

A mass spectrometry-based workflow for the proteomic analysis of in vitro cultured cell subsets isolated by means of laser capture microdissection

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Abstract This paper describes a microproteomic workflow that is useful for simultaneously identifying and quantifying proteins from a minimal number of morphotypically heterogeneous cultured adherent cells. The analytical strategy makes use of laser capture microdissection, an effective means of harvesting pure cell populations, and label-free mass spectrometry. We optimised the workflow with particular reference to cell fixation which is crucial for successful laser-based microdissection and also downstream molecular studies. In addition, we defined the minimum number of cells to be isolated and analysed for satisfactory proteome coverage. To set up this workflow, we choose human monocyte-derived macrophages spontaneously differentiated in vitro. These cells, under our culture conditions, show distinct morphotypes, reminiscent of the heterogeneity observed in tissues in various homeostatic and pathological states, e.g. atherosclerosis. This optimised workflow may provide new insights into biology and pathology of heterogeneous cell in culture, particularly when other cell selection approaches are not suitable.

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Introduction

Cell heterogeneity is widespread [1] and increasingly apparent in eukaryotic cells that differ not only in terms of function and specialisation, but also in size and morphology. The complex and highly interconnected network of signalling pathways, their spatially dependent nature, and their reliance upon low-abundance molecules leads to the stochastic behaviour that subsequently underpins the heterogeneity of cell systems.

The analysis of pure subsets of heterogeneous cells, co-existing in the same cell culture or tissue, represents an important field of research in order to link cell heterogeneity with functional changes in biology or pathology. Improvements in the sensitivity, throughput and resolution of the technologies have made it possible, allowing the definition of transient cell states, rare cells, and even the assessment of the impact of organisation and of the environment on cell behaviour, none of which can be done when studying tissue as a whole.

The last decade has seen considerable changes in the instrumental base and analytical techniques, which have led to major achievements in the area of single cell analysis. For example, the amplification of cDNA from single cells and the use of microarray technology have provided unique insights into variations in transcriptional activity at cell level [2]. However, changes in transcriptome may not correlate well with changes in protein expression, which is the ultimate

driver of biological responses. The current status of a cell is largely controlled by its functional proteome through a series of biochemical processes that include post-translational modifications. Proteomic analysis therefore has a unique capacity to snapshot the current status and composition of cell phenotypes within a defined time frame, which is highly relevant to biological functions.

Considerable efforts are being made to apply such methodologies to a limited number of cells [3], but the analytical advances in proteomics have not been as rapid or fortunate as those of single cell transcriptomics mainly because of the lack of protein amplification techniques.

Additionally, the proteome profiling of pure cell populations requires appropriate isolation techniques that become even more crucial when subsets of morphotypically distinct cells co-exist in the same context.

One practical solution to this problem is laser capture microdissection (LCM), which allows morphologically specific and/or phenotypically defined cells to be harvested from precise anatomic region, and has been used in conjunction with a variety of analytical methods [4], such as two-dimensional gel electrophoresis (2-DE) [5], surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS) [6], direct cell matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [7], or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) [8, 9]. 2-DE, the most widely used approach, is time consuming and poorly consistent, has a low dynamic range, and is deficient in the case of very large (>150 kDa), small (<15 kDa), hydrophobic or otherwise insoluble proteins. Furthermore, as more than 25–50 µg of total protein is required for each analysis, at least 50,000 cells are necessary, a number which is often impossible to be obtained in the case of less-frequent cell types or specific pathological subtypes. Although SELDI and direct cell MALDI can provide a snapshot of cells within tissues, protein identification may be limited. In this frame, LC-MS/MS approaches using label-free quantitative techniques are an attractive alternative to isotope coding because they do not require any labelling chemistries or growth conditions, and can be used for complex study designs with any number of treatment groups [10, 11].

This study was aimed at the setting up of an optimised workflow to delineate the proteomic profile of a limited number of heterogeneous primary human cells in culture. It includes sample fixation and LCM isolation, protein extraction and digestion, and analytical processing. The analysis was performed by a mass spectrometry-based method (LC/MS^E) for label-free proteomics. This approach takes advantage of data acquisition by data-independent analysis (DIA), in which fragmentation data are acquired without any precursor selection [12], allowing simultaneous identification and quantification of proteins in a complex mixture.

As a prototypical primary cell culture, we choose human monocyte-derived macrophages spontaneously differentiated *in vitro*. Under our conditions, these cells show distinct morphotypes (round-shaped, that are mostly lipid-enriched, and spindle-shaped) co-existing in the same preparation, in about the same percentage [13]. This heterogeneity may be representative of distinct subsets of tissue macrophages that have been observed under various homeostatic and pathological conditions, e.g. atherosclerosis [14]. Thus, the proposed workflow may improve our understanding of the biology of heterogeneous cell in culture, especially when other cell selection approaches are not suitable.

