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An evaluation of fixation methods: Spatial and compositional cellular changes observed by Raman imaging

Alison J. Hobro*, Nicholas I. Smith

Biophotonics Laboratory, Immunology Frontier Research Center, Osaka University, 3-1 Yamada-Oka, Suita City, Osaka 565-0871, Japan

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ABSTRACT

Although live cell imaging is desirable, it is not always feasible and in many situations cells are fixed in order to provide a 'snapshot' of the nature and distribution of molecules within a cell while minimising changes from cell movement, sample degradation etc. There is a wide range of fixation methods available that act via different mechanisms, and on different cell components. Each method has advantages and disadvantages and a choice of what fixation method to choose for a particular experiment needs to take these factors into consideration. Here we used Raman spectroscopic imaging of live cells, and compared with cells preserved with aldehyde, or organic solvent-based fixation methods to assess the chemical changes induced by each fixative, and their impact on the quality of images that can be obtained from fixed cells. Overall, aldehyde fixation methods performed significantly better than organic solvents with less severe loss of biochemical information. Aldehyde based fixatives show an altered biochemical content of the cells, attributed to adduct formation, but this can be minimised by optimising fixation temperature, or through removal of adduct formation by detergent-based permeabilization treatments as a second step (at the cost of the loss of other biochemical information). The results showed that organic solvents, on the other hand, lead to a severe loss of cell content, attributed to the loss of membrane integrity after the removal of lipids. Additionally, fixation with aldehydes prior to permeabilization with organic solvents does not provide adequate protection of cytoplasmic content. The use of Raman imaging is ideal for comparing groups of cells in terms of their molecular content, and the results show that aldehyde fixations methods are preferable for studies where the overall molecular content of the samples is important. Although there is no universal fixation method for every application, the results here allow us to provide a few general principles: where spectral similarity to live cells is important, fixation with paraformaldehyde at room temperature is preferable, at the cost of some blebbing and vacuole formation. Where preservation of cellular structure or biomolecular distribution is important, a mix of paraformaldehyde and glutaraldehyde would be more appropriate, but at the cost of some changes to spectral profile, particularly in DNA-related bands.

1. Introduction

Understanding the processes in a cell is an important step in many aspects of cell biology and medicine. Knowledge of normal cell metabolism, cell response to environmental factors and eventual cell aging and death can provide insights into how the entire organism functions. More importantly, information on how and when these normal processes go wrong is fundamental for the understanding of disease, the development of preventative measures, such as vaccines, and the progress towards more effective treatments and cures. Although the ideal situation would be to obtain information on the composition and distribution of biomolecules from measurements of living cells, in real time, in a label-free manner, there are very few techniques that can achieve this. Instead for many applications cell fixation is routinely employed to allow a snapshot of a cell state to be measured. There are many reasons why a fixation step might be needed. (1) The preparation steps required for a particular technique may be very harsh or impossible to perform on a live cell. Transmission electron microscopy, for example, requires dehydration, staining, embedding in resin and sectioning of cells prior to measurement. (2) A technique might require the removal of certain cell components for the analysis to work. Immunostaining using primary and secondary antibodies for subsequent fluorescence imaging requires the cell membrane to be disrupted to allow the antibodies access to their targets within the cell. This usually

* Corresponding author.
E-mail address: ajhobro@ifrec.osaka-u.ac.jp (A.J. Hobro).

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requires fixation and permeabilization, i.e. the creation of holes in the cell membrane. (3) There may be a considerable time between the collection of the sample and the time when it is analysed. This is particularly common in a clinical setting where cell smears may be taken during a doctor's surgery visit, or during a surgical procedure, that are then sent to a different location to be examined by a pathologist in the following hours, days or weeks. The provision of cell samples in tissue banks also requires effective fixation of samples as there could be months or years between sample collection and analysis. (4) A sample is to be measured by more than one technique. Unless measurements can be taken simultaneously in a multimodal system, fixation is necessary to ensure no cell movement or degradation between the subsequent measurements. This is particularly important when performing correlative imaging as any slight movement in cell position, or cell contents, will affect the ability to match up different images. (5) The cell phenomenon of interest occurs on a shorter timescale than the measurement can be performed. As an example, Raman imaging of a whole cell can currently be achieved in a few minutes. Phenomena occurring faster than this timescale, such as the action of molecular motors (moving at approximately 1 μm/s) or the initial stages of clatherin mediated endocytosis (occurring in approximately 1 min) [1], would then be blurred. Although fixation does not improve the ability to resolve time-varying phenomena, it can prevent the inherent blur from events that occur during the measurement process.

Fixation methods can be classed into four main groups either by their chemical nature; aldehydes, alcohols, oxidising agents and metallic fixatives [2], or by their action; cross-linking, dehydration, the effects of heat, or the effects of acid [3]. Several researchers who have evaluated fixation methods for particular cells and tissues have noted that the choice of fixation method is often a personal choice, or down to the convenience of a particular method, rather than because of the preservation characteristics of the fixative [4,5]. However, there is not a 'one size fits all' method and some fixatives are more effective at preserving particular cell constituents than others. In this paper we have concentrated on aldehyde-based methods, which act as cross-linking agents, and organic solvents, which act by precipitating proteins, as these two groups are commonly used in cell biology, immunology and medicine.

1.1. Aldehydes

Aldehyde-based fixatives, including formaldehyde (also referred to as formalin when in its liquid form [6]), paraformaldehyde and glutaraldehyde, act as cross-linking agents that react with proteins and nucleic acids in the cell [3,4]. Formaldehyde, in particular, is a widely used fixation method that results in low levels of shrinkage and good preservation of cellular structure [2] for a wide range of cells and tissues [3] and does not appear to result in significant structural changes to proteins [7]. However, there are indications that formaldehyde can result in blebbing-like effects on the cell membrane as well as indentations and vacuoles forming on or close to the nuclear and mitochondrial membranes [8]. Although all aldehyde fixatives act via crosslinking the extent and nature of the crosslinking can influence the effectiveness of the fixation, for example, glutaraldehyde has been shown to be more effective at preserving high-molecular weight DNA than formaldehyde [3] but produces much poorer immunoreactivity during immunostaining [9].

1.2. Organic solvents

Organic solvents such as methanol, acetone and ethanol preserve cells through a process of dehydration and precipitation of proteins [4,10]. These fixatives have been shown to be more effective at preserving nucleic acid content in cells than aldehyde-based fixatives are [3,11,12], but at the cost of cell membrane structure, the loss of cytoplasmic organelles and nuclear content along with damage to structural elements such as microtubules [5,10]. However, methanol in particular, is often used during immunostaining as it can provide high levels of immunostaining coupled with low background, or non-specific, staining [13].

1.3. Permeabilization

Cell permeabilization is the process by which the cell membrane is made more porous than normal to allow relatively large molecules, such as dyes or antibodies that would normally be excluded by the cell membrane, to pass into the cytoplasm and reach intracellular targets [14]. Organic solvents do this by removing lipids from the cell membrane (and have the added advantage that they also simultaneously fix cells, thereby only requiring a one-step process). Lipids and cholesterol are also removed from the cell membrane by the action of detergents such as Triton X-100, Tween 20 and Saponin [14]. When using aldehyde-based fixation methods permeabilization is performed as a second step and there is some evidence that this two-step fixation-permeabilization process, such as paraformaldehyde fixation followed by methanol permeabilization, can be particularly effective in allowing access to intracellular molecules while limiting some of the cellular damage that can occur when using methanol as a single fixation-permeablization step [5].

1.4. Fixation and Raman

The effects of some common fixation methods on the Raman spectra of biological materials have mainly focussed on tissues with only a few papers concentrating on the effects in cells, although much of the information gleaned is applicable to both. Many of the tissue-based studies have looked at the effects of formaldehyde fixation compared to unprocessed tissue and have identified the loss of carotenoids, lipids and cholesterol as well as changes in protein conformation that have been attributed to the cross-linking action of the formaldehyde [15,16] although the nuclei appear to be well preserved [17]. Coherent anti-stokes Raman studies have also found spectral differences in fixed versus unprocessed tissues, with methanol-acetone fixation having a dramatic impact on the lipid content of tissues [18]. Cell-based studies have concentrated on a small number of common fixation methods: methanol, ethanol, formalin/formaldehyde/paraformaldehyde, air-drying, cytocentrifuging, Carnoy's solution or combinations of these [19–24]. Some of these studies indicated significant morphological changes to the cells when air-dried [19] whereas others did not [20] but in both cases spectral variations between air dried and other cell treatments were apparent [19,20]. Formaldehyde fixation appears to produce minimal spectral changes compared to live cells although some changes to lipid based vibrations are observed [19,22–24]. Dehydration of cells through ethanol fixation results in more distinct changes in protein bands, something which has been attributed to the unfolding of proteins during the dehydration process [22], along with changes in the distribution of proteins and lipids within the cell [21]. The spontaneous Raman analyses in these previous studies are based on single point measurements of cells [20–24] or on raster scanning of cells producing relatively low spatial resolution images [19].

In this paper we present Raman images obtained for a wide range of aldehyde and organic solvent based fixation methods and, using principle component analysis, assess the effects of these fixation methods on both Raman spectral profile and image quality.

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Paraformaldehyde (closely related to formaldehyde/formalin solutions but without the addition of methanol as a stabilizer [6]) and glutaraldehyde were selected as 'pure' aldehydes as they are commonly used as fixation methods but have different rates of crosslinking due to the different numbers of aldehyde groups available [6]. As for many fixation methods, combinations of more than one agent are often used in an attempt to exploit the useful properties of both, or in an attempt to use one agent to mitigate the undesirable action of another [3]. A formaldehyde and glutaraldehyde mix therefore exploits the different crosslinking properties of the two molecules and is a common fixative for transmission electron microscopy. The 'pure' organic solvents selected for this study were methanol, ethanol and acetone, which have been suggested to be good fixatives for nucleic acid components of cells [3] and for subsequent immunostaining [13], although the additional action of cell permeabilization may have marked detrimental effects on the cell structure [5,10], and lipids in particular are significantly affected by the use of organic solvents [14,25]. Again, mixtures of organic solvents, methanol:ethanol, methanol:acetone and Carnoy's solution as one-step procedures, and methanol with a subsequent acetone wash as a two-step procedure were chosen as examples of mixtures of organic solvents in order to assess if the potentially undesirable consequences of single fixative agents are lessened or increased by their combinations. Finally, as two-step fixation-permeabilization procedures are also commonly used in cell biology we assessed the effects of an organic solvent based permeabilization agent, methanol, and one detergent based agent, Triton X-100, on cells previously fixed with paraformaldehyde.

