in this study show narrow CI values between -35.6465 and 41.6991, suggesting that values approximate acceptably of the true value. Therefore has an acceptable standard statistical correspondence between the feasibility of MTT method and CellProfiler.

The correlation coefficient is an association measure, not being appropriate to evaluate the concordance between methods (Altman and Bland, 1983; Bland and Altman, 1986). The Bland-Altman method is the most adequate to assess the concordance among two methods (Hirakata et al., 2009). So, the image analysis is a methodology considered promising to analyze cellular viability, presenting a statistical significance among the obtained values (Figure 3).

The CellProfiler proved to be effective and facilitator of biological analyses procedures, as for example the analysis of parameters in retina cells of mice (Boatright et al., 2015), chromosomes measurements (González et al., 2014), pilot study of cellular analysis in Bird hemogram (Beaufrère et al., 2013), identification of Chlamydia species, with a much shorter time of analyses (Osaka et al., 2012), among others. Bray and collaborators (2015) developed and used a pipeline for identification of yeast colonies in agar plates, confirming the possibility to create adjustable pipelines in several biological experiments.

Many researches performed use techniques, like immunofluorescence, that are in part onerous. The present study used only routine staining, of low cost and a method that evaluate viability and cellular parameters. And also, without the need of a fluorescent staining or any other technique that elevate the final cost, being promising to the scientific community.

The study obtained a quick analysis, was inexpensive and efficient. However, scarcity of data around this subject, denote the importance of more studies to enhance this technique.

4. CONCLUSIONS

The method for image analysis CellProfiler Software proved to be simple, low cost and fast, optimizing the cell analysis. It is a technique that allows the verification of the cellular morphology and compared to the gold standard test, MTT, presented with little variation. Image analysis is a method that can be reproduced, low cost and efficient. More studies are needed in order to improve this analysis for of cellular and biological detection.

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Compliance with ethical standards

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Ethical approval

This article does not contain any studies with human or animals participants performed by any of the authors.

Conflict of interest

The authors declare that they have no competing interests**.**

5. REFERENCES

Ali, MA. Abul, FM. Al-Hemaid, FM. Abou-Tarboush, FM. In vitro cytotoxicity screening of wild plant extracts from Saudi Arabia on human breast adenocarcinoma cells. Genet. Mol. Res., 2014, 13, 3981-3990.

Altman DG, Bland JM. Measurement in Medicine: The analysis of method comparison studies. Journal of the Royal Statistical Society. Series D (The Statistician) 1983;32(3):307-17.

Beaufrère H, Ammersbach M, Tully TN Jr. Complete blood cell count in psittaciformes by using high-throughput image cytometry: a pilot study. J Avian Med Surg. 2013 Sep;27(3):211-7. PubMed PMID: 24344512.

Bland, JM. Altman D. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet.1986;327(8476):307-1.

Bland JM, Altman DG. Measuring agreement in method comparison studies. Stat Methods Med Res. 1999 Jun;8(2):135-60. Review. PubMed PMID: 10501650.

Boatright JH, Dalal N, Chrenek MA, Gardner C, Ziesel A, Jiang Y, Grossniklaus HE, Nickerson JM. Methodologies for analysis of patterning in the mouse RPE sheet. Mol Vis. 2015 Jan 15;21:40-60. eCollection 2015. PubMed PMID: 25593512; PubMed Central PMCID: PMC4301600.

Bray MA, Vokes MS, Carpenter AE. Using CellProfiler for Automatic Identification and Measurement of Biological Objects in Images. Curr Protoc Mol Biol. 2015 Jan 5;109:14.17.1-14.17.13. doi: 10.1002/0471142727.mb1417s109. PubMed PMID: 25559103; PubMed Central PMCID: PMC4302752.

Buzin AR, Pinto FE, Nieschke K, Mittag A, de Andrade TU, Endringer DC, Tarnok A, Lenz D. Replacement of specific markers for apoptosis and necrosis by nuclear morphology for affordable cytometry. J Immunol Methods. 2015 May;420:24-30. doi: 10.1016/j.jim.2015.03.011. Epub 2015 Mar 31. PubMed PMID: 25840146.