Experimental

Monocyte isolation and culture

Monocytes were isolated from venous blood of healthy consenting volunteers using sodium citrate (0.129 mol/L) as anticoagulant. The study was conducted according to the principles expressed in the Declaration of Helsinki, after Local Research Ethics Committee approval. The mononuclear cells were isolated by means of Ficoll-Paque Plus (GE Healthcare, Milan, Italy) density centrifugation at 450×g for 20 min at room temperature (RT).

In order to remove platelet contamination, the lymphocyte-/monocyte-rich layer was washed with phosphate-buffered saline (PBS) containing 5 mmol/L EDTA, and suspended in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Lonza Milano, Bergamo, Italy), and 10 % freshly obtained autologous serum. The cells (2×10^6) were plated in 35-mm well plates (PrimariaTM, Falcon, Sacco, Como, Italy) or in DuplexDish 50 mm (5×10^6) (Carl Zeiss, Milan, Italy) for the LCM experiments and kept at 37 °C (5 % CO₂). After 90 min, the non-adherent cells were removed and the adherent cells were cultured for 7 days in Medium 199 (Lonza Milano, Bergamo, Italy) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 10 % autologous serum at 37 °C (5 % CO₂). The medium was not replaced throughout the culture period.

Cell fixation protocols

After 7 days' culture, the monocyte-derived macrophages were washed three times with PBS and fixed under one of the following conditions: (i) 70 % ethanol solution for 30 min at -20 °C; (ii) 2 % paraformaldehyde (PFA) in PBS for 15 min at RT; and (iii) air-drying for 15 min at RT.

Laser capture microdissection and catapulting

LCM was performed using a laser microdissection system from PALM® MicroLaser Technologies (Bernried, Germany) containing a PALM MicroBeam and RoboStage for high-throughput sample collection, and a PALM RoboMover (PALM RoboSoftware 2.2). The laser MicroBeam uses a pulsed UV beam (wavelength 337 nm) which is coupled via the epifluorescence beam path into an inverted light-microscope (AxioVert 200 M, Carl Zeiss, Milan, Italy) and focused through a $\times 40$ dry objective lens. A neutral density filter mounted in the laser beam path allows the laser energy to be continuously adjusted as required, without displacing the beam. The coincidence of the laser focus with the optical focus of the microscope is essential for precise laser micromanipulation and microdissection of the selected cell. The settings for laser cutting were adjusted for each sample by observing the interaction between the laser and the membrane slide (UV-Energy 60–70 and UV-Focus 42). Macrophages were selected manually and upon the cut they were captured by means of the RoboPC's autocatapulting feature (UV-Energy 90–100 and UV-Focus 42), and catapulted directly into the cap of a 0.65 mL microcentrifuge tube. The catapulting efficiency was verified by assessing the presence of cells inside the tube cap. Then lysis buffer was added to the cap, and the lysed sample was transferred into the tube by centrifugation. After this, to ensure that all cells were transferred, we examined the cap under a microscope. Adherent macrophages, which were obtained from three healthy donors, were dissected for a maximum of three consecutive days. Approximately 4,000 laser-pulsed cells of both morphotypes (about 2,000 spindle and 2,000 round macrophages) were collected, and stored at $-80\text{ }^{\circ}\text{C}$ until processing. In parallel, macrophages samples were prepared, without morphology selection, by scraping the plate in PBS and then stored at $-80\text{ }^{\circ}\text{C}$ until processing for comparison with the LCM-dissected cells from the same subjects.

Total protein assay

The protein concentration was determined by the Bradford method [15] that measures the absorbance of an acidic solution of Coomassie Brilliant Blue G-250 that shifts from 465 to 595 nm when binding to protein occurs. Briefly, the cell lysate (2 μL) was added to the Bradford reagent (Biorad, Milan, Italy) according to the manufacturer's instructions. The absorbance at 595 nm was measured by the DU 800 spectrophotometer (Beckman Coulter, Milan, Italy) and the protein concentration was calculated on the basis of a standard curve generated with bovine serum albumin.

