Research Article

Detergent addition to tryptic digests and ion mobility separation prior to MS/MS improves peptide yield and protein identification for *in situ* proteomic investigation of frozen and formalin-fixed paraffin-embedded adenocarcinoma tissue sections

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The identification of proteins involved in tumour progression or which permit enhanced or novel therapeutic targeting is essential for cancer research. Direct MALDI analysis of tissue sections is rapidly demonstrating its potential for protein imaging and profiling in the investigation of a range of disease states including cancer. MALDI-mass spectrometry imaging (MALDI-MSI) has been used here for direct visualisation and *in situ* characterisation of proteins in breast tumour tissue section samples. Frozen MCF7 breast tumour xenograft and human formalin-fixed paraffin-embedded breast cancer tissue sections were used. An improved protocol for on-tissue trypsin digestion is described incorporating the use of a detergent, which increases the yield of tryptic peptides for both fresh frozen and formalin-fixed paraffin-embedded tumour tissue sections. A novel approach combining MALDI-MSI and ion mobility separation MALDI-tandem mass spectrometry imaging for improving the detection of low-abundance proteins that are difficult to detect by direct MALDI-MSI analysis is described. *In situ* protein identification was carried out directly from the tissue section by MALDI-MSI. Numerous protein signals were detected and some proteins including histone H3, H4 and Grp75 that were abundant in the tumour region were identified.

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1 Introduction

Enhancing our understanding of the process of cancer relies heavily upon identification of diagnostic biomarkers, *i.e.* proteins or other biomolecules that are correlated to disease states or represent potential therapeutic targets. Stress proteins, the HSP families and some membrane-associated proteins have been found to be involved in tumour progression and represent potentially novel chemotherapeutic targets [1–4]. The distribution of such biomarkers and monitoring changes occurring in their expression during tumour development is of great interest. Such data can be analysed for correlation with known clinical and prognostic indices or be subjected to improved statistical analysis to yield novel tumour classification systems.

Originally described by Caprioli *et al.* [5], MALDI- mass spectrometry imaging (MALDI-MSI) is a technique that enables the generation of images and profiles of compound

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Abbreviations: ANI, aniline; **$CHCl_3$,** chloroform; **EtOH,** ethanol; **FFPE,** formalin fixed paraffin embedded; **IMS,** ion mobility separation; **MALDI-MSI,** MALDI-mass spectrometry imaging; **OcGlc,** octyl-α/β-glucoside; **SA,** sinapinic acid

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(proteins, lipids, drugs and small molecules) distribution directly from a tissue section [6–9]. Several molecules can be localised and visualised in a single experiment. Indeed the technique allows the acquisition of multiple MS spectra from a tissue section. These spectra are then compiled together so that the relative abundance or intensity of each detected ion signal within the tissue section can be represented as a molecular map or image. MALDI-MSI enables the screening of proteomic information directly from a tissue section with no requirement for specific targets and could aid the identification of target proteins whose functionality and expression are strongly related to tumour progression. Several papers have already reported the successful use of the technique for the direct analysis of proteins in biological tissue sections and it has been used to plot their spatial distribution within different regions of the section [10–12]. MALDI-MSI has also been used to investigate changes in protein profiles within tumour tissue sections [13] and changes occurring in response to therapeutic agents [14]. These studies show that MALDI-MSI can be used to distinguish between cancerous and normal tissues, thus highlighting the benefit of the technique for rapid characterisation of a disease at the protein level.

Several improvements and advances have been made in the technique including the use of robotic devices to achieve accurate matrix deposition on the tissue section. This has been found to be useful for decreasing protein delocalisation within the tissue section as well as for improving the sensitivity of the technique [15, 16]. Sample pre-treatment including *in situ* digestion has been found to increase the amount of protein information obtained by direct MALDI-MSI analysis and also improve the detection of some proteins not detected by direct MALDI-MSI due to their molecular weight. Lemaire *et al.* [17] reported a strategy combining the use of *in situ* micro-digestion and *in situ* extraction of proteins in formalin-fixed paraffin-embedded (FFPE) tissue sections. This approach facilitated and improved the identification and localisation of proteins within such samples [17, 18]. The use of *in situ* digestion on frozen tissue sections that allowed the identification and distribution of numerous signals within rat brain tissue sections has also been reported [19, 20].

