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Influence of different fixation reagents on NIH 3T3 fibroblasts morphology: An AFM study

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ABSTRACT

The Atomic force microscope (AFM) has recently become an attractive tool for biological analysis, especiallyfor the characterization of both morphological and mechanical properties. In this study, we investigatedthe influence of different fixation reagentsonNIH 3T3 fibroblast cells′ morphology. Three protocolswere selected: paraformaldehyde, methanol and acetone. Height, perimeter, area, volume and roughness of cells were measured using an AFM. As expected, different fixation protocols produce different morphological changes in cells. However, to preserve fibroblasts′ morphology, the methods applying aldehyde fixations performed much better than those using either methanol or acetone. Based on quantitative assessments, fibroblasts fixed with paraformaldehyde showed the closest volume, area and perimeter values to living cells. Roughness data and a Western blot test confirmed that methanol fixation produces larger cell membrane damage, when compared to the other two fixatives.

KEYWORDS

AFM;
Cell morphology;
Fixatives.

INTRODUCTION

Cell biology research presupposes accurate knowledge of cellmorphology. Relations between the various organs and tissues can be studied in their dependence on time if their spatial arrangement is ascertained with accuracy. Furthermore, knowing their morphology can be an additional index of cells′ vital status and surface adhesion to successfully study cell physiology and mechanical properties. Cell morphology is, therefore, a fundamental element to have a complete understanding ofcell activity. In this respect, working with living cells would be of paramount importance. However, lack of available data on the properties of living cellsmakes the use of fixed cells inevitable. The aim of fixation should be to preserve the molecular arrangement as muchas possible as found in vivo. Although even the most careful fixation does alter the cytoplasmic structure and introducesartefacts that can interfere with the interpretation of cellular ultrastructure, the choice of a given fixative often depends on the individual′s personal choice, thereby neglecting the possible drawbacks of fixatives. The practice of fixing protocols for biological samples could be useful for many reasons: (i) samples could be stored for a long period of time; (ii) fixation immediately after sample preparation stops in vitro ac-

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tivation processes; (iii) samples are immediately permeabilized and ready for intracellular staining and cell cycle analysis; (iv)fixation increases strength and rigidity and might help preserve the morphology (shape and structure) of the sample as it is processed for further analysis. However, all fixation reagents affect cell morphology, as a consequence ofdenaturation, precipitation and cross-linking of proteins.

In previous studies, microscopy methods were used to evaluate the performance of fixation methods, including light microscopy[17], reflection contrast microscopy[10], fluorescence microscopy[6], Raman microscopy[14], electron microscopy[10], as well as AFM[15]. The use of AFM overcomessome problems related to the aforementioned methods, since this technique is not bound to a wavelength and does not use electrons. Moreover, living and fixed cells can both be studied using AFMand 3-D morphological images at nanometre or sub-nanometre scales, under either dry or wet conditions[1,4,8,9,11].

For AFM cell studies, the optimization of sample preparation is essential, and this requires the determination of three factors: (i) the substrate on which the cells are cultured,(ii) the optimal concentration of cells seeded onto this substrate and (iii) the fixation method. The most common substrate used in cell cultures for AFM examination is glass coverslip. Other materials used aremicroscope slides and Petri dishes. However, to measure mechanical, thermal and electrical cell characteristics optimally, silicon-based Micro Electro Mechanical Systems (MEMS) are frequently used and, therefore, the influence of this material substrate needs to be investigated.

This paper reportsthe influence of different fixation chemicals on NIH 3T3 fibroblasts morphology, comparing the results with those of cells in living conditions. Cells were studied on two different substrates (glass and silicon) to ascertain whethernot only fixation chemicals, but also different materials'surfaces can influence their morphology.