Sample preparation for LC/MS^E

After being dissolved in 25 mmol/L NH_4HCO_3 containing 0.1 % RapiGest (Waters Corporation, Milford, MA, USA), the LCM air-dried cells were sonicated and centrifuged at $13,000\times g$ for 10 min. Each sample was heated at $80\text{ }^{\circ}\text{C}$ for 15 min, reduced with 5 mmol/L dithiothreitol (DTT) at $60\text{ }^{\circ}\text{C}$ for 15 min, and then carbamidomethylated with 10 mmol/L iodacetamide for 30 min at RT. Digestion was performed overnight at $37\text{ }^{\circ}\text{C}$ while maintaining a ratio of 1 μg of sequencing grade trypsin (Promega, Milan, Italy)/20 μg of protein, which was based on the calculated recovery for the number of processed cells. After digestion, 2 % *v/v* trifluoroacetic acid (TFA) was added to hydrolyse the RapiGest and inactivate the trypsin. The solution was then incubated for 20 min at $37\text{ }^{\circ}\text{C}$, vortexed, and centrifuged at $13,000\times g$ for 10 min. The PFA-fixed cells were processed on the basis of two different protocols. In accordance with the first (PFA-A), which has been previously described by Bagnato et al. for tissues [16], the samples were suspended and boiled in 30 % *v/v* acetonitrile (ACN) and 100 mmol/L NH_4HCO_3 buffer for 10 min to reverse the cross-linking of proteins induced by PFA fixation. Protein digestion was performed as described above. The second protocol (PFA-B) involved protein extraction using a Liquid Tissue MS Protein Prep Kit (Expression Pathology Inc., Gaithersburg, MD, USA) as instructed by the manufacturer. Briefly, the samples were incubated with the Liquid Tissue extraction buffer at $95\text{ }^{\circ}\text{C}$ for 1.5 h with intermittent mixing, and then centrifuged at $10,000\times g$ for 1 min. Digestion was performed using trypsin (1 μL , 15 U/ μL) for 1 h at $37\text{ }^{\circ}\text{C}$. The samples were centrifuged a final time at $10,000\times g$ for 1 min before being reduced with DTT (10 mmol/L) at $95\text{ }^{\circ}\text{C}$ for 5 min and then centrifuged. Peptide concentration and purification were performed using ZipTip C18 (Millipore, Milan, Italy) in accordance with the manufacturer's instructions, and the peptides were then solubilised in 0.1 % *v/v* formic acid in water.

LC/MS^E

Nanoscale LC separations of tryptic peptides for qualitative and quantitative multiplexed LC/MS^E analysis were performed using a nanoACQUITY system. The samples were mixed with 50 fmol yeast alcohol dehydrogenase (ADH) digest as an internal standard for molar estimation [17], and injected into a Symmetry C₁₈ nanoACQUITY trap column, 100 \AA , 5 μm , 180 $\mu\text{m}\times 2\text{ cm}$ (Waters Corporation, Milford, MA, USA), for pre-concentration and desalting. The peptide mixtures were subsequently directed from the pre-column to the analytical column BEH130 C₁₈, 130 \AA , 1.7 μm , 75 $\mu\text{m}\times 250\text{ mm}$ (Waters Corporation, Milford, MA, USA), and then eluted at a flow rate of 300 nL/min by increasing the organic solvent B concentration from 3 to 40 % over 90 min, using

0.1 % v/v formic acid in water as reversed phase solvent A, and 0.1 % v/v formic acid in ACN as reversed phase solvent B. All of the analyses were made in triplicate.

The precursor ion masses and associated fragment ion spectra of the tryptic peptides were measured using a SYNAPT-MS, a hybrid quadrupole orthogonal acceleration time-of-flight Q-Tof mass spectrometer (Waters Corporation, Milford, MA, USA) directly coupled to the chromatographic system. The time-of-flight analyser was externally calibrated using NaI from m/z 50 to 1990, and the data post-acquisition lock mass data were corrected using the monoisotopic mass of the doubly charged precursor of [Glu¹]-fibrinopeptide B (m/z 785.8426) delivered to the mass spectrometer at 100 fmol/ μ L through a NanoLockSpray interface using a pressure pump. The reference sprayer was sampled every 30 s. Accurate mass data were collected in DIA by alternating lower and higher energy applied to the collision cell. The spectral acquisition time in each mode was 0.6 s, with a 0.1 s inter-scan delay. In the low-energy MS mode, the data were collected at constant collision energy of 3 eV; in high energy mode, the collision energy was ramped from 12 to 35 eV during each 0.6 s scan. The radio frequency (RF) applied to the quadrupole mass analyser was adjusted in such a way that ions from m/z 300 to 2,000 were efficiently transmitted, thus ensuring that any ion with a mass of less than m/z 300 only arose from dissociations in the collision cell. The DIA data were processed and searched using ProteinLynx GlobalSERVER (PLGS) version 2.3 (Waters Corporation, Milford, MA, USA). The proteins were identified by searching a human species-specific UniProt database (release 57.0; 20,333 entries). Ion detection, data clustering, and the normalisation of the data-independent LC/MS^E data has been previously explained in detail [18–20].

The following search criteria were used for protein identification: the default search parameters included the “automatic” setting for mass accuracy (approximately 10 ppm for precursor ions and 25 ppm for product ions); a minimum of one peptide match per protein, a minimum of three consecutive product ion matches per peptide, and a minimum of seven total product ion matches per protein; up to one missed cleavage site allowed; carbamidomethyl-cysteine as fixed modification; and methionine oxidation as variable modification.