Cardoso, LF; Cordeiro, SG, Fronza, M; Endringer, DC; Andrade, TU; Lenz, D. Combination of Free Software, Light Microscopy and Non-specific Dyes for a High-throughput Analysis in Live/Dead Cell Count. Current Pharmaceutical Analysis, 2015, 11(4): 233-238.

Campaner, Adriana Bittencourt; Galvao, Maria Antonieta Longo; Santos, Roberto Euzébio dos; Aoki, Tsutomu. Células glandulares atípicas em esfregaços cervicovaginais: significância e aspectos atuais. J. Bras. Patol. Med. Lab. 2007, vol.43, n.1 p.37-43.

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA,Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol. 2006;7(10): R100. Epub 2006 Oct 31. PubMed PMID: 17076895; PubMed Central PMCID:PMC1794559.

De Jesus Raposo MF, de Morais AM, de Morais RM. Marine polysaccharides from algae with potential biomedical applications. Mar Drugs. 2015 May 15;13(5):2967-3028. doi:

10.3390/md13052967. Review. PubMed PMID: 25988519; PubMed Central PMCID: PMC4446615.

Fronza M, Murillo R, Ślusarczyk S, Adams M, Hamburger M, Heinzmann B, Laufer S, Merfort I. In vitro cytotoxic activity of abietane diterpenes from Peltodon longipes as well as Salvia miltiorrhiza and Salvia sahendica. Bioorg Med Chem. 2011 Aug 15;19(16):4876-81. doi: 10.1016/j.bmc.2011.06.067. Epub 2011 Jun 29. PubMed PMID: 21775156.

Giavarina D. Understanding Bland Altman analysis. Biochemia Medica. 2015;25(2):141-151. doi:10.11613/BM.2015.015.

González JE, Romero I, Gregoire E, Martin C, Lamadrid AI, Voisin P, BarquinerJF, García O. Biodosimetry estimation using the ratio of the longest:shortest length in the p remature chromosome condensation (PCC) method applying autocapture and automatic image analysis. J Radiat Res. 2014 Sep;55(5):862-5. doi: 10.1093/jrr/rru030. Epub 2014 Apr 30. PubMed PMID: 24789085; PubMed Central PMCID: PMC4202288.

Guo Y, Wang Y, Cao L, Wang P, Qing J, Zheng Q, Shang L, Yin Z, Sun Y. A Conserved Inhibitory Mechanism of a Lycorine Derivative against Enterovirus and Hepatitis C Virus. Antimicrob Agents Chemother. 2015 Nov 23;60(2):913-24. doi:10.1128/AAC.02274-15. PubMed PMID: 26596952; PubMed Central PMCID: PMC4750679.

Hirakata, Vânia Naomi, and Suzi Alves Camey. "Análise de concordância entre métodos de Bland-Altman." Revista HCPA. Porto Alegre. Vol. 29, no. 3 (2009), p. 261-268. (2009).

Jones Tr, Kang Ih, Wheeler Db, Lindquist Ra, Papallo A, Sabatini Dm, Golland P, Carpenter Ae. CellProfiler Analyst: data exploration and analysis software for complex image-based screens. BMC Bioinformatics. 2008 Nov 15;9:482.

Kamentsky L, Jones TR, Fraser A, Bray MA, Logan DJ, Madden KL, Ljosa V, RuedenC, Eliceiri KW, Carpenter AE. Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. Bioinformatics.2011 Apr 15;27(8):1179-80. doi: 10.1093/bioinformatics/btr095. Epub 2011 Feb 23. PubMed PMID: 21349861; PubMed Central PMCID: PMC3072555.

Krajewska M, Smith LH, Rong J, Huang X, Hyer ML, Zeps N, Iacopetta B, Linke SP, Olson AH, Reed JC, Krajewski S. Image analysis algorithms for immunohistochemical assessment of cell death events and fibrosis in tissue sections. J Histochem Cytochem. 2009 Jul;57(7):649-63. doi: 10.1369/jhc.2009.952812. Epub 2009 Mar 16. PubMed PMID: 19289554; PubMed Central PMCID: PMC2699321.