MATERIALS AND METHODS

Cells and substrates

In this study, NIH 3T3 fibroblasts were used. Cells werecultured in Dulbecco's modified Eagle's medium (DMEM+GlutaMAX-I)supplemented with (i)1g/l D-Glucose pyruvate (ii)5%vol. of FCS (Foetal Calf Serum Invitrogen, Carlsbad, CA, USA) and (iii) 5µg/ml gentamicinand streptomycin (DMEM, Sigma Aldrich, Missouri, USA). Initially, cells were grown in flasks up to the 100% confluence. They were then detached with 1 mL of 1X trypsin prepared in PBS (Phosphate Buffered Saline, Sigma-Aldrich, Missouri, USA) and left for a few minutes at 37 °C. Cells were then harvested in 5 mL of complete DMEM and centrifuged at 1200 rpm for five minutes. This solution was re-suspended in a 5 mL volume of medium. Cells were plated on a $20x20mm^2$ substrate, at a concentration of $5x10^4$cells/cm^2 with 2 ml DMEM supplemented with 5% FCS. They were left to spread for 24 h. Concentration varied depending on the type of study to be carried out. Cells needed to be well isolated to analyse theirmorphologyand evaluate their geometrical dimensions with greater accuracy.

Experiments were performed on two different types of substrates: $20x20mm^2$glass cover slips (Marienfeld, Lauda-Königshofen, Germany) and $20x20mm^2$monocrystalline silicon samples (University Wafer, Boston, Ma., USA).

Living and fixed cells were studied on both substrates. For each experimental condition, 40 cells were tested (living and fixed).

Fixation

Chemical fixatives[2] are classified according to their action on proteins and subdivided into: (i) coagulants, (ii) non-coagulants. The most common coagulants are methanol, acetone, solutions of bi-chloride of mercury, acetic acid, tri-chloroacetic acid, picric acid, which act very rapidly, penetrating inside the cell, producing large masses of chromatin. The most common non-coagulants are, instead, formaldehyde and osmic acid, resulting in a very fine precipitation of proteins. These are also known as cross-linking.[5]. Organic solvents, such as alcohols and acetone, remove lipids and dehydrate cells, precipitating proteins on the cellular architecture. Cross-linking reagents (as paraformaldehyde) form intermolecular bridges, usually through free amino groups, thus creating a network of linked antigens.

For this work, cells were rinsed witha phosphate buffered saline solution (PBS - Sigma Aldrich, Missouri, USA) and then fixed with either:

1) 3.5% paraformaldehyde (Sigma Aldrich, Missouri,

USA) for 20 minutes at room temperature or
2) acetone (5 minutes) or
3) methanol (4 minutes)
Then washed twice in PBS and stored at 4 °C.

Atomic force microscope (AFM)

Single cells (Figure 1) were analysed usinga Solver-Pro M (NT-MDT, Moscow, Russia) atomic force microscope. Fixed and living cells were scanned in a contact mode. The AFM cantilever was equipped with a PNP-DB silicon nitride tip (Nanoworld, Neuchâtel, Switzerland). Scan time was 10 minutes for 100x100 ?m^2, witha resolution of 256x256 points and thus pixel size 0,4x0,4µm^2. The AFM was equipped with liquid scanning set-up and measurements were performed at room temperature.

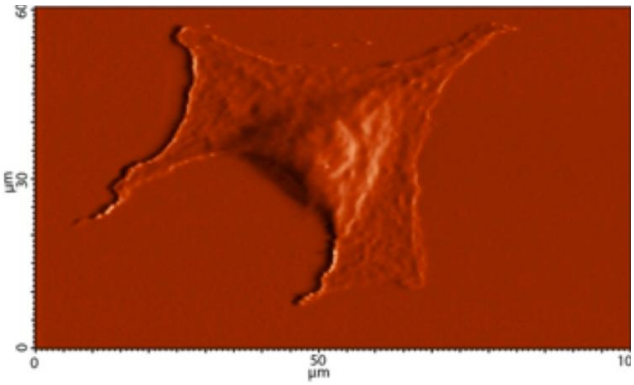

Figure 1 : AFM acquisition of 3T3 living cell on glass

Only well-spreadand isolated cellswere investigated. Those with a round shape and a dark edge were discharged. Living cells were kept in an incubator at 37 °C, but tested at room temperature.

With a Solver-Pro controller it is possible to have multiple types of measurements during a single scan. For the purposes of this study, the variation of displacement along the z-axishas been recorded during the outward and return scan. This way, two sets of measures wereanalysedfor every single cell.