The initial protein false-positive rate (FPR) of the identification algorithm was set at 4 % using a randomized database that was five times larger than the original database, leading to a peptide FPR that was typically smaller than 1 %. In addition, using replication as a filter, the false-positive rate of the experiment is minimised because false-positive identifications have a random nature and do not tend to replicate across injections.

The molar estimation of each well-characterised protein in the mixture was determined by dividing the average MS signal response of the three most abundant tryptic peptides of each protein by the signal response factor, as previously

described [12, 21]. The average MS signal response from the internal standard protein(s) is used to determine a universal signal response factor (counts/mol of protein), which is then applied to the other identified proteins in the mixture to determine their corresponding molar estimation. The absolute quantity of each well-characterised protein in the mixture is determined by dividing the average MS signal response of the three most intense tryptic peptides of each well-characterised protein by the universal signal response factor described above.

Mass measurement errors for identified proteins are expressed as root mean square (RMS) errors for precursors and products ions.

Gene ontology analysis

The identified proteins were classified on the basis of the Gene Ontology (<http://www.geneontology.org>) hierarchy using AmiGO v.1.8 (<http://amigo.geneontology.org/cgi-bin/amigo/go.cgi>) for the gene ontology (GO) annotation searches [22], and CateGORizer for the GO classification terms in GO slim (<http://www.animalgenome.org/tools/catego/>) [23].

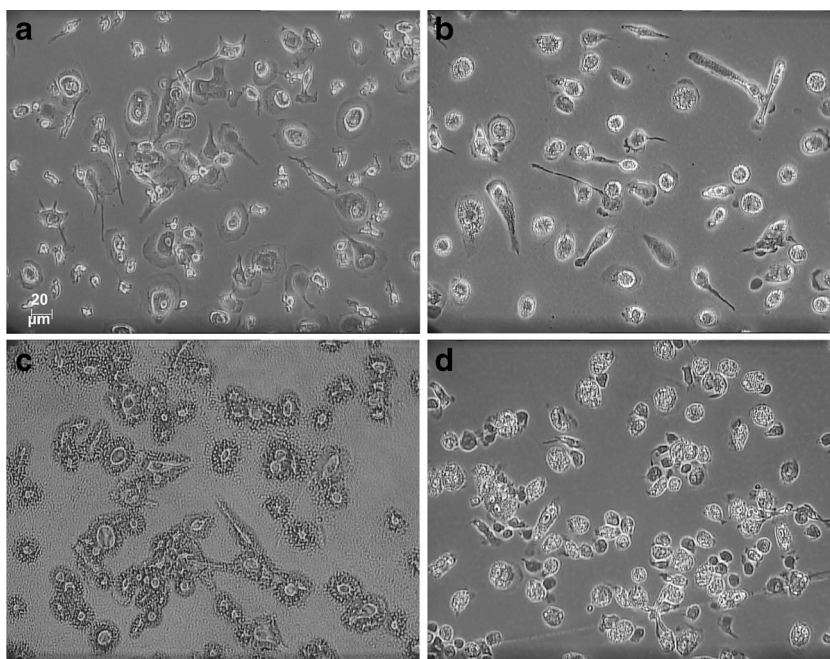
Results and discussion

LCM is a well-known means of isolating single cell populations and cell areas from tissues. In the field of proteomics, it has been extensively applied to tissues but there is little information concerning its use in the isolation of cultured cells. Herein, we describe a nanoscale LC-MS workflow that we have set up and used to characterise the proteomic profile of heterogeneous cultured human macrophages collected by means of LCM.

Effect of different fixation protocols on macrophage morphology

Optimal fixation protocols of samples to be collected by LCM facilitate the selection of distinct cell subsets in the case of heterogeneous populations, but fixation affects both the quality and reproducibility of downstream proteomic analyses. We therefore compared the efficiency of various fixation protocols and their compatibility with subsequent proteomic analysis. Cross-linking (aldehyde-based) fixatives generally have slight effects on genomic DNA but can profoundly affect RNA and proteins [24]. However, some protocols have been developed to process formalin-fixed, paraffin-embedded tissues [16]. Alternatively, air fixation, in the absence of any fixative, could be used. As shown in Fig. 1, both PFA and air fixation (panels b and c) preserved the macrophage morphologies observed in living cells (panel a). It has been shown that these fixation approaches have only minor effects on nucleic

Fig. 1 Effects of fixation on macrophage morphology. Representative contrast images (200x original magnification) of macrophage cultures under living conditions (a), and after fixation with PFA (b), air (c) and ethanol (d). $n=3$ for each condition



acid and protein spectral profiles, and on the distribution of these macromolecules, as deduced from Raman spectral images [25]. Precipitating reagents such as ethanol are the best choice for protein studies. Ethanol, however, could not be used under our conditions because it markedly altered the morphological heterogeneity typical of living macrophages (Fig. 1, panel d). Given this, the next step was to compare protein recovery from PFA- and air-fixed cells (150,000 cells/sample).