2. Materials and methods

2.1. Sample preparation

Spontaneously immortalized wild-type mouse embryonic fibroblasts (MEF) were kindly donated by Dr. S. Akira (Osaka University, Japan) and cultured in Dulbecco's modified eagle medium (DMEM, Nacalai Tesque, Japan) cell culture media supplemented with 10% foetal bovine serum (FBS, Gibco, Japan) and 1% Penicillin-streptomycin (Sigma-Aldrich, Japan). Cells were plated onto poly-L-lysine (Sigma-Aldrich, Japan) coated quartz bottomed culture dishes (FPI, Japan) and incubated at 37 °C, 5% CO_2, for at least 18 h prior to measurement/fixation to allow cells time to adhere to the quartz substrate. For live cell imaging, cells were rinsed twice with PBS (Nacalai Tesque, Japan) at 37 °C and then covered with 2 mL of PBS at 37 °C before being transferred to the Raman microscope for immediate measurement. For fixed cell imaging, cells were rinsed twice with PBS at 37 °C before fixation (see Table 1 for the parameters for each fixation method). All chemicals used for fixation; methanol, acetone, ethanol, acetic acid, chloroform, Triton ×100, formaldehyde, paraformaldehyde and glutaraldehyde were purchased from Sigma-Aldrich, Japan. The aldehydes and Triton were diluted to the appropriate concentrations in PBS. After fixation, cells were rinsed three times with PBS at 4 °C and stored at 4 °C for 1 or 24 h. Cells were removed from the fridge and allowed to warm to room temperature for five minutes before being transferred to the Raman microscope to avoid condensation forming on the culture dish during measurement.

2.2. Raman spectroscopy

Raman spectra were recorded on a Raman-11 spectrometer (Nanophoton, Japan) operating in line scanning mode (pseudo-line mode, covering a distance of approximately 133.4 μm), using 532 nm excitation. Both live and fixed cells, immersed in PBS, were imaged using 5 s per line and a laser power of approximately 180 mW at the sample using a CFI Apo 60x NIR water immersion objective with a numerical aperture of 1.00 and a working distance of 2.8 mm (Nikon, Japan). 180 mW excitation power was selected for all measurements as live cells are still viable after Raman spectral collection using these parameters. Fixed cells were also measured at the same laser power in order to allow direct comparison for this study. Measurements at higher laser powers should be possible for fixed cells if desired, an upper limit for fixed cells was not investigated in this study. Spectra were projected onto a PIXIS 400 (Princeton Instruments, USA) camera with a 1340 × 400 pixel array with 20 × 20 μm pixels, via a spectrograph employing a 600 g/mm grating, resulting in a spectral range of 530–2981 cm^{-1}. The slit width was 60 μm. The detector readout speed was 2 MHz and operated using high gain. Pixels in the recorded images are 335.1 (height) × 335.6 (width) nm and each image has a fixed width of 400 pixels (total width 1334.4 μm) while the height is defined by the user (as a number of lines, hence the final image will be a multiple of 335.1 μm). Depending on the size of the cell, individual images took between 10 and 25 min to collect.

2.3. Data processing and principal component analysis (PCA)

Averaged Raman spectra were obtained by extracting regions of 15 × 15 pixels, as a single averaged spectrum, from random regions

Table 1
Parameters for cell fixation.

Preparation name	First treatment			Second treatment (where applicable)		
	Chemical composition	Length of exposure (minutes)	Temperature (°C)	Chemical composition	Length of exposure (minutes)	Temperature (°C)
PFA 4	4% Paraformaldehyde in PBS	10	4			
PFA room	4% Paraformaldehyde in PBS	10	22			
Gluteraldehyde	2.5% Gluteraldehyde in PBS	10	4			
Formaldehyde & Gluteraldehyde	2% Formaldehyde, 2.5% Gluteraldehyde in PBS	10	4			
PFA Triton	4% Paraformaldehyde in PBS	10	4	0.5% Triton	5	4
PFA methanol	4% Paraformaldehyde in PBS	10	4	Methanol	5	−20
Methanol	Methanol	10	−20			
Acetone	Acetone	10	−20			
Ethanol	95% Ethanol, 5% Acetic Acid	10	4			
Methanol:Acetone	50% Methanol, 50% Acetone	10	−20			
Methanol:Ethanol	50% Methanol, 50% Acetone	10	−20			
Methanol Acetone wash	Methanol	10	−20	Acetone	1	−20
Carnoy's Solution	60% Ethanol, 30% Chloroform, 10% Acetic Acid	10	4			

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within each cell analysed. In all cases, 3 regions of the nucleus and 5 regions of the cytoplasm were selected and the process carried out in the Raman-11 data viewer (Nanophoton, Japan). Data pre-processing and principal component analysis (PCA) were performed using the Eigenvector PLS toolbox (Version 7.5, Eigenvector Research Inc., USA). The averaged spectra were baseline corrected (4th order weighted least squares), the spectral regions that were not of interest removed (1810–2799 cm^{-1} and 2973–2981 cm^{-1}) and smoothed (Savitzky-Golay, 5 point window, zero order and no derivative). PCA was performed on mean centred data and cross-validated using random subsets with 10 data splits and 5 iterations.

Raman images were cropped, using the Raman-11 data viewer, to remove excess regions of the image where cells were not present. No pixel binning or aspect ratio alterations were made during this process. Due to the size of the images all preprocessing steps were carried out using the Eigenvector PLS Toolbox accessed via the Matlab (version R2010b, Mathworks, USA) command line rather than via the graphical user interface. In the spectral domain, images were baseline corrected, the spectral regions cropped and smoothed in the same manner as for the averaged extracted spectra. In order to remove any spikes due to cosmic rays in the image, an additional step of filtering the spectra in the image domain (box-filter, 3 point window) was also applied. This box filter has the effect of smoothing in the image domain. After this, images were concatenated using the Eigenvector MIA toolbox (Version 2.8, Eigenvector Inc., USA) to form the combined images used for PCA. (The PCA results in Figs. 1 and 2 were based on images from a single treatment for each analysis, i.e. the PCA was performed on an image created from the five images obtained for each treatment method, and the different treatment methods were analysed separately providing independent information on the effects of each treatment method on the cell composition and biochemical distribution. The PCA results in Figs. 3 and 5 are from images containing both the five live cell images and the five images obtained from a single fixation method. This provides information on the biochemical composition and spatial distribution of biochemical in the fixed cells, relative to the information that is also present in the live cells). The data from the concatenated images was mean centered prior to PCA (meaning the first principle component reflects spectral variation rather than spectral intensity). PC1 and cross validated using random subsets with 10 data splits and 5 iterations. Again, due to the size of the images analysed, PCA and cross validation were carried out by accessing the Eigenvector Toolbox routines 'pca' and 'crossval' via the Matlab command line.

PCA scores images were imported into ImageJ as individual images (negative PC scores were imported as an inverted image) and converted to a stack where each layer was assigned a different colour. The colour contrast was adjusted so that contrast from the cells was still visible but the background did not exhibit strong intensity. For the combined images shown in Fig. 3 scores plots from all samples were concatenated in the MIA-toolbox before importing into ImageJ to produce the overlaid images. The overlaid images have then been split into three separate images for presentation purposes. The original PCA scores plots for each analysis are shown in the Supplementary information (SI 1 and SI 2).

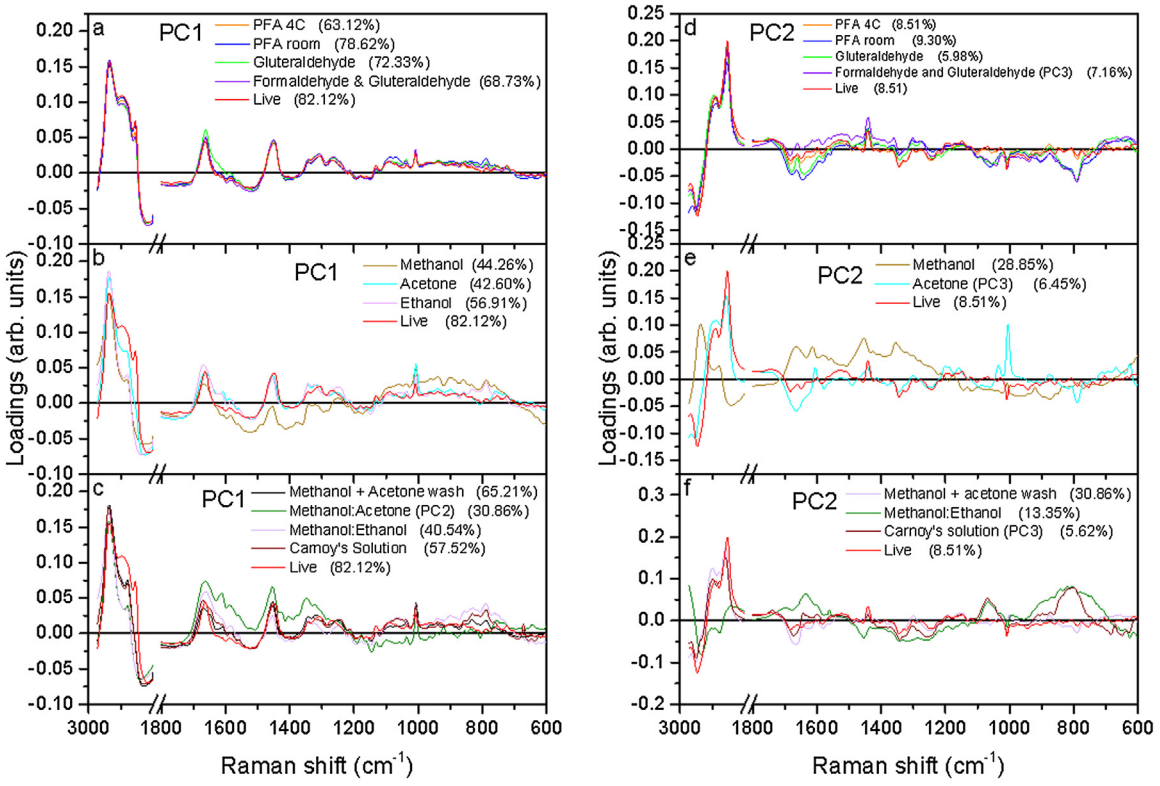

Fig. 1. Loadings for PCA analyses for live cells and all fixation methods analysed independently. Loadings have been grouped into (a and d) aldehyde based, (b and e) 'pure' organic solvent based and (c and f) mixed organic solvent based fixation methods. Loadings from live cells are shown in each panel (PC1 in panels (a–c) and PC2 in panels (d–f)) for ease of comparison to loadings obtained from fixed cells. The loadings from fixed cells have been grouped together based on their spectral similarity to the loadings from live cells with panels (a–c) showing mainly PC1 loadings and (d–f) showing selected PC2 and PC3 loadings from fixed cells (for completeness all PCs, including those not discussed in the text, are included in SI 1). These are discussed further in the text. The percentage variance captured for each PC is given in brackets in the key. Band positions are noted only for where the spectral profile differs between live and fixed cells.