Lamoral-Theys D, Andolfi A, Van Goietsenoven G, et al. Lycorine, the Main Phenanthridine Amaryllidaceae Alkaloid, Exhibits Significant Anti-Tumor Activity in Cancer Cells that Display Resistance to Proapoptotic Stimuli: an Investigation of Structure-Activity Relationship and Mechanistic Insight. Journal of medicinal chemistry. 2009;52(20):6244-6256. doi:10.1021/jm901031h.

Lamprecht Mr, Sabatini Dm, Carpenter Ae. CellProfiler: free, versatile software for automated biological image analysis. Biotechniques. 2007 Jan;42(1):71-5.

Lee W, Lee J, Kulkarni R, Kim MA, Hwang JS, Na M, Bae JS. Antithrombotic and antiplatelet activities of small-molecule alkaloids from Scolopendra subspinipes mutilans. Sci Rep. 2016 Feb 24;6:21956. doi: 10.1038/srep21956. PubMed PMID: 26905699.

Li X, Zhao T, Cheng D, Chu C, Tong S, Yan J, Li QY. Synthesis and biological activity of some bile acid-based camptothecin analogues. Molecules. 2014 Mar24;19(3):3761-76. doi: 10.3390/molecules19033761. PubMed PMID: 24662087.

Liu J, Hu WX, He LF, Ye M, Li Y. Effects of lycorine on HL-60 cells via arresting cell cycle and inducing apoptosis. FEBS Lett. 2004 Dec 17;578(3):245-50. PubMed PMID: 15589827.

Luo Z, Wang F, Zhang J, Li X, Zhang M, Hao X, Xue Y, Li Y, Horgen FD, Yao G, Zhang Y. Cytotoxic alkaloids from the whole plants of Zephyranthes candida. J NatProd. 2012 Dec 28;75(12):2113-20. doi: 10.1021/np3005425. Epub 2012 Nov 28. PubMed PMID: 23190013; PubMed Central PMCID: PMC3580115.

Mabhiza D, Chitemerere T, Mukanganyama S. Antibacterial Properties of Alkaloid Extracts from Callistemon citrinus and Vernonia adoensis against Staphylococcus aureus and Pseudomonas aeruginosa. Int J Med Chem. 2016; 2016:6304163. doi: 10.1155/2016/6304163. Epub 2016 Jan 20. PubMed PMID: 26904285; PubMed Central PMCID: PMC4745602.

Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K. Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. BMC Complement Altern Med. 2010 Sep 28;10:55.

Mans, DR, da Rocha AB, Schwartsmann G. Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds. Oncologist. 2000;5(3):185-98. PubMedPMID: 10884497.

Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983 Dec 16;65(1-2):55-63. PubMed PMID: 6606682.

O'Rourke A, Kremb S, Bader TM, Helfer M, Schmitt-Kopplin P, Gerwick WH, Brack-Werner R, Voolstra CR. Alkaloids from the Sponge Stylissa carteri Present Prospective Scaffolds for the Inhibition of Human Immunodeficiency Virus 1(HIV-1). Mar Drugs. 2016 Feb 4;14(2). pii: E28. doi: 10.3390/md14020028. PubMed PMID: 26861355.

Osaka I, Hills JM, Kieweg SL, Shinogle HE, Moore DS, Hefty PS. An automated image-based method for rapid analysis of Chlamydia infection as a tool for screening antichlamydial agents. Antimicrob Agents Chemother. 2012 Aug;56(8):4184-8. doi: 10.1128/AAC.00427-12. Epub 2012 May 21. PubMed PMID: 22615279; PubMed Central PMCID: PMC3421616.

Patra S, Muthuraman MS, Prabhu AR, Priyadharshini RR, Parthiban S. Evaluation of antitumor and antioxidant activity of Sargassum tenerrimum against Ehrlich ascites carcinoma in mice. Asian Pac J Cancer Prev. 2015;16(3):915-21. PubMed PMID: 25735382.

Perviz S, Khan H, Pervaiz A. Plant Alkaloids as an Emerging herapeutic Alternative for the Treatment of Depression. Front Pharmacol. 2016 Feb 15;7:28. doi: 10.3389/fphar.2016.00028. eCollection 2016. Review. PubMed PMID: 26913004; PubMed Central PMCID: PMC4753303.