Protein retrieval

In order to establish the minimum cell number required for adequate proteome coverage, preliminary experiments were performed to assess protein recovery from 150,000 air-fixed cells. To this end, adherent macrophages were lysed in buffer containing 25 mmol/L NH_4HCO_3 and 0.1 %w/v RapiGest. Protein recovery was 50 μg , detected by the Bradford assay, as described in the “Experimental” section. After digestion with trypsin, 500 ng of protein peptides (equivalent to about 1,500 cells) were analysed using a label-free DIA method of data acquisition, LC/MS^E (see “Experimental” section).

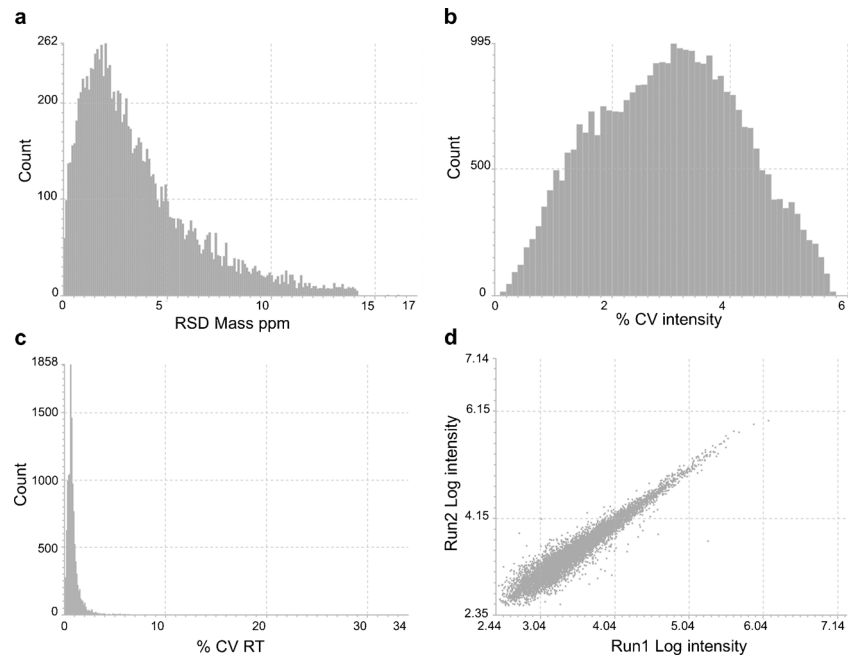
In order to establish analytical reproducibility, a number of quality control evaluations were made using PLGS 2.3 software (Waters Corporation, Milford, MA, USA) with the Identity^E algorithm for exact mass and retention time (EMRT) data analysis and database search. The mass precision of the extracted peptide components was typically within 2.5 ppm (interquartile range 1.5–7.4 ppm), and the variability of the intensity measurements between replicated injections showed a coefficient of variation (CV) of <5.8 % at sample level (Fig. 2, panels a and b). The reproducibility of the

retention time assigned to the accurate mass-retention time components across replicated injections was also very high (Fig. 2, panel c), with an average CV for the identified peptides of <0.2 %. The variations in intensity of all of the matched peptide components between replicates were evaluated by comparing their corresponding values, as shown in Fig. 2, panel d, in which the data are distributed along a diagonal line with minor intensity deviations between matched cluster components. These results confirm the findings of previous studies indicating the highly consistent reproducibility of intensity measurements using label-free DIA [12, 26].

A total of 194 proteins (validated in two out of three technical replicates) were identified and quantified in 1,500 air-fixed macrophages. Table S1 (Electronic Supplementary Material) shows the molecular weights (mW), isoelectric point (pI), PLGS scores, the unique identified peptide number and sequence coverage of each protein, precursor RMS mass errors, product RMS mass errors, retention time errors, and their absolute quantification. Interestingly, protein quantity ranged over about three orders of magnitude (from 20 pg for Ig λ chain C to 12 ng for α actin). Examples of spectra for three selected proteins with different abundance (high, cathepsin D; medium, cofilin1; and low, phosphoglycerate mutase 1) are shown in Figs. S1, S2, and S3 (Electronic Supplementary Material).