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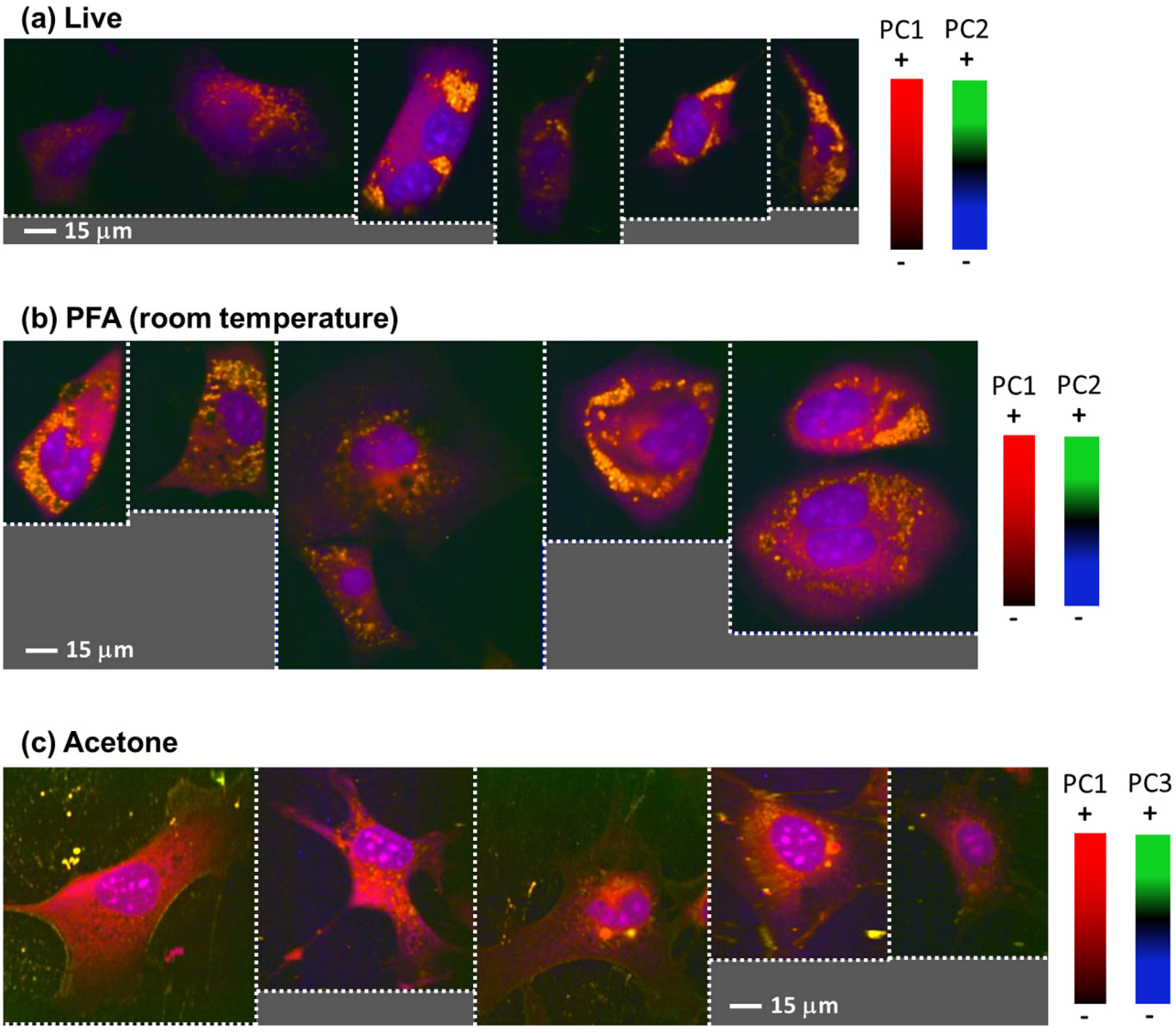

Fig. 2. Example PCA scores plots shown as overlaid images for (a) live (PC1 82.12%, PC2 8.51%), (b) PFA room temperature (PC1 78.62%, PC2 9.3%) and (c) acetone (PC142.6%, PC2 9.95%) fixed cells corresponding to the loadings plots in Fig. 1. Scores plots for all other fixation methods are shown as single component images in Supplementary information (SI 1). Dotted white lines show the boundaries of each image and the grey areas show where no image information is present as a result of differing image sizes. The scale bar represents 15 μM. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)

3. Results

3.1. Fixation alters the Raman spectral profile and image quality from that of live cells

In live and in all fixed cells the greatest amount of variation was in PC1, which is heavily influenced by the spectral differences between the cells and the background regions in the images. (The single exception to this is Methanol:Acetone where PC1 is dominated by the effects of background differences between the five images analysed (see SI 1) and, instead, the cell versus background information is described by PC2). Fig. 1(a–c) indicates that a notable proportion of this variation is attributable to lipid vibrations as shown by the intense bands at 1450 and 1662 cm^{-1}, as well as in the high wavenumber region between 2800 and 3000 cm^{-1} [26–29]. In addition, bands from other biomolecules including Amide III protein vibrations at 1341, 1308 and 1265 cm^{-1}, amino acids particularly phenylalanine (with potential contributions from other molecules including tryptophan) at 1006 cm^{-1} and, potentially, carbohydrate or nucleic acid based vibrations in the 1000–1200 cm^{-1} region are also present [29,30]. The scores plots for PC1 for each method investigated (SI 1) show that, with a few exceptions which will be discussed below, PC1 is found throughout the cytoplasm and in the nucleoli, although the rest of the nucleus usually does not reflect high scores for PC1. The highest scores for PC1 are found in the cytoplasm close to the nucleus, often with small dots forming the regions of highest PC1 intensity. Given the general nature of the spectral profile for PC1 it is not easy to assign this to a specific organelle or component of the cell, rather these regions of high intensity are likely to reflect lipid-rich regions of the cell which may be lipid-droplets, or vesicles including exosomes, endosomes and lysosomes which would contain a high proportion of lipids in their membranes.

Although the loadings vector profiles of the PC1 loadings vectors are broadly similar there are noticeable differences compared to that of live cells for all fixation methods studied. The closest loadings vector profiles to live cells are found when using aldehyde-based fixation methods; PFA, gluteraldehyde and a formaldehyde/glutaraldehyde mix (Fig. 1a). Only a few differences are observed in the fingerprint region, and the changes in intensity

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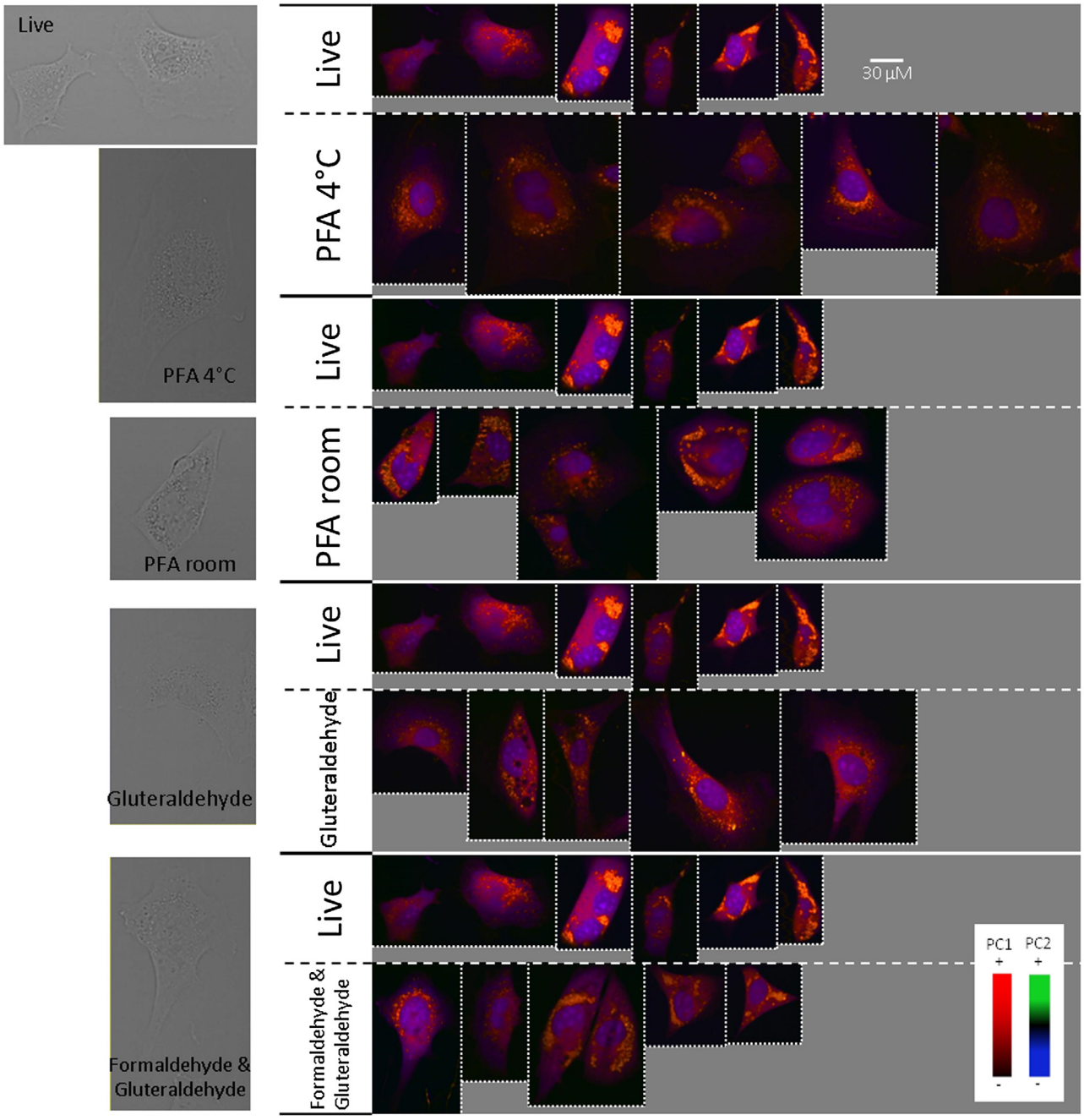