Here we report the development and the improvement of the *in situ* methodology by incorporating a surfactant in the trypsin digest protocol in a study of frozen and FFPE breast tumour tissue sections. The methodology developed in this work aims at enhancing the detection and identification of proteins that are of low abundance or difficult to detect by direct MALDI-MSI. To achieve this direct protein analysis by MALDI-MSI followed by *in situ* enzymatic cleavage was performed. The methodology was shown to be successful for the identification of numerous protein signals and was able to differentiate between tissue regions within the section. The selectivity and benefits of the methodology employed are discussed. The signal overlap generally encountered after performing on-tissue digestion making direct protein identification challenging is here addressed by using ion mobility separation (IMS) prior to MALDI-MSI and MALDI-MS/MS. The advantages of using the combination of these technologies following *in situ* digestion are discussed.

2 Materials and methods

2.1 Chemicals and materials

Modified sequence-grade trypsin was purchased from Promega (Southampton, UK). All other materials, including sinapinic acid (SA), CHCA, aniline (ANI), ethanol (EtOH), chloroform ($CHCl_3$), xylene, octyl-α/β-glucoside (OcGlc), TFA, haematoxylin, eosin and indium-tin-oxide glass slides were purchased from Sigma-Aldrich (Dorset, UK).

2.2 Tissue samples

2.2.1 MCF7 breast tumour xenograft samples

Animals: Investigations and all animal procedures were carried out under a project licence issued by the UK Home Office and guidelines from the United Kingdom Co-ordinating Committee on Cancer Research were followed [21]. Female Balb/C immunodeficient nude mice (Harlan UK, Blackthorn, UK) aged 6–8 wk were used. Mice received CRM diet (SDS, Witham, UK) and water *ad libitum*. Animals were kept in cages in an air-conditioned room with regular alternating cycles of light and darkness.

Transplantation and treatment of tumour samples: MCF7 breast tumour xenografts were obtained from the Institute of Cancer Therapeutics, Bradford, UK. The transplantation of tumour is described as follows: briefly 24 h prior to tumour transplantation, in order to stimulate tumour growth, a slow-release oestrogen pellet (Innovative Research, Sarasota, FL, USA) was implanted subcutaneously in the upper dorsal area of the animal under brief general inhalation anaesthesia. MCF-7 human mammary adenocarcinoma tumours were excised from a donor animal, placed in sterile physiological saline containing antibiotics and cut into small fragments of approximately 2 mm^3. Under brief general inhalation anaesthesia tumour fragments were implanted in the left and right flanks of each mouse using a trocar. Once the tumours had reached a volume of 200–400 mm^3, as measured by callipers, mice were sacrificed. Tumours were excised and immediately snap-frozen in liquid nitrogen and stored at $-80°C$ to minimise protein degradation and maintain the original sample morphology until further analysis.

2.2.2 Human FFPE breast tumour samples

Ex vivo human breast tumour tissue samples were obtained following fully informed patient consent and local ethical

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committee approval. Tissue samples were fixed in 10% buffered formalin for 24 h, dehydrated in 70% EtOH and paraffin embedded; 5 μm sections were cut using a cryostat (Leica Microsystems, UK) and mounted onto a histological glass slide. FFPE tissue sections were stored at room temperature until further analysis.

2.3 Tissue preparation

Frozen MCF7 xenograft tissue samples were cut using a cryostat (Leica Microsystems) operating at $-20^{\circ}C$. Five 10 μm sections were cut and thaw-mounted onto either a TLC foil, from which the stationary phase had been previously removed, or an indium-tin-oxide glass slide. Rinsing procedures were performed to increase the MS data quality. For frozen tissue sections, the rinsing steps consisted of immersing them for approximately 1 min in EtOH solutions at different concentrations (from 50 to 90%) followed by a 30 s wash in $CHCl_3$. The use of $CHCl_3$ for washing tissue sections was first reported by Lemaire *et al.* and aims at decreasing the amount of lipids from tissue sections prior to direct protein analysis with MALDI-MSI [22]. Sections were then allowed to dry at room temperature before digestion and matrix deposition. For FFPE tissue sections, paraffin wax was first removed according to the procedures described previously [16, 23]. Briefly tissue sections were twice immersed in xylene for 5 min and then rehydrated for 2 min in EtOH solutions (from 100 to 70%). After rehydration the section was washed for 30 s in $CHCl_3$ in order to remove lipid residuals.