Data analysis

We focused the analysis on themaximum cell height, perimeter, area, volumeand roughness respectively. Area and perimeter were calculated usingImageJsoftware (NIH, USA). In this case it was important to increase the contrast of the two-dimensional cell images, to emphasize the boundaries (see Figure 2 a, b, c). Volume was calculated directly with Nova (NT-MDT, Russia) AFM controlling software. Height images were also used to calculate the roughness of cell surface based on root mean square (RMS) values, i.e., the standard deviation of all the height values within the given area.

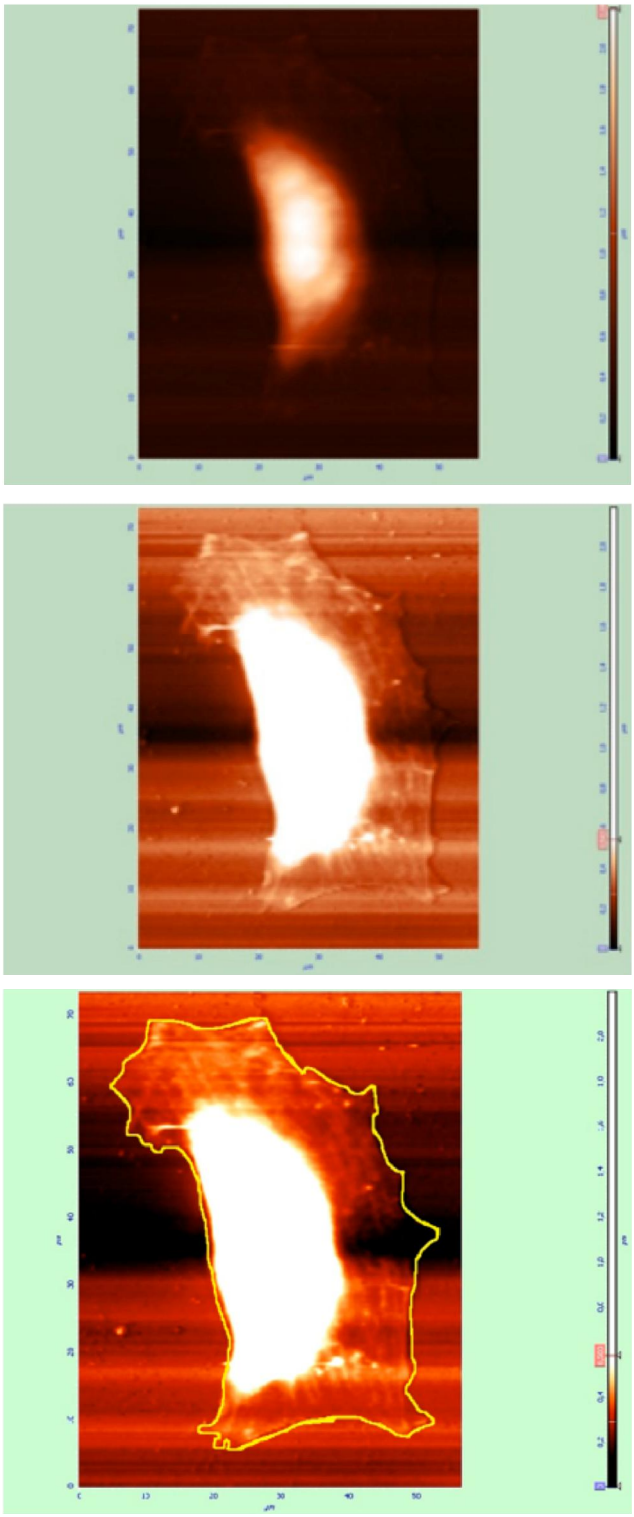

Figure 2 : (a) Living 3T3 cell on Silicon, scanned during outward AFM cantilever movement, (b) same cell with gain in contrast; (c) same cell with highlighted perimeter.

The calculated error (due to calibration plus op-

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erator) is around 2.5 % for area and perimeter, while for volume calculation (operator only) it is around 3%.

Data wereanalysed using R statistical software. They were schematically plotted as a quartiles graph. The boxplot is presented as a box, whose endpoints are the first quartile (i.e. the smallest observation (Q_1)) and third quartile (i.e. the largest observation (Q_3)), with a horizontal line corresponding to the second quartile (Q_2, median)[12]. The whiskers are placed between Q_1 and Q_3at a distance (interquartile distance)equal to 1.5 times the distance between Q_1 and Q_3. The spacing between the different parts of the box helps indicate the degree of dispersion (spread) and skewness in the data, and identify outliers. Data that fall outside the range [Q_1-1, 5(Q_1-Q_3), Q_3 +1.5(Q_1-Q_3)] are individually marked with dots and called outliers.