Fig. 3. PCA scores plots shown as overlaid images for all fixative methods when compared directly to live cells. For each fixation method PCA was performed on a combined image containing five live and five fixed cell images. Original scores plots are shown in SI 2. Resulting scores plots were then concatenated to produce a combined image for the overlay and false colour steps to ensure the contrast for each layer was applied uniformly for each fixation method. PC1 positive scores are shown in red, PC2 positive scores are shown in green and PC2 negative scores shown in blue. For methanol and methanol:acetone, where PC2 was affected by differences in background between different images (SI 2), PC3 scores are shown instead (positive in green and negative in blue). Percentage variances are as follows: PFA 4 °C (PC1 74.38, PC2 7.91), PFA room temperature (PC1 80.17, PC2 8.88), Gluteraldehyde (PC1 77.42, PC2 7.36), Formaldehyde & Gluteraldehyde (PC1 75.83, PC2 8.15), Methanol (67.83, PC3 8.38), Acetone (PC1 75.72, PC2 8.09), Ethanol (PC1 75.58, PC2 9.33), Methanol:Acetone (PC1 66.31, PC3 8.10), Methanol:Ethanol (PC1 73.49, PC2 8.73), Methanol Acetone wash (PC1 75.52, PC2 8.7) and Carnoy's Solution (PC1 73.17, PC2 8.99). Solid white lines indicate the boundaries for each of the PCA analyses. Dotted white lines show the boundaries of each Raman image used, and the grey areas show where no information is present as a result of the differing image sizes. The scale bar represents 30 µm. Corresponding microscope images for the first cell of each treatment are shown on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and band shifts that do occur are primarily in bands positioned between 600 and 1000 cm^{-1}, that are too weak to interpret with any reliability. Therefore, the main changes in loadings vector profile between live and aldehyde fixed cells are a small relative loss in intensity of the band at 2856 cm^{-1} (CH_2 symmetric stretching) and a loss of intensity and band profile change of the two bands at 2879 and 2881 cm^{-1} (CH_2 and CH_3 stretching) [26–28]. The corresponding PC1 scores images (live (2a) and PFA room temperature (2b) given as examples, all others shown in SI 1) also show little, if any, change in distribution between live and aldehyde fixed cells. The possible exceptions being the formation of vesicles present in some cells, and potential blebbing on the upper left side of cell 1, of the PFA room temperature fixed cells and

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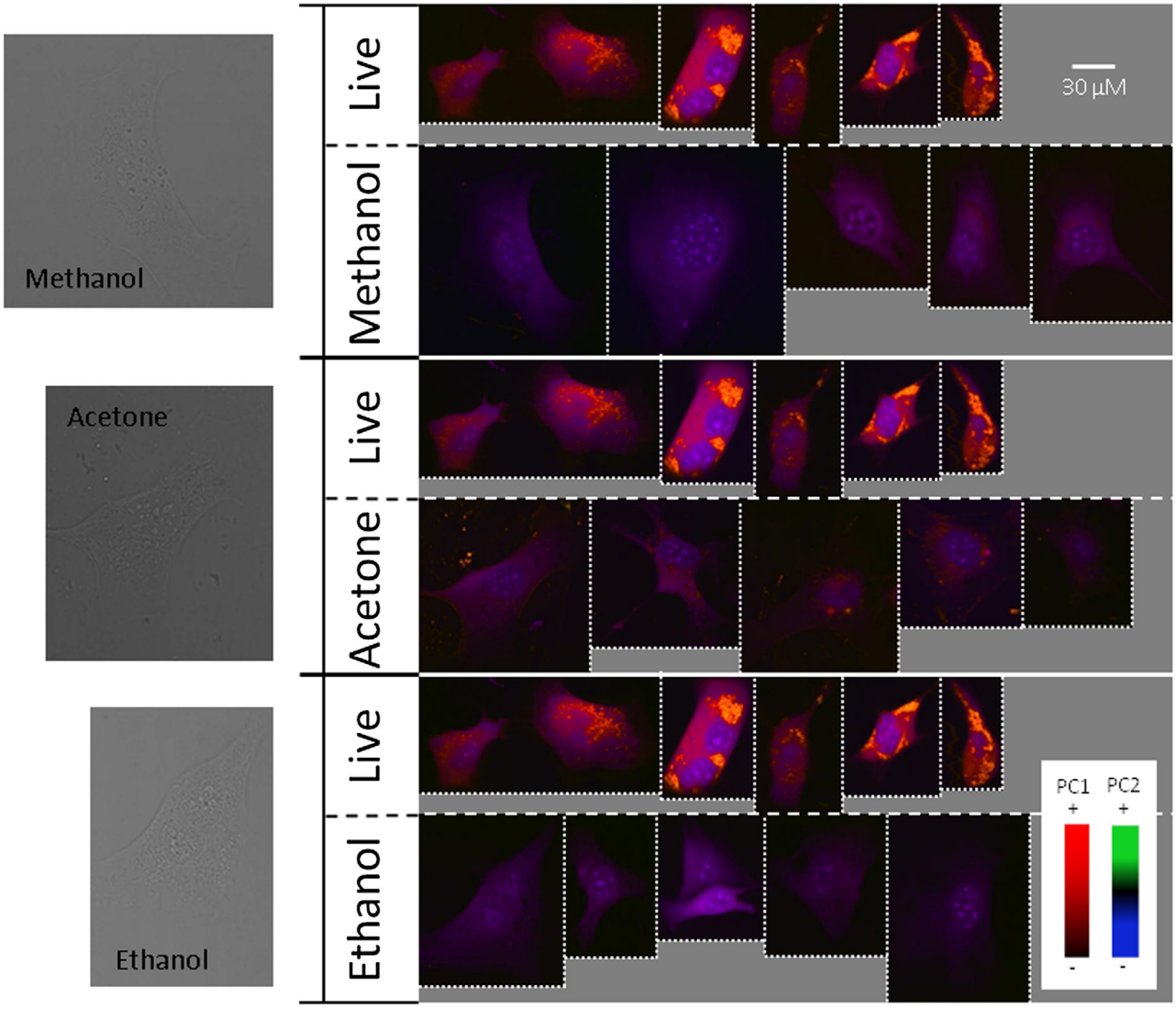

Fig. 3. (Continued)

glutaraldehyde, where only one cell reflects the dots of high intensity in the cytoplasm.

The aldehyde-based fixation methods also give rise to similar loadings as the live cells for PC2 (Fig. 1d) (PC3 for formaldehyde & glutaraldehyde as PC2 is affected by background differences between the images analysed), although there are more differences observed than for PC1. The two regions between ~875–770 cm^{-1} and 1120–1025 cm^{-1} show a series of negative peaks in PFA 4 °C, glutaraldehyde and formaldehyde & glutaraldehyde that are not present in the live or PFA room temperature fixed cells. The peaks in these regions, positioned at 1101, 1057 (PFA 4 °C), 1041 (glutaraldehyde and formaldehyde & glutaraldehyde), 856, 829 and 790 cm^{-1}, are all associated with nucleic acids originating from the phosphate backbone, ribose moiety and pyrimidine base vibrations [31,32]. These two regions are also present in the PC2 loadings, shown by the dotted cyan line (Fig. 1d), for formaldehyde & glutaraldehyde although the other features in this component do not reflect the other bands present in the live loadings. The bands at 856 and 829 cm^{-1}, along with a lack of the Raman marker band for RNA at approximately 814 cm^{-1}, indicate that these bands are illustrative of the presence of DNA in particular [32]. In all three fixation methods, the scores plots indicate that these negative bands are found in the nucleus, and are further concentrated in the nucleoli visible in many of the PC2 scores images. Although the scores plots still show a nucleus and nucleoli with strong negative values for PC2, PFA fixation at room temperature does not give rise to the DNA based negative bands in the PC2 loadings plot, indicating the temperature at which the fixation process is carried out also influences the biochemical changes occurring in the cell. (This will be discussed in more detail in Section 3.4). Additional differences between live and PFA 4 °C fixation are seen at ~1624 cm^{-1} where the shoulder is more pronounced in PFA 4 °C, also reflected in the loadings for glutaraldehyde, and in the bands at 2891 and 2854 cm^{-1} where PFA 4 °C has lower relative intensity than the live loadings, indicating a small loss in lipid-rich molecules upon fixation.

The effect of the organic solvent fixation methods as visualised by the PC1 scores plots suggests a dramatic effect on the quality of Raman images that can be obtained after fixation. The PC1 scores plots for all fixation methods that involve acetone suffer from high intensity spots, often outside the cells suggesting a 'residue' of some of the fixation agent or cell content that has been released from the cell, that then results in relatively low scores for the cells themselves. This is particularly noticeable in methanol + acetone

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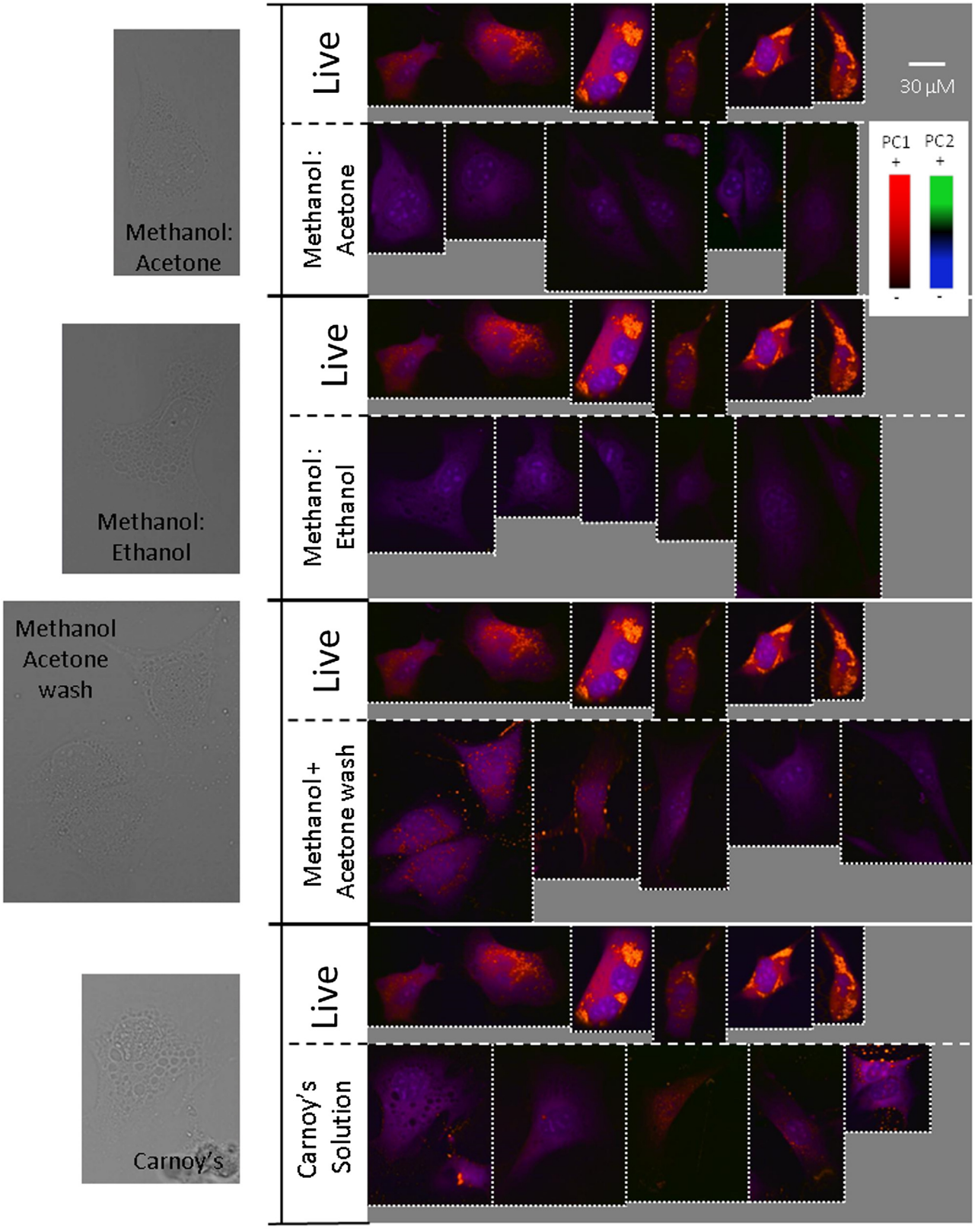