The same number (1,500) of PFA-fixed cells was analysed using the two protein extraction protocols (PFA-A and PFA-B). Using the method described by Bagnato et al. [16] (PFA-A), we identified only 31 proteins (Fig. 3, panel a and Table S1, Electronic Supplementary Material). The second approach (PFA-B) allowed the identification of 59 proteins

Fig. 2 Analytical LC/MS^E reproducibility details of the detected exact mass-retention time (EMRT) components in the sample replicates obtained from 1,500 air-fixed cells. **a** Mass precision (RSD Mass) measurement of all EMRT clusters. **b** Coefficient of variation (CV) of the intensity measurement of all EMRT clusters. **c** CV of retention time (RT) of all EMRT clusters. **d** Binary comparison of the intensity measurements of the matched EMRT components of the replicate injections



(Fig. 3, panel a and Table S1 in the Electronic Supplementary Material), a number which is still far from that identified in air-fixed cells. The overlapping among the three lists of identified

proteins is shown in the Euler-Venn diagram (Fig. 3, panel b) and in Table S1 (Electronic Supplementary Material). It is noteworthy that proteins identified from PFA-fixed samples

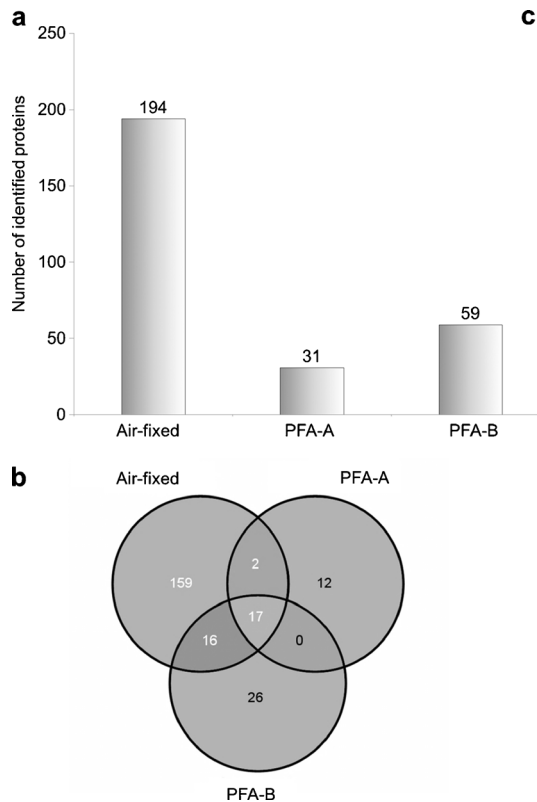
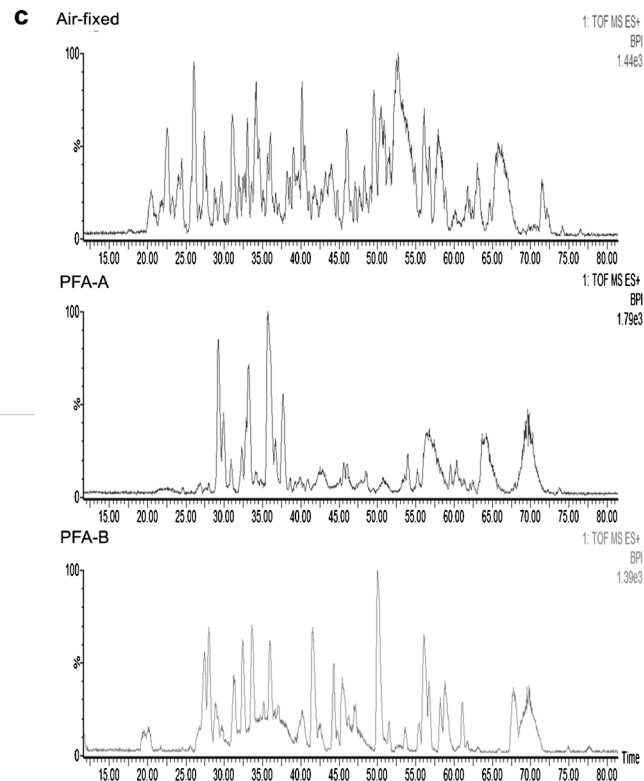
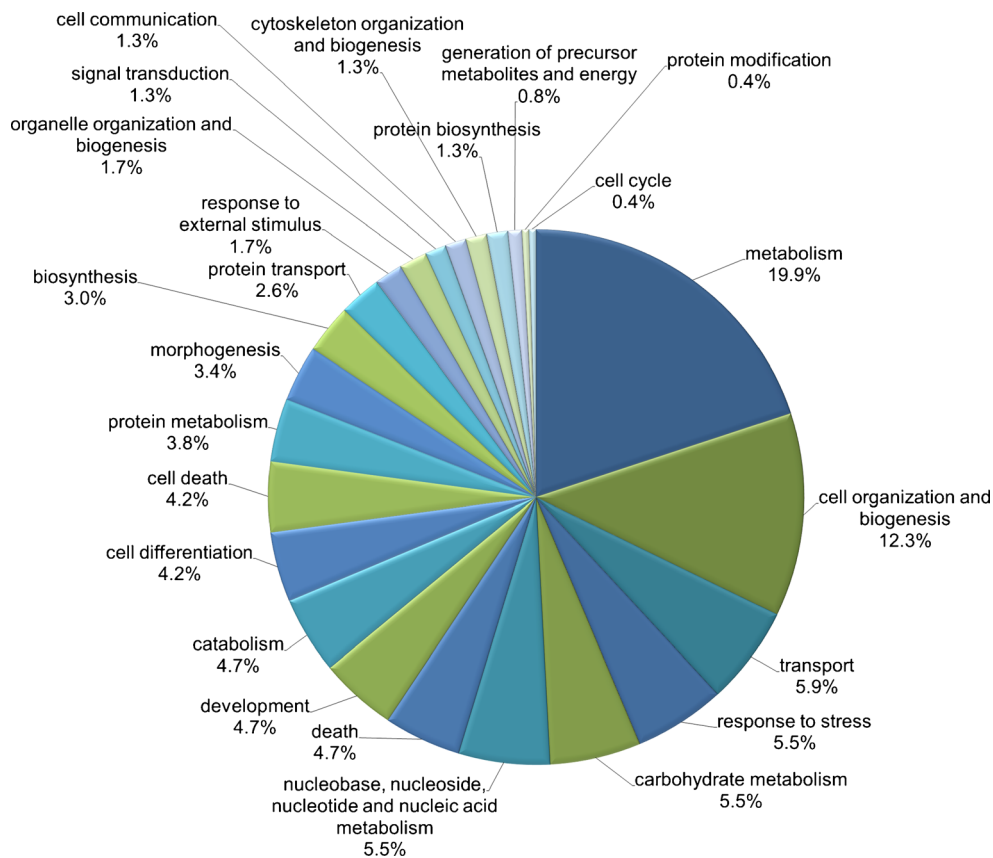