Qualitative assessments

Fixation and imaging artefactsmight affect the morphological analysis. Qualitative assessments were therefore considered when analysing AFM images. Fixation artefacts are causedby the presence of salt crystals, which might be due tothe washing buffer (PBS) andto drying[3]. However, all our measurements were done in solution and drying was not needed. Other artefacts are dueto inappropriate AFM operation or tip contamination, which might lead to inaccurate measurements and consequently interfere with the morphological analysis. In the present study, the contaminated AFM tip was cleanedafter every cell scan and replaced when image quality was poor. This way, salt crystals and contamination artefacts can be considered avoided, as far as we are concerned. Another situation occurs when material adheres to and is removed from the AFM tip or when the tip does not follow the cell surface's contour accurately. This could be due to the force applied to the tip, which makes it difficult for the tip itself to disengage from the sample. Even fixed chemicals may be exerting adhesive forces onto the tip, preventing it from disengaging. In this case, the AFM set up was adjusted, and the same sample area was re-scanned, until optimization was achieved.

RESULTS AND DISCUSSION

Glass and silicon substrates

In our experiments, fixed fibroblasts showed no statistical differences for all acquired conditions, when measured on glass or silicon substrates.

The boxplot of volume values' distribution for living and fixed cellsis shownin Figure 3 and a similar trend is recognizable for perimeter and area respectively. Tests on normal distribution were performed with no positive results.

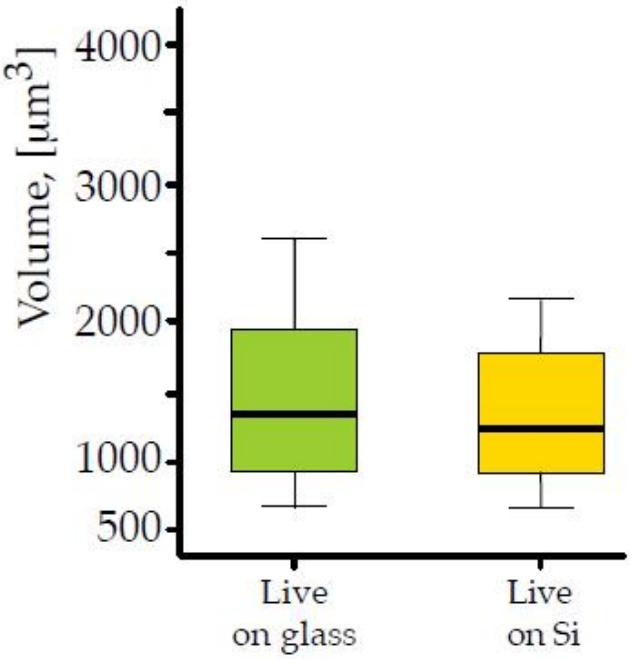

Figure 3 : Volume data for living cells on different substrates: glass and silicon

For living fibroblasts, qualitative results show that they are flattened and spread on both glass and silicon surfaces. However, if the maximum cell height is used as an index to reflect the "healthy" and adherentcell condition, the silicon/glass height ratio is H" 1.12 ± 0.09 suggesting that cells on silicon tend to be more rounded and therefore a little less prone to spreading.

Fixative chemicals

Figure 4 (a, b, c) shows the perimeter, area and volume data for the living and fixed cells respectively. We used the mediansfor a numerical comparison between samples, because the data are not normaland the average values do not reflect their true trend. If, for example, a group is considered, comprising many low-value data but two much larger data, outliers significantly influence the average value, while the median gives more weight to the homogeneous group.

TABLE 1 shows the data obtained as a ratio between the living and fixed cells for perimeter, area and

volume medians, respectively. Graphical methods, such as histograms and box plots, give just an indication of data distribution. To find out if data follow a normal distribution, seen as Gaussian, the Shapiro-Wilk test was used[16]. The values of analysedcells did not follow a normal distribution, however, and this result is in agreement with what was expected: the shape of a given cell is highly variable and very sensitive to environmental conditions, making it difficult to have a normal distribution.