Fig. 3. (Continued)

wash and Carnoy's solution, but also appears in the scores plots for acetone and methanol:acetone fixed cells. The PC1 scores plots for methanol, methanol:ethanol and ethanol based fixation methods still show relatively clear cells, but with the majority of the intense regions associated with the nucleoli and, to a certain extent, the rest of the nucleus while the cytoplasm is comparatively weak. This is in contrast to the live cells where the PC1 scores plots show greatest intensity in cytoplasmic regions and lower intensity in the nucleus. The PC1 loadings vectors (Fig. 1) also support the idea that the use of organic solvents for fixation induces more substantial

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changes in the biochemical composition of the cells than seen with aldehyde-based fixation. All of the organic solvents analysed result in a loss of lipid-based molecules compared to live cells as shown by the change in band profile between 3000 and 2800 cm^{-1} where the bands at 2897 and 2856 cm^{-1} for live cells are replaced by a single band at approximately 2880 cm^{-1} in fixed cells. The loss of relative intensity is particularly noticeable for methanol, ethanol, methanol:acetone and methanol:ethanol while acetone, methanol + acetone wash and Carnoy's solution fixatives give rise to a less severe reduction in relative intensity compared with live cells.

A Raman band at approximately 1660 cm^{-1} is usually associated with lipid C=C stretching while bands between 1680 and 1670 and 1655–1650 cm^{-1} are usually attributed to β-sheet/β-barrel and α-helix conformations of proteins [29]. Therefore it is likely that a shift in peak position from 1662 to 1669 cm^{-1} accompanied by a band broadening for the majority of the organic solvents may indicate a loss of lipids, resulting in an overall relative increase in the proportion of protein present in the fixed cells, as well as protein secondary structure changes. This is also consistent with the PC1 scores plots showing significant intensities in the nucleoli of the cells, particularly in methanol fixation based images, as these areas will be rich in histone proteins. Methanol + acetone wash and Carnoy's solution in particular, also show the emergence of a shoulder at approximately 1649 cm^{-1} which may be indicative of a greater degree of α-helical protein content than seen for the other fixation methods and live cells. The increase in relative intensity of protein associated bands in the Amide I region is also reflected in the Amide III region between 1400 and 1200 cm^{-1} where the loadings for live cells shows three bands at 1341 (CH_2 end-gauche wagging mode, Tryptophan), 1308 and 1265 cm^{-1} (Amide III vibrations) while the loadings from all organic solvent based fixation methods show an increase in intensity for the band at 1342 and a band at 1323 cm^{-1}, both assigned to Amide III α-helix, as well as 1251 cm^{-1}, assigned to Amide III β-sheet vibrations and phosphate vibrations [26,27,29]. Other profile changes between the organic solvent and live cell loadings are in low intensity bands so interpretation should be treated with caution, but may still be of interest. The use of any single solvent from methanol, ethanol or acetone appears to produce more pronounced bands at approximately 1611, 1587 and 788 cm^{-1}, potentially from, amino acid or nucleic acid vibrations [29,31], with the exact position dependent on the solvent used. The use of ethanol, also contained in Carnoy' solution and the methanol: ethanol mix, also appears to contribute to a small band at approximately 1060 cm^{-1}. Although this band corresponds quite closely to the ethanol band at ~1055 cm^{-1} it is unlikely to originate from ethanol residue as there is no evidence of the much stronger ethanol band positioned at 883 cm^{-1}. This is in agreement with Meade et al. who also did not observe any evidence of the fixative spectra within the Raman spectra obtained from fixed cells [23] Finally, cells fixed with Carnoy's solution produce a low intensity but well defined band at 671 cm^{-1} that is not present in the loadings of any of the other organic solvents or the live cells. As Carnoy's solution contains ethanol and acetic acid, whose influence on the loadings vectors have already been accounted for in the ethanol fixed cells, this band originates from the action of the chloroform component of Carnoy's solution. While this band can be attributed to guanosine in nucleic acids, with its intensity proportional to the amount of ordered structure present [31], the loadings vector for Carnoy's solution does not show an increased intensity for other bands that would be associated with nucleic acids.

The scores plots and loadings vectors for PC2 and 3 (where applicable) are less consistent with that of live cells than for the aldehyde based fixation methods. In the case of ethanol PCA only identified one component, and for methanol:acetone the two components identified have already been discussed and so these two treatments are not included in the following discussion. PC2 scores plots from acetone and methanol:ethanol as well as both PC2 and PC3 scores from Carnoy's solution all exhibit relatively low positive scores (much of the contrast on these images originates from the negative scores contributions). The corresponding loadings vectors all exhibit a relatively intense broad positive feature with a maximum close to 800 cm^{-1} and a lower intensity broad band at 1070 cm^{-1}, indicating the positive bands in these loadings originate from the quartz substrate the cells are plated on. PC2 loadings for both methanol:ethanol and Carnoy's solution show strong negative bands in the high wavenumber region that both differ from the loadings vector of live cells. With peaks at 2935 and 2878 cm^{-1} and the corresponding scores plot strongly highlighting the nucleoli, methanol:ethanol fixation appears to affect the C—H stretching components of molecules, primarily proteins within the nucleoli. The negative PC2 scores for Carnoy's solution, on the other hand, are found in the isolated spots outside the cell suggesting they originate from the fixative solution itself (or possible extracted cell components). The corresponding loadings show a relatively broad band envelope with discernible peaks at 2900 and 2864 cm^{-1} and a shoulder at 2940 cm^{-1}.

Acetone (PC3), methanol + acetone wash (PC2) and Carnoy's solution (PC3) all reflect the main bands present in the live loadings vector between 3000 and 1100 cm^{-1}. At lower wavenumber the methanol + acetone wash loadings vector remains similar to that of live cells, although the bands are generally very low intensity, while the other organic solvent based fixation methods show very different loadings vector band profiles to that of live cells. The PC3 loadings vector for acetone contains moderately intense negative bands at 819 and 790 cm^{-1} and a weak shoulder at 837 cm^{-1}, originating from pyrimidine nucleotides, RNA backbone and DNA backbone, respectively [31,32]. These bands are not so prominent in the loadings vectors from live or other organic solvent fixation methods, but are somewhat similar to the loadings vector profiles of some of the aldehyde fixation methods (Fig. 1d). The corresponding scores plots also indicate relatively strong negative scores in the nucleus and nucleoli. In addition, the PC3 loadings vector of acetone also shows two strong positive bands at 1035 and 1004 cm^{-1} (slightly shifted from that of the other loadings vectors at 1006 cm^{-1}) that are assigned to phenylalanine [29,30]. Fig. 2b indicates that the highest positive scores, therefore those regions strong in phenylalanine and some lipid-based vibrations (shown by the bands in the loadings plot at 2900, 2887 and 2857 cm^{-1}), are located throughout the cytoplasm and are particularly concentrated on the outer edges of the cell (particularly clear in cells 1 and 3 where a yellow line, formed from the overlap of PC1 (in red) and PC3 scores (in green), can be seen surrounding the cell). This may indicate a bias towards preserving phenylalanine rich membrane protein complexes in the outer membrane when using acetone fixation.

3.2. Fixation leads to a loss of Raman intensity in the cytoplasm

The PCA results discussed in the previous section describe the differences in spectral profile and distribution of molecules as a result of the different fixation methods. It is also useful to directly compare each fixation method to the ideal situation, the measurement of live cells. Fig. 3 shows the PCA scores plots obtained when the analysis is performed on a combined image containing the data from live cells and the fixed cells of interest. These images are created from an overlay of the principle components of interest (originals are shown in SI 2). The most striking difference is a slight loss of intensity for PC1 scores for the aldehyde based fixation methods (Fig. 3a) and the large overall loss

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of intensity for all principle components for the organic solvent based fixation methods (Fig. 3b and c). The loss of scores intensity for the aldehyde based methods is only slight for PFA room temperature, glutaraldehyde and formaldehyde & glutaraldehyde where most of the fixed cells give a similar intensity to the lower intensity live cells, but do not show the very intense scores values seen in the live cells (depicted in yellow due to the overlap of PC1 positive (in red) and PC2 positive (in green) scores). The negative PC2 scores, concentrated in the nuclei, appear very similar between all three fixation methods and the live cells, indicating little change in these parts of the cell upon fixation. PFA 4 °C fixed cells scores are generally a little lower in intensity for all PCs, including the negative PC2 scores, suggesting a slight detrimental effect on image quality when using PFA 4 °C fixation than when using the other aldehydes.

The effect of organic solvent-based fixation on the scores plots is dramatic. The overall intensity of all PCs is noticeably reduced compared to that of the live cells. Fig. 3 shows that all major classes of biomolecules are affected (PC1 accounts for a great deal of overall cell content with bands from lipids, proteins and nucleic acids, while PC2 (or PC3 where appropriate) contains other lipid and nucleic acid vibrations). Although all organic solvent fixed cells show a reduced intensity in the PCA scores plots this is particularly marked in methanol, ethanol, methanol:acetone and methanol: ethanol fixed cells where the cell scores are weak relative to the scores obtained from the background regions that is it difficult to identify the cell edges clearly and the contrast between the cytoplasm and nucleus is poor. Acetone, methanol + acetone wash and Carnoy's solution provide marginally better contrast between the cytoplasm and background regions, but do provide slightly better contrast between the cytoplasm and nucleus for the majority of cells analysed.