2.4 Direct protein analysis by MALDI-MSI

Direct protein analysis was performed only on frozen tissue sections. HE staining was performed for a comparative histological study. For direct protein profiling SA (prepared as 25 mg/mL in ACN:water:TFA, 50%:50%:0.2% in volume) was used as a matrix. Several spots (300 nL each) of the matrix solution were deposited onto specific regions of the section that was differentiated by the histological study. For imaging experiments matrix solution (SA prepared at 25 mg/mL in 100% EtOH:0.5% TFA) was spray coated onto the section using a gravity fed pneumatic air spray gun set to 40 psi. Approximately 15–20 spray cycles were required to give a homogeneous matrix coverage. MALDI-MSI data were acquired in the linear mode using an Applied Biosystems Voyager DE™ PRO equipped with a 337 nm N_2 laser operating at a repetition rate of 20 Hz. Full scan mass spectra were recorded in positive ion mode from 2000 to 50 000 Da. About 200 laser shots were averaged to create a spectrum from each matrix spot. Calibration of the mass spectrometer was performed using ion signals from α and β chains of haemoglobin. Protein ion images were generated with Biomap 3.7.5 software, which can be used to measure the amplitude of selected mass signals and to reconstruct 2-D ion density maps. Profiles were preprocessed using *SpecAlign* [24, 25] (which has the ability to perform spectral alignment as an additional pre-processing step).

2.5 *In situ* digestion

In situ digestion was performed using a 0.05 μg/μL trypsin solution made up in deionised water containing 0.1% of OcGlc. The trypsin solution was deposited on the section using an automatic pipette or a Sun-Collect MALDI-Spotter (SunChrom). Using the Sun-Collect spotter 100–150 nL of trypsin solution/spot was deposited onto the section. The spot-to-spot distance was set at 400 μm. The section was then incubated for 2 h in a humid chamber at 37°C with 5% CO_2 to perform enzymatic digestion. Following enzymatic digestion, matrix deposition was performed using either an automatic pipette or a robotic printer. An ionic matrix [26–28], CHCA mixed with ANI (CHCA/ANI), was used as the matrix for MALDI-MS analysis of the resulting peptides. Matrix solutions were made at 10 mg/mL in ACN:water:TFA (1:1:0.1 in volume).

2.6 *In situ* peptide analysis by MALDI-MSI and direct protein identification with IMS MALDI-MS/MS

MALDI-MSI data were acquired in the reflector and positive mode using either a MALDI SYNAPT™HDMS system (Waters Corporation, Milford, MA) operating with a 200 Hz Nd:Yag laser, or an Ultraflex™II MALDI-TOF/TOF instrument (Bruker Daltoniks, Bremen, Germany) equipped with a Smartbeam™ laser. When using the MALDI SYNAPT™HDMS system, full scan mass spectra were recorded from 600 to 2500 Da. Images were generated and reconstructed using Biomap 3.7.5 software. Standards consisted of a mixture of poly(ethylene glycol) standards ranging between *m/z* 400 and 3000 Da. When using the Ultraflex™II MALDI-TOF/TOF instrument, full scan mass spectra and images were recorded from 600 to 5000 Da. FlexImaging™ 2.0 software (Bruker Daltoniks) and Biomap 3.7.5 software were used for image reconstruction. Standards for spectral calibration consisted of a mixed solution of peptides ranging between 900 and 3500 Da.

MALDI MS/MS analyses were acquired using the MALDI SYNAPT™HDMS operating in IMS mode directly from the digested tumour tissue sections. A full description of the MALDI SYNAPT™HDMS instrument has been previously reported [29]. Briefly the configuration of the instrument allows the separation of ions in the Trap T-Wave™ (pre-IMS) and the Transfer T-Wave™ (post-IMS), operating as two separate collision cells. Ion fragmentations were performed in the Transfer T-Wave™ after IMS and optimised based on the precursor ion mass [30].