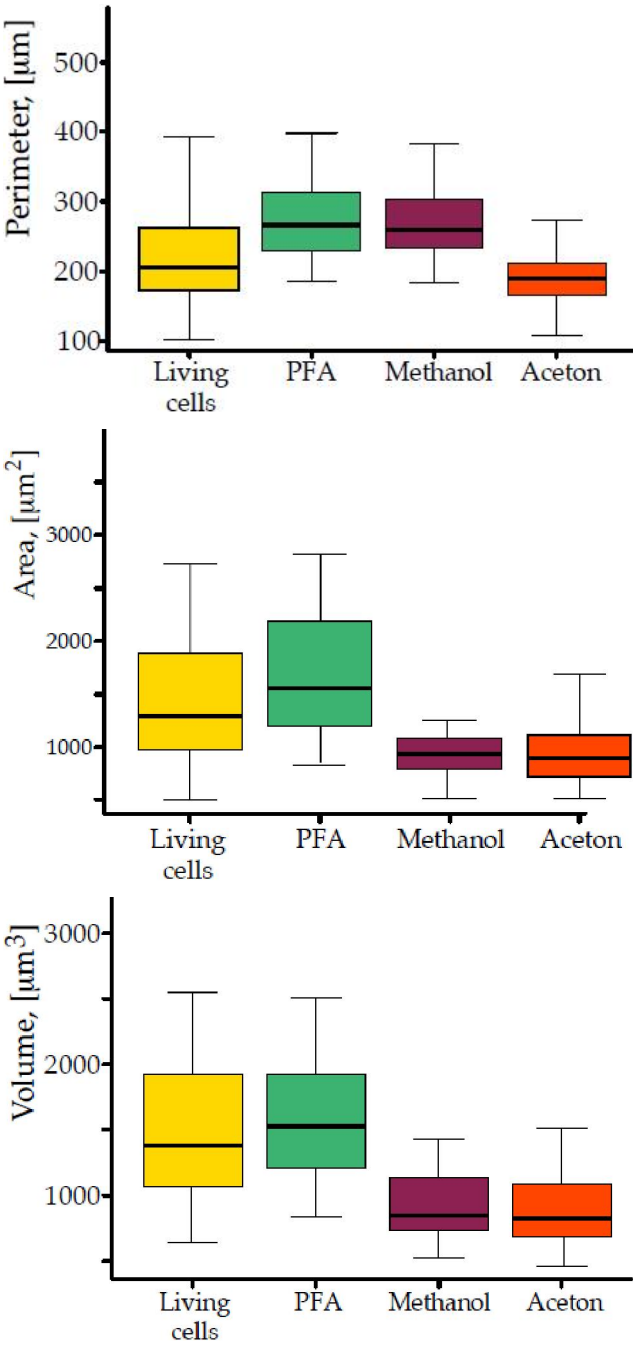

Figure 4 : (a) perimeter data for living and fixed cells. (b) area data for living and fixed cells, (c) volume data for living and fixed cells

TABLE 1 : Perimeter, area and volume data as ratio between different chemical fixative and living 3T3 fibroblasts

Perimeter	Area	Volume
$P_{PFA}/P_l=1.12$	$A_{PFA}/A_l=1.08$	$V_{PFA}/V_l=1.10$
$P_M/P_l=1.09$	$A_M/A_l=0.66$	$V_M/V_l=0.63$
$P_A/P_l=0.87$	$A_A/A_l=0.73$	$V_A/V_l=0.68$

The subscript "PFA" stands for Paraformaldehyde, "l" for living cells, "M" for Methanol and "A" for Acetone.

Living cells

The data distribution for living cells shows higher spread compared to those of fixed cells. The living cells' volume values distribution is reported in Figure 5, where data for living cells on glass andon silicon are shown. Data dispersion is mainly due to environmental conditions. If cells' morphology was related to biological variety only, then the same data dispersion would also be found in the fixed cells data. This result shows the strong influence of temperature during the acquiring procedure:while the AFM scan was performed, the temperature of the cell culture mediumdecreasedfrom 37 °C to room temperature and living cells therefore slightly contracted. Small temperature variations during testing, instead, didnot affect fixed cells and thedata distribution is narrow (Figure 4a, b, c). This is the reason why in TABLE 1 the entire ratio values for paraformaldehyde (perimeter, area, volume) are larger than 1 when compared to living cells.