Although only based on a subset of the image data, PCA based on average spectra extracted from the cytoplasm and nucleus of each cell analysed (Fig. 4) also reflect the loss of cellular material in fixed cells. In this case, the positive region of the PC1 loadings plot (Fig. 4c) is dominated by peaks associated with lipid-based vibrations, although there will also be some protein associated contributions at these positions. Conversely, the negative region of the loadings plot does not show any strong bands, suggesting that datapoints that give rise to strongly positive scores reflect regions of the cell with strong Raman spectra and, hence, are rich in biomolecules while strongly negative scores reflect regions with much weaker, and less well defined Raman spectra, and are therefore low in biomolecular content. The scores for PC1 (Fig. 4a) then suggest that live cells reflect the greatest/richest biomolecular content and that these scores show relatively large variation but are found as one main group. Fixed cells, on the other hand, all show a more bimodal distribution with one group of scores centered around −500 and another either between 0 and 1000 for aldehyde fixed cells, or 0–500 for organic solvent fixed cells. This indicates that while aldehyde fixatives do not result in quite the loss of information that organic solvent methods do, certain regions of the cell primarily the cytoplasm (as shown by the large

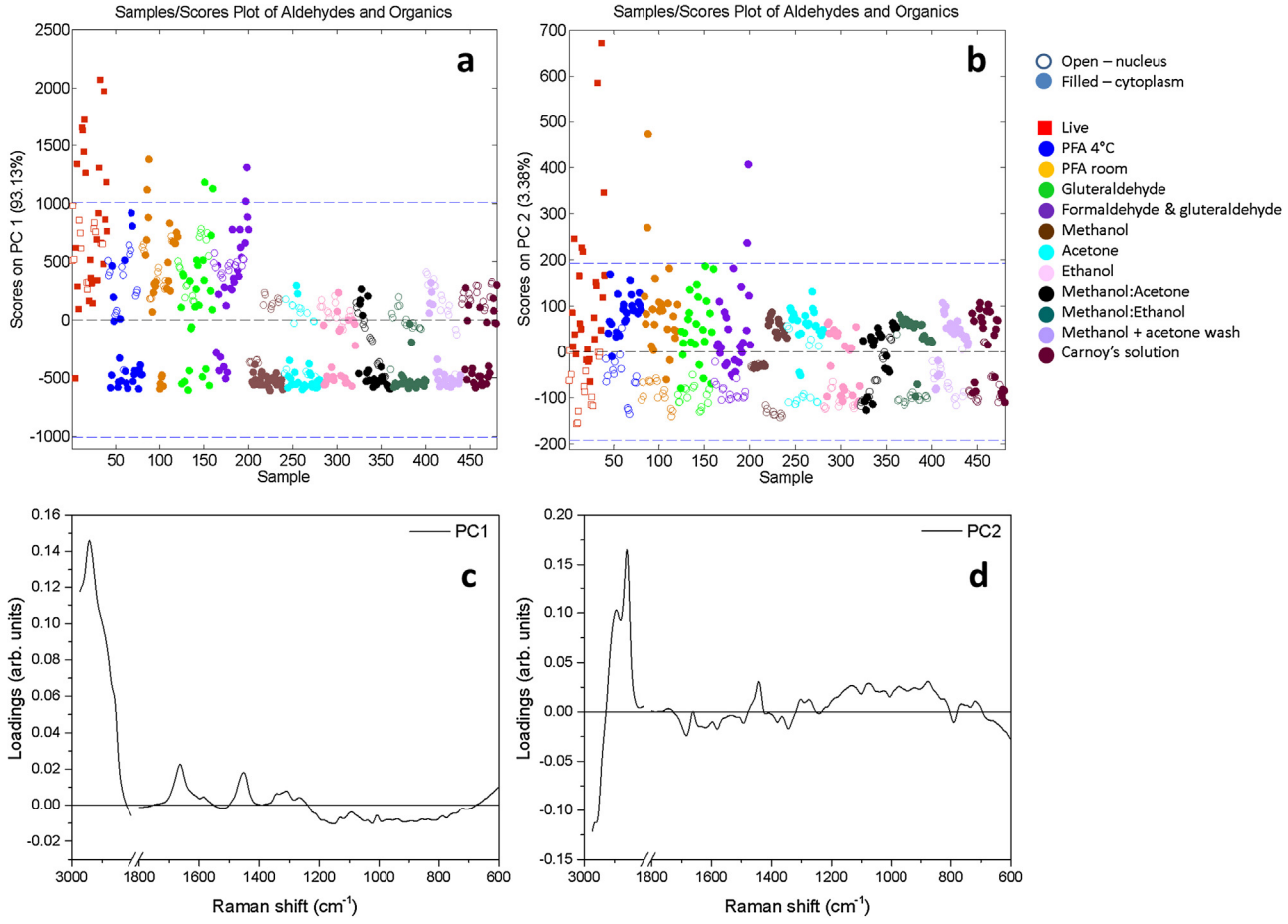

Fig. 4. PCA scores plots (a, b) and loadings vectors (c, d) for the PCA analysis performed on spectra extracted from the Raman images of live and fixed cells. Data from live cells are shown with squares and data from fixed cells are shown with circles. Datapoints obtained from the nucleus are represented by open circles/squares and those from the cytoplasm are shown as filled circles/squares. The blue dotted line on the scores plots represents the 95% confidence limit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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number of solid circles with negative scores), suffer from a loss of biochemical information as a result of fixation.

3.3. Nuclei are less affected by fixation

As discussed before, the majority of the variation identified by PCA in the average spectra extracted from the cell images (Fig. 4), is associated with overall cell content. As indicated, the greatest loss in PC1 scores for fixed cells, compared to live cells, is found in the cytoplasm. In general, the nuclei do not show the same reduction in PC1 scores. Although a few nuclei derived datapoints have negative PC1 scores most still show positive PC1 scores. For the aldehyde based methods the nuclei scores do not differ that much from those of live cells, indicating little change in the nuclear content between live and aldehyde fixed cells. Organic solvent fixed cells do show an overall reduction in PC1 scores for the nuclei derived datapoints compared to live cells, suggesting more significant alterations to the nucleus, but these changes are not as marked as for the cytoplasm derived datapoints.

The scores plots for PC2 clearly show that, despite the loss of information described in PC1, all fixation methods show a clear difference between the nucleus and cytoplasm derived datapoints. In general, cytoplasm derived datapoints reflect positive scores and the corresponding loadings plots exhibit peaks associated with lipids, proteins and broad weak bands from carbohydrates, phosphates etc. The nuclei derived datapoints almost exclusively exhibit negative scores and the corresponding loadings plot shows peaks associated with nucleic acids (the spectrum is not detailed enough to identify if only RNA, only DNA, or both are present although the lack of a marker band at 813 cm^{-1} for RNA [31,32] would suggest RNA levels are relatively low) that would be associated with components of the nucleus.

Although it is not so clear in the PCA scores images shown in Fig. 3, due to the dominating influence of the scores from live cells, this clear difference between cytoplasm and nucleus is also visible in the image based analyses. When cells for a particular fixation method are analysed without the comparison to live cells (SI 1) the nucleus is always clearly visible. For example, acetone fixed cells only show a muted contrast between cytoplasm and nucleus when analysed in conjunction with the live cells (Fig. 3b), but when analysed separately although the overall scores intensity may be low (SI 1), they still give rise to clear contrast between the cytoplasm and nucleus (Fig. 2c). Together these results show that although fixation causes a wide range of effects on both the spectral profile and the image quality, the nucleus appears to be less significantly affected than the cytoplasm, even for the harsher organic solvent based fixation methods. This is supported by previous research that suggests intact DNA and RNA of sufficient quality for amplification can be extracted from tissues that have been fixed with organic solvents [11,12].

3.4. The temperature of fixation influences the fixation process

There are a number of factors that will affect the efficiency of a fixation method. In particular, the rate at which the fixative can penetrate the sample, which is influenced by parameters such as concentration, temperature, pH etc. [33] is particularly important. This is true even for fixatives such as formaldehyde, which act to cross-link proteins significantly slower than the rate at which they penetrate a sample [6]. The temperature of formaldehyde based fixatives is known to affect the preservation of DNA within tissues, with 4 °C thought to be most effective as increasing losses of DNA content occur with increasing fixation temperature [3].