Rexhepaj E, Agnarsdóttir M, Bergman J, Edqvist PH, Bergqvist M, Uhlén M, Gallagher WM, Lundberg E, Ponten F. A texture based pattern recognition approach to distinguish melanoma from non-melanoma cells in histopathological tissue microarray sections. PLoS One. 2013 May 17;8(5):e62070. doi: 10.1371/journal.pone.0062070. Print 2013. PubMed PMID: 23690928; PubMed Central PMCID: PMC3656869.

Romanoff Am, Cohen A, Schmidt H, Weltz Cr, Jaffer Sm, Nagi Cs, Bleiweiss Ij, Port Er. Breast Pathology Review: Does It Make a Difference? Ann Surg Oncol. 2014.

Shen JW, Ruan Y, Ren W, Ma BJ, Wang XL, Zheng CF. Lycorine: a potential broad-spectrum agent against crop pathogenic fungi. J Microbiol Biotechnol. 2014 Mar 28;24(3):354-8. PubMed PMID: 24346469.

Tozetti Pb, Lima Em, Nascimento Am, Endringer Dc, Pinto Fe, Andrade Tu, Mittag A, Tarnok A, Lenz D. Morphometry to identify subtypes of leukocytes. Hematol Oncol Stem Cell Ther. 2014; 7 (2): 69-75

Wang YH, Wan QL, Gu CD, Luo HR, Long CL. Synthesis and biological evaluation of lycorine derivatives as dual inhibitors of human acetylcholinesterase and butyrylcholinesterase. Chem Cent J. 2012 Sep 8;6(1):96. doi:10.1186/1752-153X-6-96. PubMed PMID: 22958411; PubMed Central PMCID: PMC3782361.

Yuan H, Ma Q, Ye L, Piao G. The Traditional Medicine and Modern Medicine from Natural Products. Molecules. 2016 Apr 29;21(5). pii: E559. doi:10.3390/molecules21050559. Review.

Zorofchian Moghadamtousi S, Karimian H, Khanabdali R, Razavi M, Firoozinia M, Zandi K, Abdul Kadir H. Anticancer and antitumor potential of fucoidan and fucoxanthin, two main metabolites isolated from brown algae.ScientificWorldJournal. 2014 Jan 2;2014:768323. doi: 10.1155/2014/768323.eCollection 2014. Review. PubMed PMID: 24526922; PubMed Central PMCID:PMC3910333.

6. TABLES AND FIGURES

Table 1: Configuration Sequence - pipeline in CellProfiler. From these data it is possible to identify the core in the charged objects, as well as intensity, area and shape.

Load Images (1)
Color to gray (2)
Image Math (3)
IdentifyPrimaryObjects - Cell (4) (4.1) Typical diameter of objects (Min-Max) (20 – 200 pixels) (4.2) Threshold Strategy (Adaptive) (4.3) Thresholding Method (RosbustBackground)
MeasureObjectIntensity (5)
MeasureObjectSizeShape (6)

Table 2: Cellular viability of tested substances at maximum concentration evaluated by cell profiler analyses (CP) and MTT assay. Results are expressed as mean (%) ± standard deviation (SD) of three independent experiments.

		Hepa-1c1c7		**L929**	
	Concentration	MTT	CP	MTT	CP
1	19 (μM)	101.90 (2.13)	96.36 (2.05)	111.14 (1.80)	101.95 (9.32)
2	46 (μM)	103.28 (2.60)	74.75 (4.33)	103.56 (6.52)	108.63 (2.44)
3	56 (μM)	103.85 (3.07)	125.23 (4.78)	112.29 (9.22)	102.56 (3.66)
4	28 (μM)	98.30 (1.32)	87.50 (4.23)	105.22 (5.28)	98.92 (2.36)
5	30 (μM)	34.87 (1.90)	52.59 (4.99)	142.54 (9.50)	154.64 (1.39)
6	83 (μM)	6.71 (1.12)	27.05 (1.77)	6.71 (1.12)	22.87 (1.03)
7	100 (μM)	65.42 (0.59)	19.54 (1.22)	9.12 (8.14)	13.45 (7.26)
8	312.5 (μg/mL)	100.07 (9.17)	99.18 (1.20)	99.40 (11.39)	119.47 (7.33)
9	312.5 (μg/mL)	108.35 (2.39)	81.73 (1.36)	102.05 (6.60)	105.15 (3.62)
10	312.5(μg/mL)	17.52 (0.61)	17.23 (1.99)	12.75 (030)	26.15 (1.67)

(1) 3-epimacronine (2) Albomaculine (3) Galanthine (4) Trisphaeridine (5) Tazettine (6) Lycorine (7) Camptothecin (8) *Padina* sp. (9) *Sargassum* sp. (10) *Habrantus itaobinus* Ravenna.