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The obtained spectra were processed in MassLynx^{TM}. Spectral processing consisted of smoothing, baseline correction and peak centroiding. Spectra were then processed with the MaxEnt 3 algorithm, which aims at enhancing the resolution and the S/N ratio [31]. MS/MS spectra were submitted to a MASCOT (Matrix Science, Boston, MA) query search and searched against the Swiss-Prot database. Within the MASCOT search engine, the parent and fragment ion tolerances were set at 30ppm and ± 0.2 Da, respectively. The criteria also included up to two missed cleavages and the variables modifications allowed were histidine/tryptophan oxidation and methionine oxidation. *De novo* sequencing was performed manually and using the PepSeq^{TM} *de novo* interactive MS/MS sequencing tool. The parent and fragment ion tolerances were set at 0.1 Da and the threshold was set at 1%. Protein Blast searches against the Swiss-Prot database were also performed to confirm tryptic sequences.

3 Results and discussion

3.1 Direct protein analysis in breast tumour xenografts with MALDI-MS profiling and MALDI-MSI

The ability to use mass spectral protein profiling and imaging to differentiate tissue section regions has the

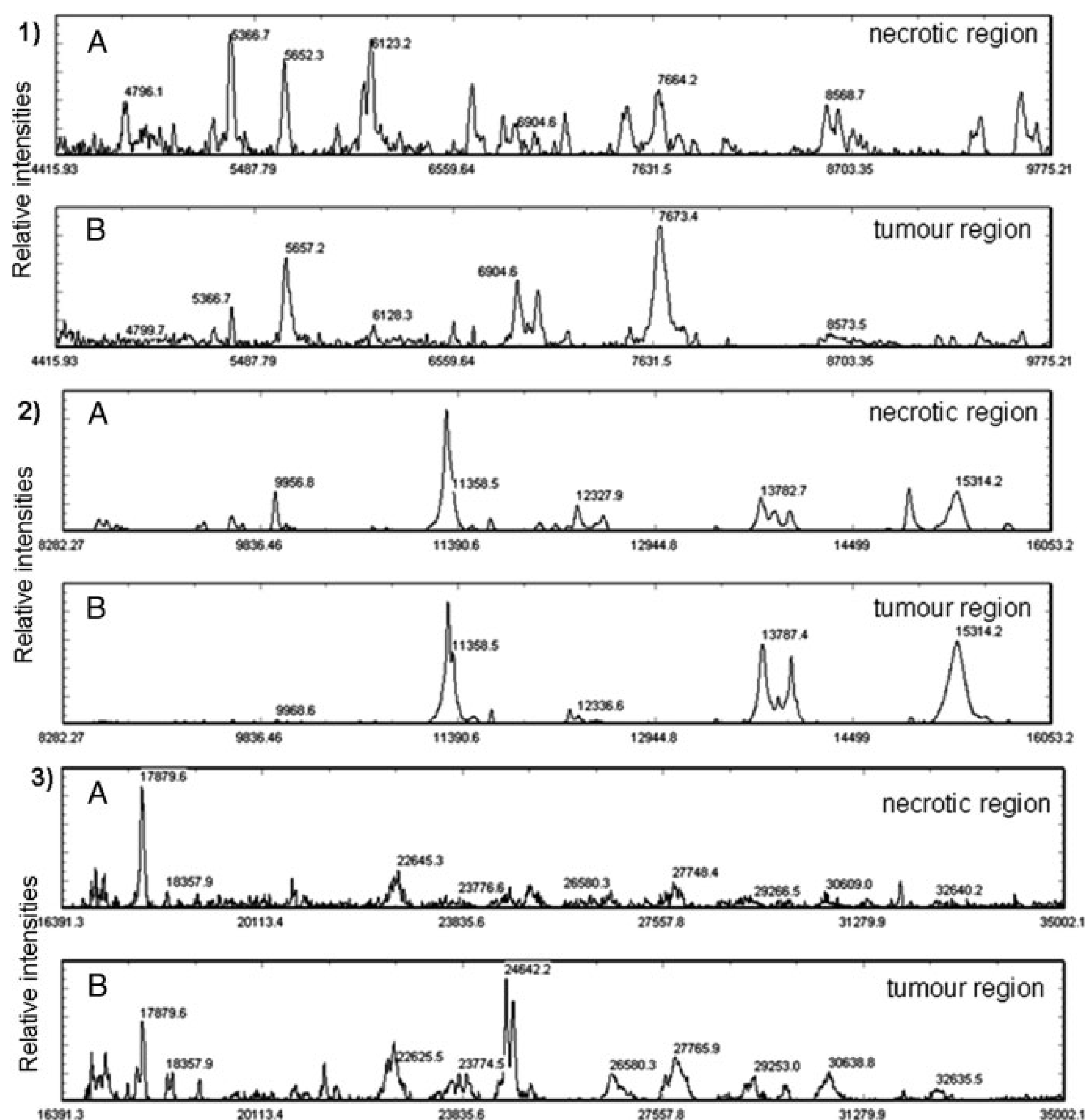

Figure 1. MALDI-MSI direct protein profiles obtained after data acquisition and processing. Spectra are displayed for both necrotic and tumour regions in the *m/z* range from 4400 to 35 000.