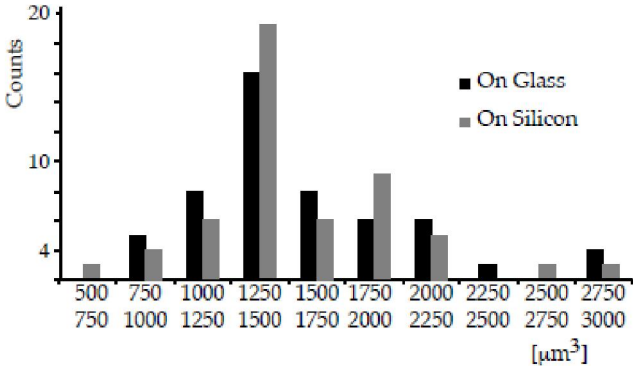

Figure 5 : Volume value distribution for living cells on different substrates

Paraformaldehyde

The fixation methods applying PFA showed good preservation ability for cell morphology, from qualitative and quantitative observations. In general, it is known that PFA fixation performs better than alcohols, since aldehydes fix cell by forming covalent chemical bonds between proteins, thus maintaining the integrity of membrane lipids as well as the surface macromolecules[7].

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From the data presented here, fibroblasts fixed with paraformaldehyde are slightly larger than the living cells. This could be related to the environmental sensitivity of living cells to temperature. On this basis, it can be concluded that cells fixed with paraformaldehyde could replace living cells in several experiments from a morphological point of view.

Methanol and acetone

From the fibroblasts analysedafter fixation with ei ther methanol or acetone, we noted that they werealways smaller than the living cells. However, while data for area and volume weresimilar (see TABLE 1), the perimeter showed an interesting result: cells fixed with methanol showed a larger perimeter than those fixed with acetone and almost comparable to that of the paraformaldehyde fixed cells. Figure 6 (a, b, c) shows the box plots only for methanol and paraformaldehyde, while the ratios for volume and area are 1.75 and 1.65 respectively, for perimeter it is only 1.02.

This can be due to considerable cell membrane damage. Alcohols, such as methanol, have indeed been shown to preserve nucleic acids, yet having strongly denaturing effects on protein[13]. To confirm this hypothesis, another two testswere carried out: (i) roughness cell assessment and (ii)Western blot.

Fibroblasts surface roughness measured in terms of the Root Mean Square (RMS = roughness average) was evaluated. Results showed that the fixed cells have anaccentuated roughness compared to living cells. RMS was the lowestfor living cells, varying between 7.9 and 20.0 nm. PFAand acetone RMS roughness values fell into similar ranges - i.e. 8.7–20.0 and 10.5 – 22.0 nm respectively. However, roughness was in a higher range when methanol was used, varying from 23 to 41 nm.

Our results strongly suggest that the fixation methods can significantly affect fibroblasts' morphology as well as their surface characteristics. The increase in cell surface roughness suggests damage to cell membrane, andmethanol fixation indeed dissolves some of the membrane lipids, creating large pores in the cell surface[18].

If cell membrane damage does occur, substantial amounts of Bcl-2 proteins will be detected by a Western blot test carried out on PBS medium, since Bcl-2 is mainly associated with membranous structures. Indeed, the amounts of Bcl-2 recovered afterPFA and acetone fixation were substantially lower than the amounts recovered after methanol fixation, as in previous stud-