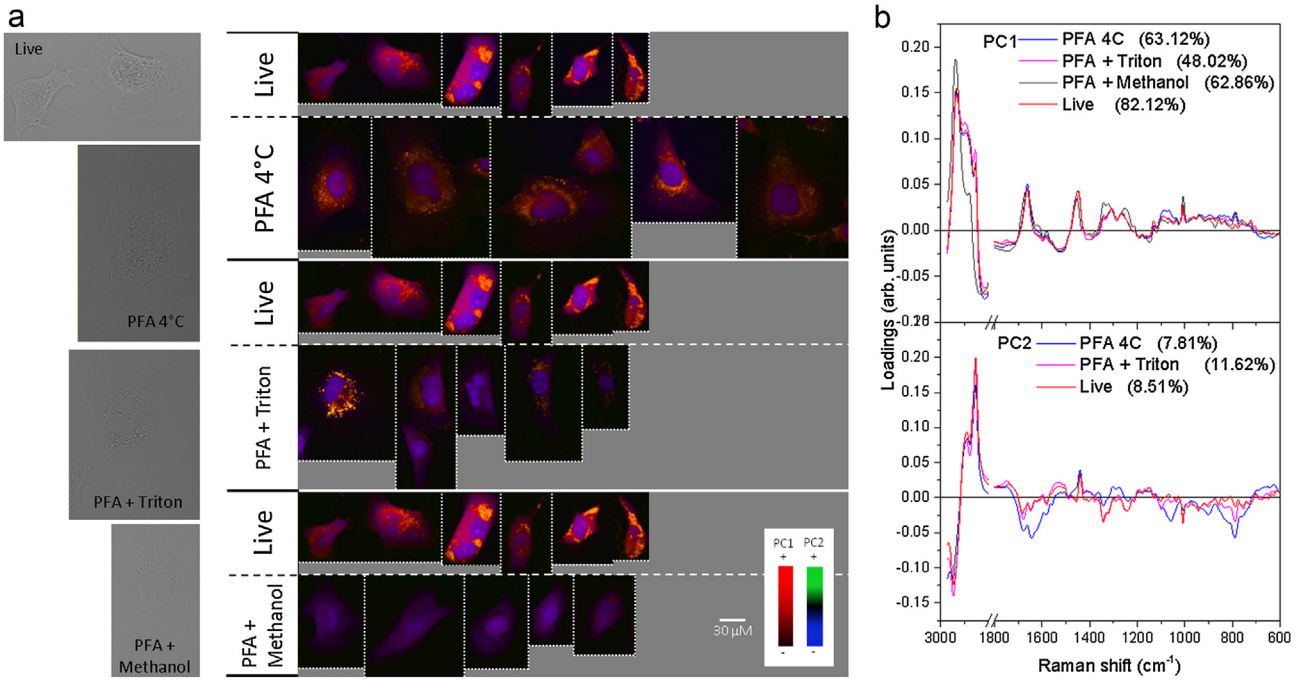

Fig. 5. PCA scores plots (a) and loadings vectors (b) obtained for live cells, PFA 4 °C, and PFA fixed cells after treatment with Triton X-100 and methanol for cell permeabilization. Loadings are taken from PCA analysis based on individual treatments (see SI 1 for corresponding scores plots). The percentage variance captured for each PC is given in brackets in the key). Scores are taken from PCA based on live versus each fixation method (to allow clear comparison between the fixation/permeabilization treated cells and the case of live cells). Scores plots were then concatenated to produce a combined image for the overlay and false colour steps to ensure the contrast for each layer was applied uniformly for each fixation method. PC1 positive scores are shown in red, PC2 positive scores are shown in green and PC2 negative scores shown in blue. Solid white lines indicate the boundaries for each of the PCA analyses. Dotted white lines show the boundaries of each Raman image used, and the grey areas show where no information is present as a result of the differing image sizes. The scale bar represents 30 μm. Corresponding microscope images for the first cell of each treatment are shown on the left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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The results presented in this paper also show that the Raman spectra and obtained images are also influenced by the fixation temperature, as shown by the results obtained with PFA fixation at 4 °C and at room temperature. The two most noticeable differences between these two sets of cells are the quality of the Raman images obtained and the spectral profiles. Overall, the image contrast (Fig. 3) is better at room temperature than at 4 °C, however, this comes at a cost of cell morphology changes, with blebbing and vacuole formation present in many of the cells fixed at room temperature. In terms of loading vector profile, the room temperature fixed cells are closer to that of live cells (Fig. 1), as they do not show additional bands associated with nucleic acids 1120-1025 and 875–770 cm^{-1}, or proteins 1800–1500 cm^{-1} These bands (also present in cells fixed with other aldehydes at 4 °C) suggest a greater contribution of proteins and nucleic acids, particularly DNA, to the Raman images. As aldehydes can form cross-links with both proteins and DNA, and adduct formation is much more prevalent at cold fixation temperatures [11,34] it is likely that the appearance of these bands in the cells fixed at 4 °C is reflecting structural changes associated with this crosslinking. Fig. 4 also shows that although the reduction of scores on PC1 is similar for the two fixation methods, cells fixed at 4 °C have more cytoplasm derived spectra in the negative half of the scores plot compared to room temperature fixed cells, indicating that the room temperature fixation is slightly better at preserving the cytoplasmic contents.

3.5. Permeabilization treatments further affect the Raman spectral profile and image quality

A number of cell biology protocols, such as immunostaining with primary and secondary antibodies for fluorescence imaging, require permeabilization of the cell, i.e. disruption of the cell membrane, to allow the antibody access to the intracellular proteins. While organic solvents fix and permeabilize at the same time, a separate permeabilization step employing either organic solvents, e.g. methanol, or detergents, e.g. Triton, are required when using aldehyde fixation methods.

In terms of image quality (Fig. 5a), triton permeabilization does appear to reduce the overall contrast of the cytoplasm, but regions of high intensity in PC1 and 2 (particularly rich in lipids as well as some contributions from proteins) are still visible throughout the cytoplasm as is the case in live and PFA 4 °C fixed cells. The nucleus is still clear in all PFA + triton cells, although the nucleoli are not quite as distinct as for the live cells, indicating some effects from the detergent on the nucleus. Methanol permeabilisation results in much more drastic loss of contrast in the cells, indicating a loss of cellular components in both the cytoplasm and nucleus. The nucleus is no longer as well defined as for the other cells shown in Fig. 5, and nucleoli are no longer discernible. However, this is slightly different to the images recorded from methanol alone (Fig. 3b) where, as a single step fixative, methanol results in cells that, while only giving feint overall cellular contrast, do clearly show contrast between the nucleoli and the surrounding nucleus. Therefore, the action of methanol on previously fixed cells is different from the action on living cells, although arguably it is just as, if not more, detrimental to the cell contents.

Fig. 5b shows the loadings vectors obtained for PC1 and PC2 for live, PFA fixed and methanol or Triton permeabilized cells. As discussed previously, the differences between live and PFA fixed at 4 °C are relatively small. The additional step of permeabilization with Triton also does not induce noticeable changes in the spectra below 1800 cm^{-1}, again, the main changes are in the relative intensities of the two bands at 2897 and 2856 cm^{-1}. One interesting point is that the additional bands seen in the loadings for PFA 4 °C that are not seen in the live cells (as discussed previously) are also not observed in the PFA + Triton treated cells, indicating the detergent acts to remove the adducts and cross-linking formed during PFA fixation at low temperatures. Methanol permeabilization on the other hand, introduces a number of changes in the loadings vector for PC1. Most noticeable is the loss of the bands at 2897 and 2856 cm^{-1} which are replaced with a much lower intensity band at 2881 cm^{-1}, previously only seen as a weak shoulder in the live and PFA fixed loadings. This change is also accompanied by a relative increase in intensity of the band at 2937 cm^{-1}. In the fingerprint region the majority of band changes are found in the 1800–1000 cm^{-1} range. The band at 1662 cm^{-1} shows a slight broadening along with a slightly increased intensity of the bands at 1624 and 1608 cm^{-1}, although these are still weak bands. The band at 1450 cm^{-1} in live and PFA fixed cells shifts to 1454 cm^{-1} with methanol fixation. The band at 1341 increases in intensity and the peaks found at 1308 and 1265 cm^{-1} in live and PFA fixed cells are now positioned at 1321 and 1253 cm^{-1}, respectively. Although many vibrations can contribute to bands in this region, the changes in these three bands suggest a shift in emphasis from proteins, as exemplified by Amide III vibrations [29], to nucleic acids where base vibrations from adenine, guanine and cytosine can give rise to bands in these three positions [31]. These spectral changes are very similar to those seen for methanol alone, indicating that the action of methanol is similar for both living and pre-fixed cells. Together with the image information it would appear that although pre-fixing with paraformaldehyde has some effect on the action of methanol, ultimately it provides minimal protection against the severe loss of biomolecules from the cell.

3.6. Short term storage after fixation does not further alter the Raman spectra or image quality obtained for most fixation methods

If a cell (or tissue) is not adequately fixed it will still be subject to changes based on the surrounding environment, and may degrade rapidly if stored for any length of time before analysis. The potential for changes in Raman spectra and image quality were investigated by fixing duplicate plates and measuring one plate after one hour storage in the fridge (the data presented in all other sections is based on this protocol) and the second plate after 24 h storage in the fridge. Although this does not consider the effect of long-term storage such as might be needed in tissue banks it does assess the effectiveness of the fixation methods and times used for complete fixation of the cell contents.

Fig. 6 shows the scores plots for PC1 and PC2 obtained from spectra extracted from the live cells, cells fixed and stored for one hour and fixed and stored for 24 h. As could be expected, the inclusion of the data from cells after 24 h storage before measurement does not greatly affect the overall trends seen in the data comparing live and one hour stored cells (Fig. 4). The aldehydes show slightly reduced PC1 scores and all methods involving organic solvents (including PFA fixed methanol permeabilized cells) show a marked reduction in PC1 scores compared to both live and aldehyde fixed cells. PC2 scores also reflect this difference in aldehyde and organic solvent fixed cells with aldehydes reflecting very similar scores and distributions to that of live cells, and organic solvents reflecting slightly more negative scores for the nucleus based spectra and less variation in positive scores for the cytoplasm.

In almost all cases, there is little difference in these results for cells that were stored for one hour and cells that were stored for 24 h prior to measurement. The exception is for Methanol:Acetone fixed cells where the cells stored for 24 h show little variation in scores, and where the spectral differences between the cytoplasm and nucleus are not apparent in either PC1 or PC2. Although PCA analysis on the whole image data (SI 3) only shows a small

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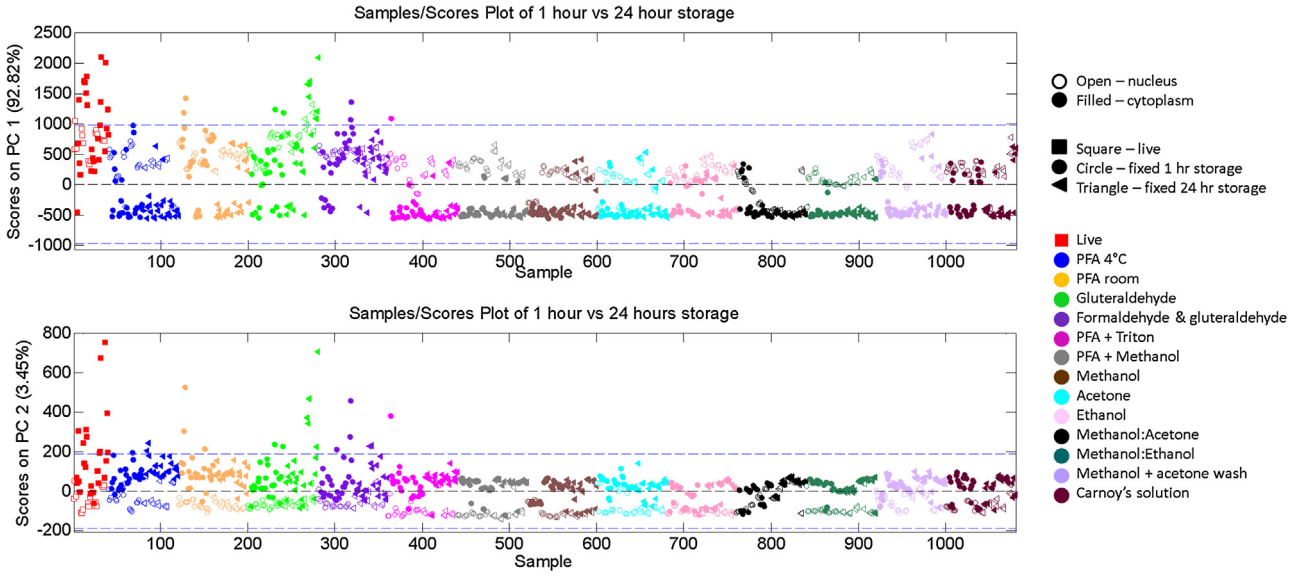

Fig. 6. PCA scores plots for the PCA analysis performed on spectra extracted from the Raman images of live (represented with squares), fixed cells stored for 1 h (circles) and fixed cells stored for 24 h (triangles). Datapoints obtained from the nucleus are represented by open shapes and those from the cytoplasm are shown as filled shapes. The blue dotted line on the scores plots represents the 95% confidence limit. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

reduction in image quality between one and 24 h stored cells it should be noted that the overall image quality for Methanol: Acetone cells is poor, regardless of the storage time. Similar treatment methods, i.e. Methanol, Acetone and Methanol + Acetone wash, do not reflect the same reduction in PC1 and 2 scores (Fig. 6) for one hour vs 24 h data, highlighting a difference in effectiveness of fixation when using each substance in isolation (including sequential applications of the two substances) compared with a 1:1 mixture.