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potential to improve the selection and identification of those proteins whose expression is closely associated with tumour growth and/or progression and thus improve the development of chemotherapeutic agents. In this study, for direct protein analysis with MALDI-MS profiling and imaging, xenograft tissue samples were used. Xenografts are human tumours or human cell lines grown in immunodeficient mice, which are extensively used in cancer research. The progression of a large number of easily observable synchronized tumours can be monitored, so that initiation of treatment can begin when the tumours reach an optimal size [32].

Five consecutive MCF7 xenograft tissue sections were used for direct MALDI-MS profiling analysis. Matrix spots were deposited onto the section using an automatic pipette in order to generate average mass spectra of the different regions (see Supporting Information). Hence protein profiles were generated from both tumour and necrotic regions. Figure 1 shows the resulting MALDI mass spectra after data pre-processing using SpecAlign software (*i.e.* baseline correction, noise removal, normalisation and spectral alignment). SpecAlign can also be used to generate average spectra: spectra are displayed in a mass range from 3000 to 35 000 Da, as signals were not detected above 35 000 Da. Many protein signals were observed and different profiles were obtained from tumour and necrotic regions in terms of signal intensities. The necrotic area was found to be data rich in the *m/z* range between 4000 and 10 000 compared with the tumour region. Necrosis is a non-specific mode of cell death in which lysis of cells takes place. The