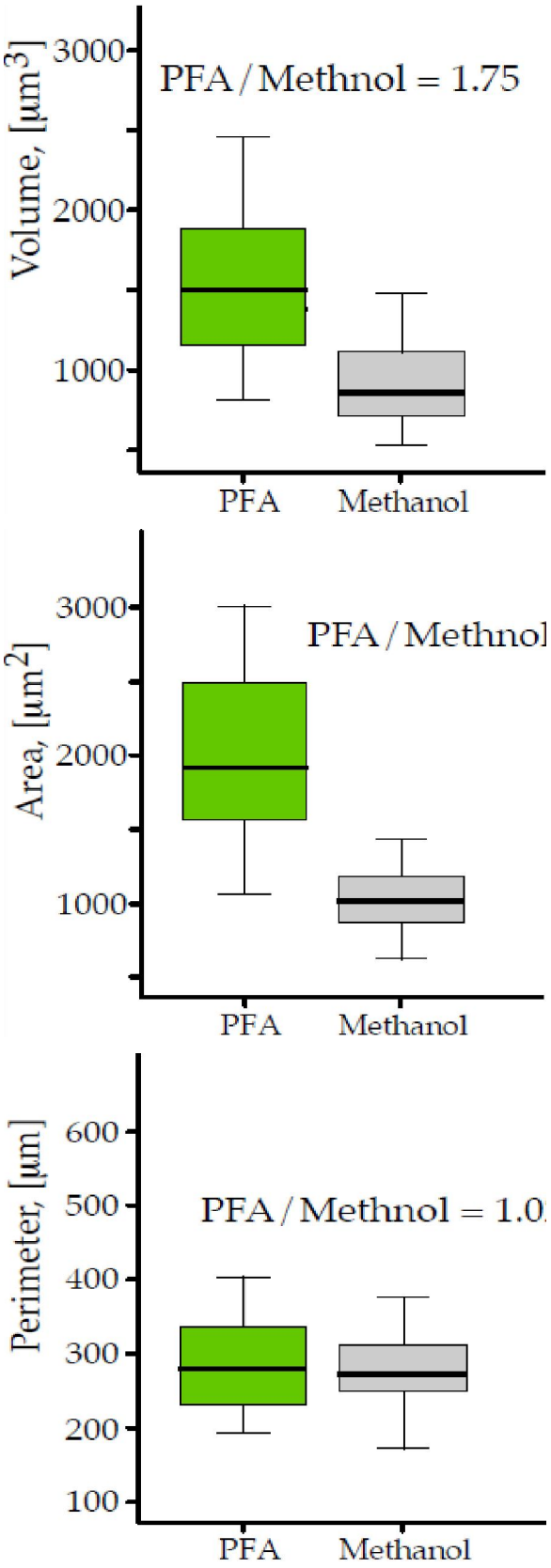

Figure 6 : (a) volume data comparison between PFA and methanol fixed cells, (b) area data comparison between PFA and methanol fixed cells, (c) perimeter data comparison between PFA and methanol fixed cells

ies[2]. This finding suggests that for Bcl-2, methanol has a greater extraction capacity than PFA and acetone, leaving numerous holes in the cells' membranes. Virtually no Bcl-2 seemed to be lost using PFA fixation.

CONCLUSIONS

Fixed NIH 3T3 fibroblasts morphology showed no statistical differences for all acquired conditionswhen they were measured on glass or silicon substrates. Living fibroblasts were flattened and spread on both glass and silicon surfaces. However, when the maximum cell height is used as an index, fibroblasts on silicon tend to be more rounded and therefore a little less prone to spreading.

To evaluate comprehensively severalfixation methods for fibroblasts and determine which was the most promising method for morphological studies, quantitative assessments were also conducted. Quantitative assessments were based on morphology preservation, comparing height, perimeter, area and volume of NIH 3T3 fibroblasts fixed using different methods. Data variability for fixed cells could be attributed to the biological nature of samples. For living cells, instead, environment conditions (such as temperature) heavily affect measurements. These results point out how important it is to work at precise and controlled environment conditions when working with living cells.

Paraformaldehyde-fixed cells show no major morphological changes in their shape or size when compared to living cells. Methanol- and acetone-fixed cells, on the other hand, tend to look shrunken and smaller in size, with the cells' outer membrane appearing uneven. Using methanol, the fibroblasts'shape was less symmetric compared to the PFA and even acetone-fixed cells. AFM roughness data revealed additional information on the integrity of the fibroblasts' membrane. A change in the cell surface morphology was apparent, especially if the cells' roughness was taken into account, and also in this respect PFA-fixed cells displayed more intact membranes. A Western blot test confirmed that proteins were presents in the medium after methanol fixation, suggesting larger cell membrane damage compared to the other two fixatives. From our data, paraformaldehyde appears to be the best fixative to optimally assess morphological data in the case of NIH 3T3 fibroblasts.

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