Fig. 3 Effect of fixation on the efficiency of proteomic analysis by LC/MS^E. **a** Number of proteins identified in 500 ng of total proteins, equivalent to approximately 1,500 cells, fixed in air or in paraformaldehyde (PFA), and processed according two different protocols (PFA-A and PFA-B). Only the proteins whose identification was validated in two out of



three replicates are considered. **b** Venn diagram of the proteins identified with the three fixation protocols. **c** Representative base peak chromatograms of tryptic peptide mixtures obtained from air-fixed cells or cells fixed with PFA and processed using protocols PFA-A and PFA-B. $n=3$ for each condition

Fig. 4 Gene ontology (GO) terms distribution obtained from air-fixed macrophages isolated by means of LCM. The proteins identified by LC/MS^E were clustered on the basis of biological processes



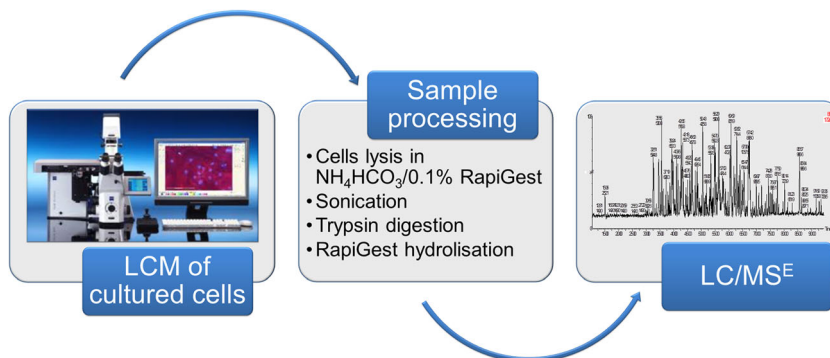
only are in general identified with a limited number of peptides, thus making really difficult any type of quantitative comparison.

Furthermore, as shown in Fig. 3, panel c, the chromatograms of PFA-fixed cells were characterised by a number of intense peaks that were not attributable to tryptic peptides but probably due to non-protein contaminants. One obvious concern when examining peptides extracted from PFA-fixed samples is the possibility of covalent modifications due to PFA fixation. The LC/MS^E data were therefore analysed to search for lysyl-formylated peptides. Under our conditions, the number of identified proteins remained unchanged, differently from what was reported by Hood et al. [27], on the presence

of lysyl-formylated residues in about 6.5 % of the identified peptides in PFA-fixed prostate cancer tissue. However, as the chemistry of PFA fixation is still not fully understood, we could not make a complete investigation of the possible peptide modifications induced by PFA.