Table 3: Comparison of IC50 of the substances tested in MTT and Image Analysis method with mean (μM) and standard deviation (SD).

	Hepa-1c1c7		**L929**	
Samples	MTT	CP	MTT	CP
Lycorine	2.88 (0.03)	1.87 (0.06)	0.62 (0.02)	0.45 (0.01)
Camptothecin	0.47 (0.01)	0.58 (0.01)	0.07 (0.005)	0.06 (0.01)
Habrantus itaobinus	0.78 (0,09)	6.88 (0.69)	0.14 (0.003)	0.15 (0.01)
Tazettine	10.40 (0.39)	14.14 (0.51)	-	-

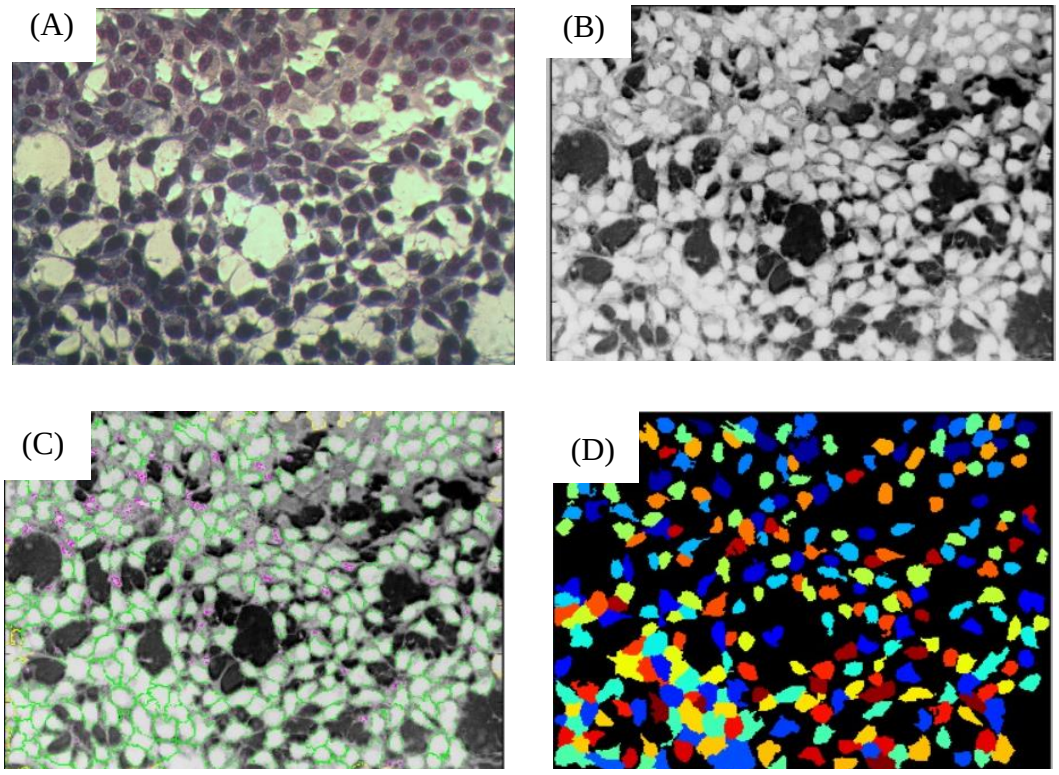
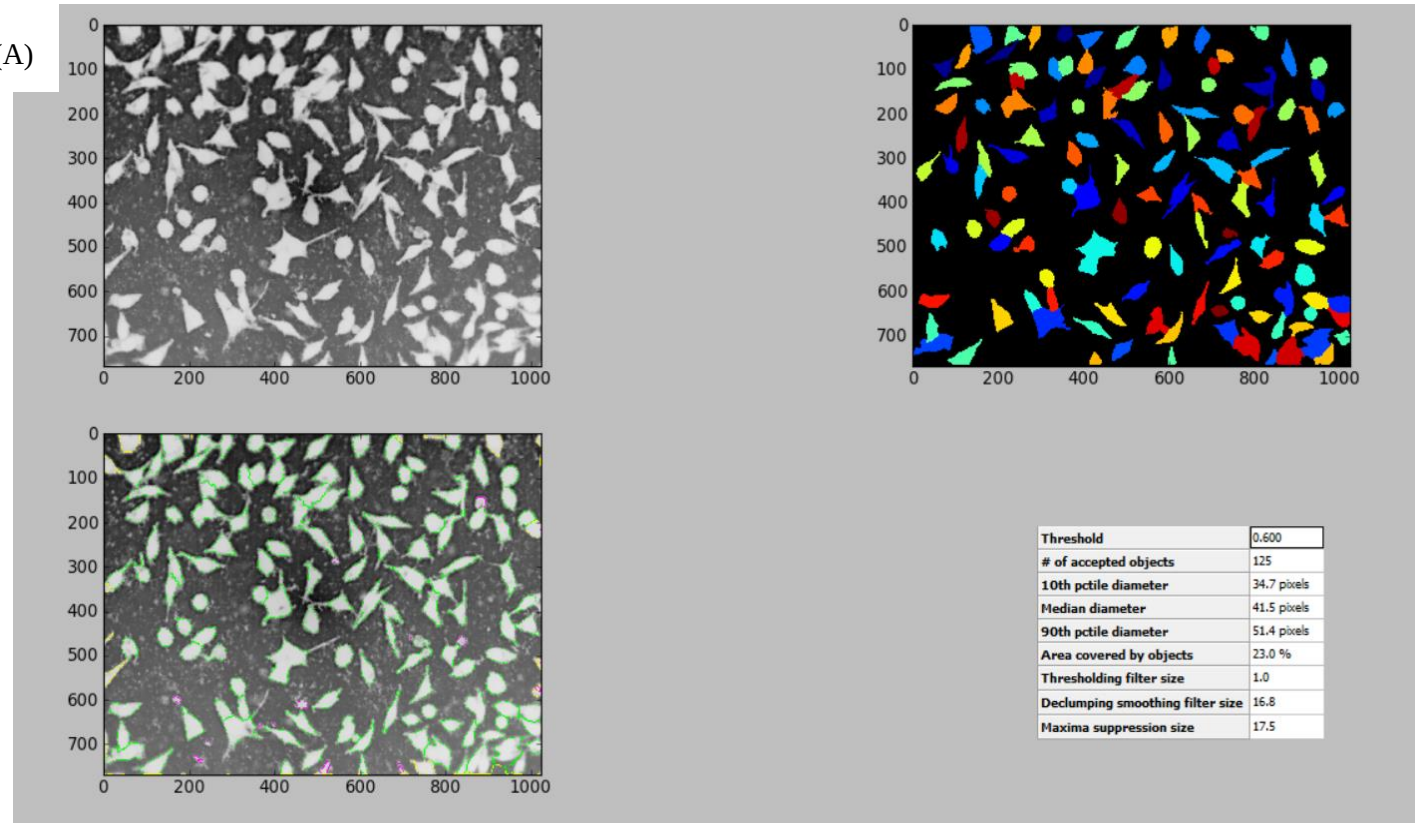


