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, especially for the characterization of both morphological and mechanical properties. In this study, we investigated the influence of different fixation reagents on NIH 3T3 fibroblast cells’ morphology. Three protocols were 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.

INTRODUCTION

Cell biology research presupposes accurate knowledge of cell morphology. 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 of cell activity. In this respect, working with living cells would be of paramount importance. However, lack of available data on the properties of living cells makes the use of fixed cells inevitable. The aim of fixation should be to preserve the molecular arrangement as much as possible as found in vivo. Although even the most careful fixation does alter the cytoplasmic structure and introduce artefacts 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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In this study, NIH 3T3 fibroblasts were used. Cells were cultured 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 gentamicin and 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 20x20mm² substrate, at a concentration of 5x10⁴ cells/cm² 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 their morphology and evaluate their geometrical dimensions with greater accuracy.

Experiments were performed on two different types of substrates: 20x20mm² glass cover slips (Marienfeld, Lauda-Königshofen, Germany) and 20x20mm² 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 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. 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 with 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.

Only well-spread and isolated cells were 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-axis has been recorded during the outward and return scan. This way, two sets of measures were analysed for every single cell.

Data analysis

We focused the analysis on the maximum cell height, perimeter, area, volume and roughness respectively. Area and perimeter were calculated using ImageJ software (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.

Atomic force microscope (AFM)

Single cells (Figure 1) were analysed using a 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 ?m², with a resolution of 256x256 points and thus pixel size 0,4x0,4μm². 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

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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 were analysed 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)$^{112}$. The whiskers are placed between $Q_1$ and $Q_3$ at 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 $[Q_1-1.5(Q_3-Q_1), Q_3+1.5(Q_3-Q_1)]$ are individually marked with dots and called outliers.

Qualitative assessments

Fixation and imaging artefacts might affect the morphological analysis. Qualitative assessments were therefore considered when analysing AFM images. Fixation artefacts are caused by the presence of salt crystals, which might be due to the washing buffer (PBS) and to drying$^3$. However, all our measurements were done in solution and drying was not needed. Other artefacts are due to 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 cleaned after 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 cells shown in 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](image)

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 adherent cell condition, the silicon/glass height ratio is $H$ $1.12 \pm 0.09$ 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 medians for a numerical comparison between samples, because the data are not normal and 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\cite{16}. The values of analysed cells 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](image1)

**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

<table>
<thead>
<tr>
<th>Perimeter</th>
<th>Area</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{PFA}/P_l = 1.12$</td>
<td>$A_{PFA}/A_l = 1.08$</td>
<td>$V_{PFA}/V_l = 1.10$</td>
</tr>
<tr>
<td>$P_{M}/P_l = 1.09$</td>
<td>$A_{M}/A_l = 0.66$</td>
<td>$V_{M}/V_l = 0.63$</td>
</tr>
<tr>
<td>$P_{A}/P_l = 0.87$</td>
<td>$A_{A}/A_l = 0.73$</td>
<td>$V_{A}/V_l = 0.68$</td>
</tr>
</tbody>
</table>

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 and on 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 medium decreased from 37 °C to room temperature and living cells therefore slightly contracted. Small temperature variations during testing, instead, did not affect fixed cells and the data 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](image2)

**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\cite{7}. 
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 analysed after fixation with either methanol or acetone, we noted that they were always smaller than the living cells. However, while data for area and volume were similar (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 tests were 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 an accentuated roughness compared to living cells. RMS was the lowest for living cells, varying between 7.9 and 20.0 nm. PFA and 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, and methanol 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 after PFA and acetone fixation were substantially lower than the amounts recovered after methanol fixation, as in previous stud-
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 conditions when 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 several fixation 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 present 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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REFERENCES