4. Discussion

In this study we have built upon previous analyses of Raman spectra obtained from fixed cells by including a wider range of chemical fixation protocols and extended this to the effect of these chemical fixation methods on Raman images with relatively high special resolution. The effects of fixation on cells observed in this paper can be easily divided into two groups, aldehydes (cross-linkers) and organic solvents (precipitation/dehydration), dependent on the nature of the chemicals used for fixation. According to the Raman images and spectra presented here, the use of aldehydes for cell fixation results in relatively minimal changes to the cells, compared to that of organic solvent based methods, which is in agreement with several previous single point Raman studies [21,23,24]. All aldehydes result in a small loss of cellular components compared to live cells, primarily lipids (Figs. 1 and 4). The appearance of vacuoles in a few cells, particularly for PFA 4 °C and gluteraldehyde fixation, and some blebbing in the PFA 4 °C fixed cells has been noted before, where the blebs were shown to contain liquid that is not attributable to the fixation solution alone [8]. Together, this indicates that the aldehyde fixation methods do lead to some membrane damage, and loss of cytoplasmic material, but that this loss is relatively small. In addition, there are some modifications to proteins within the cells as evidenced by changes in intensity of the Amide III bands at 1341, 1308 and 1265 cm^{-1}. However, similarly to previous cell studies we do not see the changes in Amide I peaks at 1490 or 1667 cm^{-1} that would indicate the formation of methelyne bridges between proteins and formaldehyde-based fixatives [23].

Several researchers have noted that formaldehyde based fixation may not be particularly effective at preserving high molecular weight DNA and that the temperature of fixation is highly influential, with 4 °C preserving more high molecular weight DNA than room temperature fixation [3,11,12]. Our results could suggest that DNA is preserved when fixing at 4 °C as nucleic acid bands are found in the negative region of the PC2 loadings plot for PFA °C, gluteraldehyde and formaldehyde & gluteraldehyde, but not for PFA fixed at room temperature. However, it should also be noted that the loadings plot from live cells also does not reflect these nucleic acid bands, suggesting that PFA room temperature fixation is, in fact, closer to the live cell case, and the fixation methods at 4 °C are 'over-emphasising' the nucleic acid contributions. One of the known issues with using aldehyde based fixation methods is the formation of adducts (between proteins and formaldehyde) that can reduce or prevent molecules e.g. immunostains from binding to intracellular antigens [34], with different amino acids more amenable to formaldehyde-induced modifications than others [35]. Although formaldehyde adduct formation is most often associated with proteins it has been suggested that formaldehyde-DNA crosslinking can occur, affecting the rigidity of the DNA [11], and we may expect that to affect the nucleic acid bands at 1120–1025 and 875–770 cm^{-1}, producing more intense Raman bands in this region that may then result in an overestimation of the cellular DNA content. It has also been noted that the adduct formation is relatively weak in that it is also reversible at room temperature [34] explaining why PFA room temperature fixation and, indeed, live cells do not reflect these adduct-based contributions. Permeabilization, as well as disrupting the cell membrane to allow large molecules to enter, has also been shown to reverse this adduct formation [34]. Our study showed that the strong nucleic acid bands (and also those attributed to proteins) in the negative region of the PC2 loadings for aldehyde fixation at 4 °C (Fig. 1d) were not present after treatment with the detergent Triton (Fig. 5b), indicating the observed increase in DNA contributions is indeed due to byproducts of the fixation process.

In terms of Raman image quality, PFA fixed at 4 °C shows some loss in contrast, although the overall distribution of biomolecules

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does not seem to be greatly affected. Fixation at room temperature appears to be much better in terms of relative contrast of the PC scores, but at the cost of more noticeable vacuole formation and blebbing. Similarly for gluteraldehyde, the image contrast is close to that of the live cells, although the strong lipid contributions seen in live cells are not so noticeable in gluteraldehyde fixed cells. There are also some regions where vacuole formation is visible in the gluteraldehyde fixed cells (Fig. 3a, especially cells 2 and 5). The combination of formaldehyde & gluteraldehyde fixation appears to combine the benefits of formaldehyde fixation at cold temperatures (lack of blebbing, good preservation of cytoplasmic lipids) with the advantages of gluteraldehyde fixation (good retention of biomolecules responsible for cellular contrast), making this the ideal choice in terms of image quality. However, the spectral analysis does indicate the presence of formaldehyde adducts which, depending on the particular experiments to be performed, may need to be removed by detergent or organic solvent at the cost of some of the image contrast.

Organic solvents, on the other hand result in a marked loss of cellular content, and a bias in the signal obtained from the cytoplasm versus the nucleus. Much of the loss of cellular content is associated with lipids and is consistent with both previous Raman studies [21,23,24] and cell biology studies [25]. The loss of lipid-based bands in the loadings of organic solvent based fixation methods is not surprising as such solvents are often used to permeabilize cells to allow the entry of large molecules, such as fluorescence stains or antibodies, into the cell. In order for such molecules to pass into a cell the cell membrane must be disrupted and this is usually achieved through the removal of lipids or cholesterol from the lipid bilayer [14]. This is likely to also explain the significant loss of lipid based intensity in the cytoplasm in the scores plots of organic solvents versus that of live cells, as the lipid-rich membranes from organelles and vesicles in the cytoplasm will also be stripped of their lipid components.

Although we might expect lipid-based vibrations to be severely affected by the use of organic solvents as a result of the removal of lipid components of cell membranes [14] and changes to lipid droplets/core lipids [25] our results show that proteins and nucleic acids are also affected. Some researchers have shown that organic solvents are effective at preserving nucleic acids and result in more uniform fixation with less structural changes than for aldehyde based methods [3,4,12,13]. However, it should be noted that the majority of these studies have been performed on tissue or bone, which may well respond differently to fixation as a result of the increased tissue architecture present, or they have been primarily interested in the identification of a particular cellular component after fixation (e.g. via immunostaining or RNA/DNA extraction) and do not take into account changes in other molecules that were not of specific interest. Those studies that have investigated the effects of organic solvent fixation on cells have generally noted that organic solvent based fixative methods have a significant detrimental effect on the cells [21,23,24], particularly with regard to membrane integrity [5]. This, coupled with the fact that it is challenging for any fixation methods to preserve soluble proteins and those proteins with weak associations with cellular architecture [5], may go some way to explaining the overall lack of intensity seen in the PCA scores plots for the organic solvent fixatives. Although the disruption of the cell membrane is often exploited to allow the passage of immunostains into the cell, the large holes produced [5] are also likely to allow the passage of many molecules from the cytoplasm out of the cell and into the surrounding media. Hence why organic solvent fixed cells show such a dramatic loss of cytoplasmic contrast (Fig. 3), although the nuclei (which have one more membrane separating them from the rest of the cell) do not seem to be as severely affected (Fig. 4). Although several papers have suggested organic solvent fixation is desirable before immunostaining [10,11,13] our results indicate that this might not always be the case, depending on the target, as many potential staining targets may be removed from the cells during organic solvent fixation.

Most of the organic solvent based fixation methods also affect the remaining protein and nucleic acid content of the cells. Organic solvents are known to preserve cells by dehydration and precipitation/aggregation of proteins [3]. Our results show that organic solvent fixed cells exhibit shifts in Raman bands in the Amide III region (1341, 1308, 1265 cm^{-1} in live cells and 1342, 1323 and 1251 cm^{-1} in organic solvent fixed cells), which are associated with protein secondary structure elements such as α-helix and β-sheet conformations, suggesting a significant change in lipid to protein ratios as well as changes in protein structure when the cells are exposed to the solvents. Our results also indicate that acetone fixation, although not fixation methods where acetone is mixed with other solvents, also affects the nucleic acid content of the cells, possibly indicating some of the same crosslinking, or DNA rigidity similar to that introduced by the aldehyde fixation methods.

The use of organic solvents as permeabilization steps after aldehyde fixation is also commonly suggested as a way of removing adducts and other potential antigen masks prior to steps such as staining [4,5,34]. Although Hoetelmans et al. found that PFA fixation followed by methanol permeabilization was preferable to methanol fixation alone [5] our results indicate that there is little difference between the two methods. Although the PFA fixation results in relatively good preservation of cellular components and their distribution, the addition of methanol still results in a substantial loss of cytoplasmic material, and the aggregation of the remaining proteins.

5. Conclusions

Aldehyde fixation is most effective at preserving the cell content and distribution of cellular components. At room temperature cells may form vacuoles or may lose some cellular content through blebbing. Although this can be minimised by fixing at 4 °C this can lead to other undesirable effects, namely the formation of adducts. Therefore, for Raman spectroscopy, the choice of aldehyde fixation will be dependent on whether the image quality, where formaldehyde & gluteraldehyde is best, or spectral quality, where PFA room temperature fixation is best, is most important. In terms of Raman images, the use of organic solvents is best avoided due to the loss of cellular material and the more severe spectral changes observed. However, where organic fixation methods are needed in order to carry out additional analyses such as immunostaining, Acetone or Carnoy's solution are the better options as they still provide some cellular contrast both between the cell and the surrounding area and across the cytoplasm and nucleus. However, due to the acetone, cells fixed using these protocols should be washed more thoroughly than for other fixation methods in order to remove the spots or debris present on some images. Finally, where permeabilization is required, detergents such as Triton are preferable to organic solvents as they remove the potential adduct formation without significantly altering the Raman spectra from that of live cells, with only a relatively small loss in overall cell contrast.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vibspec.2016.10.012.

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