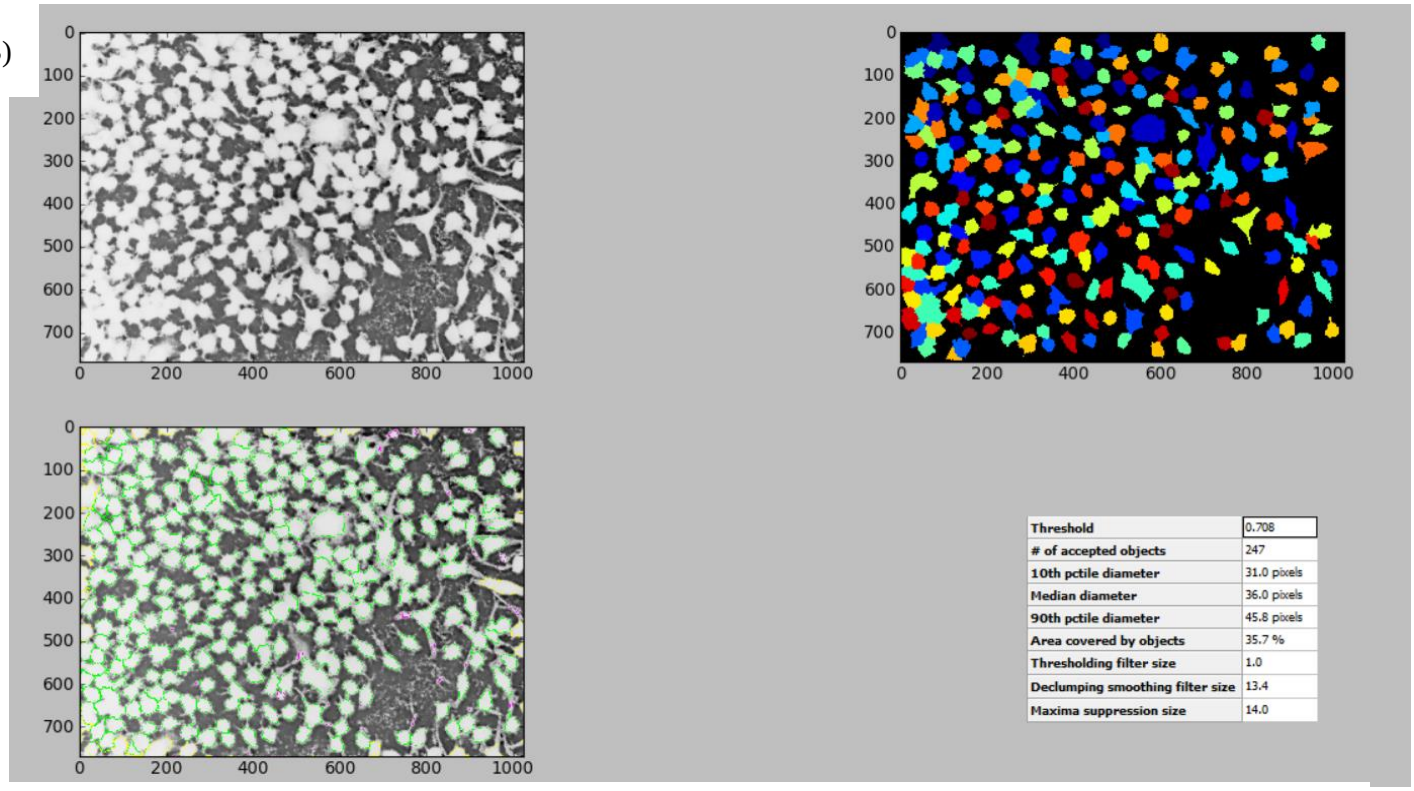
Figure 1. Cell identification in CellProfiler. The original image (A) was converted to grayscale (B) cells were identified for measured objects, and nuclei in green and pink, identifying objects deleted (C). Then, was showing the cell (D).

(A)