Overall, the comparative analysis of protein identification using the three different protocols and the same number of cells showed that PFA fixation markedly reduces (by about one third) the number of identified proteins and proteome coverage. Reduced proteome coverage has also been observed by others when comparing frozen and PFA-fixed tissues [16]. We conclude that, under our conditions, the small number of identified proteins in PFA-fixed cells does not allow a

Fig. 5 Flowchart of the steps of proteomic analysis by means of LC/MS^E of in vitro cultured macrophages isolated by LCM after air fixation



comprehensive analysis of macrophage proteome, and that air fixation should be preferred when making proteomic analyses of cultured cells captured by means of LCM.

Determination of the minimum number of cells for proteomic analysis

In order to reduce the time required for cell isolation by means of LCM, we first established the minimum cell number necessary for satisfactory proteome coverage by comparing the number of identified proteins in samples of 1,500, 1,000 and 500 air-fixed macrophages. This number increased from 142 (500 cells) to 191 when 1,000 cells were processed, and no further increase was observed when 1,500 cells were analysed (194 proteins). The overlapping between proteins identified from 1,000 and 1,500 cells was 70 %. Overall, these data indicate that the analysis of samples containing 1,000 cells allows good proteome coverage, thus limiting the time required for cell capture.

Protein identification in air-fixed cells captured by means of LCM

For these experiments, monocytes were cultured in DuplexDish 50 mm culture plates characterised by a polyethylene-naphthalate membrane, and optimised for cell isolation by LCM. Under these conditions, macrophage morphology was preserved and was fully comparable to that of cells cultured on Primaria™. Having established that 1,000 cells was the minimum number required for proteomic analysis, adequate number of cells necessary for three technical replicates was collected by means of LCM (Fig. S4, Electronic Supplementary Material). A total of 136 proteins distributed over three orders of magnitude were identified in the samples (Table S2, Electronic Supplementary Material), which is slightly lower than that recovered from the same number of cells collected by scraping (191) (Table S3, Electronic Supplementary Material). This difference may be due to partial protein degradation during microdissection, the time of which should be therefore minimised as much as possible [28], or to some differences in the percentage of morphotypes captured by LCM (50 % spindle and 50 % round macrophages) with respect to that of the whole lysate obtained by scraping.

Gene ontology in cells captured by means of LCM

In order to identify the GO terms that were over-represented in both the cell component and functional categories, the list of identified proteins was analysed using AmiGO for the GO annotation search and CateGORizer for the classification of GO terms [22, 23, 18]. The classification by cell localisation showed that 62 proteins related to the cytosol, 38 to the plasma

membrane, 35 to the cytoskeleton, 17 to the cytoplasmic vesicles, 7 to the endoplasmic reticulum and 6 to the signalosome (Table S4, Electronic Supplementary Material). The annotation of biological processes revealed that most of the proteins were involved in metabolism (19.9 %), cell organisation and biogenesis (12.3 %), transport (5.9 %), response to stress (5.5 %) and carbohydrate metabolism (5.5 %) (Fig. 4). Furthermore, there were small but representative classes of proteins involved in cell differentiation, cell communication and signal transduction.

Conclusions

To the best of our knowledge, this is the first proteomic analysis of *in vitro* cultured cells collected by LCM and analysed by means of LC/MS^E. The workflow, summarized in Fig. 5, was set up both in terms of cell fixation and number of cells required for the analysis: 136 proteins were identified in 1,000 air-fixed cells, providing a better proteome coverage than that found in PFA-fixed cells, with preservation of the cell morphology.

We acknowledge that a limited number of proteins have been identified in our analysis, likely depending on several factors, such as MS instrument limitation, sample preparation, but also cell type. Specifically, the enhanced sensitivity and improved duty cycle of the new instruments will certainly enable a great increase of identified proteins with higher sequence coverage and confidence. In addition, a partial protein degradation occurring during microdissection can reduce the number of identified proteins, even if in our conditions this effect is negligible, as shown by the comparison of the results obtained when cells are collected by LCM (136 proteins) with those obtained with cell lysate (191 proteins). Lastly, the nature of the cell population under investigation could be relevant, probably as the result of different proteome dynamic ranges.

Although these limitations exist, the application of the present workflow to MDMs showing different morphotypes allowed the identification of proteins that were differentially expressed in the two cell subsets (in preparation).

We expect our proposed workflow to be useful for profiling the proteome of a minimum number of heterogeneous cultured cells when the preservation of morphology is required for selection and other approaches such as sorting are unsuitable because of the absence of specific antigens distinguishing between two distinct morphotypes of the same cell type. In addition, notwithstanding obvious limitations exist for the extrapolation of *in vitro* data to an *in vivo* situation, the proposed workflow might be useful to investigate how changes of the microenvironment as well as pharmacological manipulations can affect the proteome profile of the distinct cell phenotypes that reside within the tissue.

Finally, this protocol may offer a technical advance in the “microproteomic” field, in which the ambitious goal is to dissect and characterise specific anatomical regions within a single cell, i.e. cell body or cell projections [29].

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