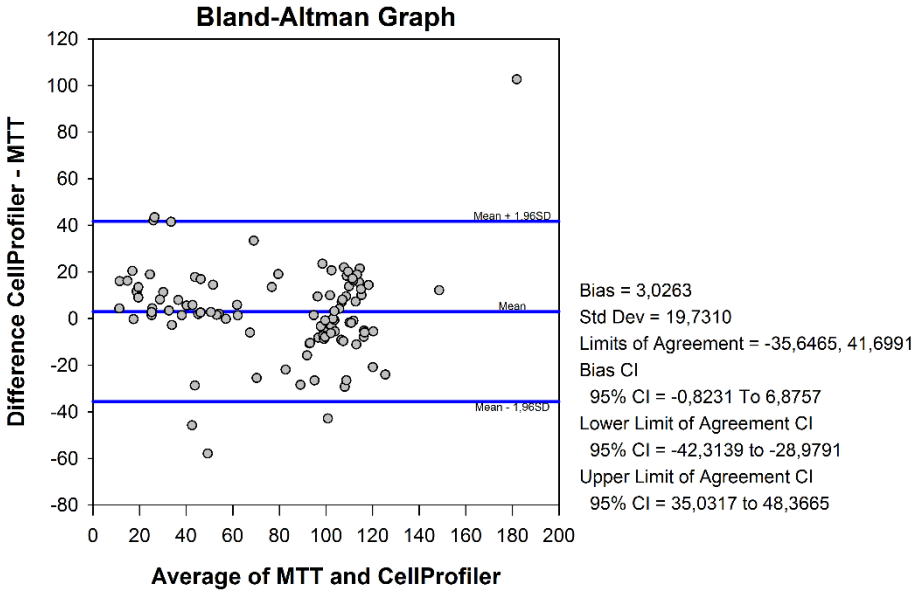
Threshold	0.600
# of accepted objects	125
10th pctile diameter	34.7 pixels
Median diameter	41.5 pixels
90th pctile diameter	51.4 pixels
Area covered by objects	23.0 %
Thresholding filter size	1.0
Declumping smoothing filter size	16.8
Maxima suppression size	17.5

(B)



Threshold	0.708
# of accepted objects	247
10th pctile diameter	31.0 pixels
Median diameter	36.0 pixels
90th pctile diameter	45.8 pixels
Area covered by objects	35.7 %
Thresholding filter size	1.0
Declumping smoothing filter size	13.4
Maxima suppression size	14.0

Figure 2. The objects (cells) were identified and quantified by CellProfiler software separately in each selected image in the L929 cell (A) and Hepa-1c1c7 cell (B).



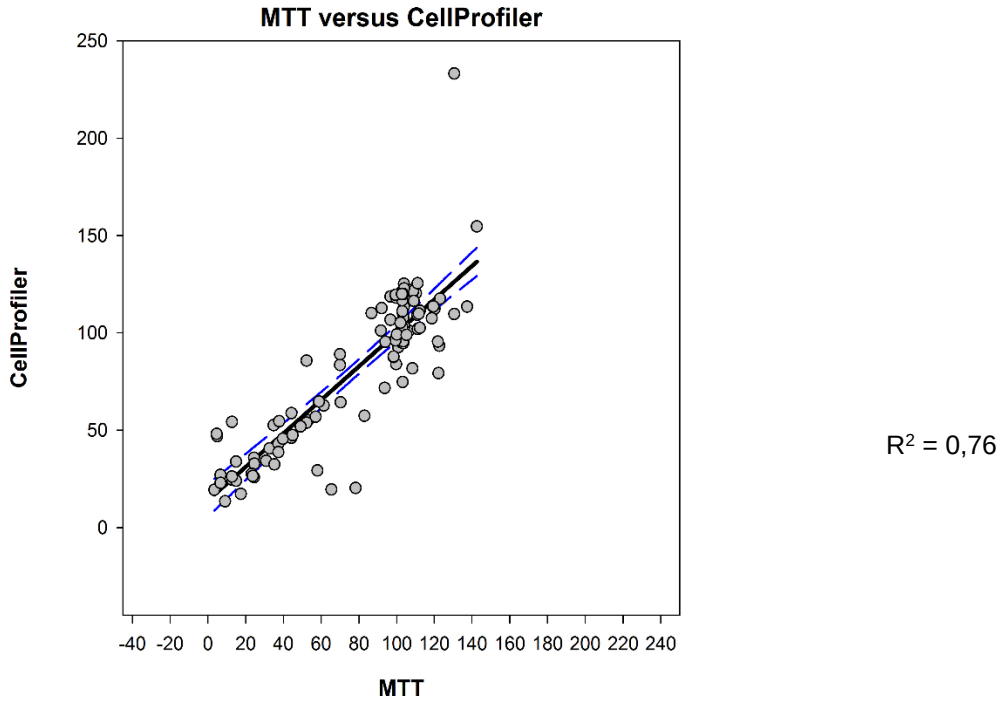


Figure 3. Statistical analysis Bland Altman. This figure shows the correlation between two different methodologies.