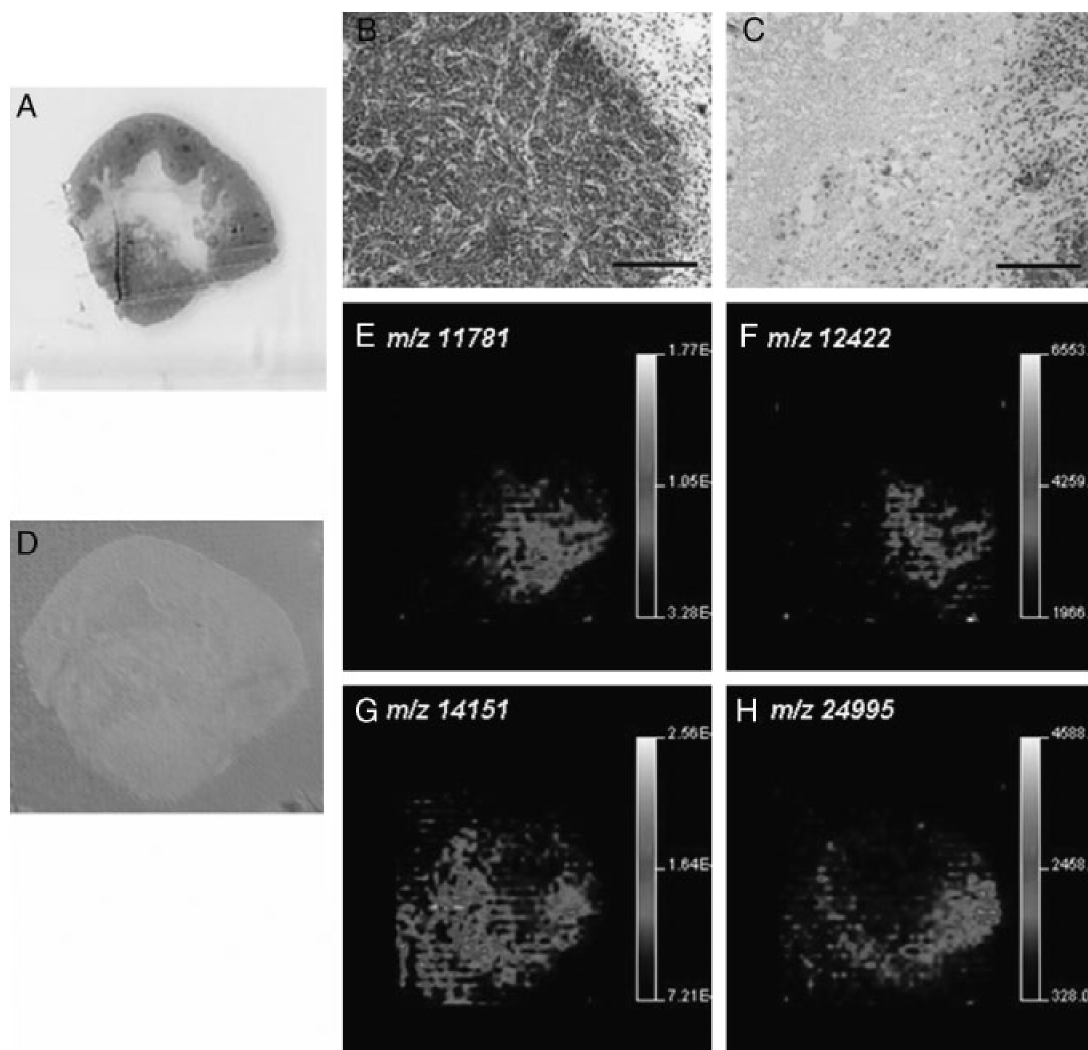

Figure 2. Optical and MALDI-MSI images of an MCF7 breast tumour xenograft section. (A–C) The HE images of a xenograft section, tumour cells and necrotic region, respectively; the scale bars in (B) and (C) correspond to 200 μm. (D) The section after matrix coverage before acquiring MALDI images. (E–H) MALDI-MS images of the distribution of *m/z* 11 781, 12 422, 14 151 and 24 995, respectively, within the section. These signals were observed in all tissue sections analysed ($n = 5$).

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increased number of observed signals in the necrotic protein profiles could relate to proteolysis occurring during cell death. Some signals were mostly detected in the tumour area including those at *m/z* 6904.6 and 7673.4. The protein at *m/z* 7673.4 may correspond to the doubly charged species of haemoglobin alpha. These data are in agreement with those already reported by Reyzer *et al.* [14], in which this signal was found to be abundant in transgenic breast tumour samples.

It has been reported that histones represent good targets for MALDI-MSI in tumour tissue sections [14, 33]. The obtained profiles from the tumour region were also data rich in the histone mass range ($m/z = 10\,000$ to 15 000). Signals at $m/z = 11\,358$, 13 787 and 15 314 are detected in the tumour area and may correspond to histone species.

Although the S/N ratio of protein signals decreased considerably in the mass range above 30 000 Da, the obtained protein profiles allow the detection of protein signals in the tumour region. This can clearly be seen in the zoomed-in spectra (*m/z* from 17 000 to 35 000 Da). Signals at *m/z* 24 642.2, 24 772.72, 27 765.9 and 30 638.8 were detected only in the tumour region. Using MALDI-MS for direct protein profiling and imaging within the tissue section it was possible to correlate protein abundance to a specific area of the section (see Supporting Information).

MALDI-MS imaging was also performed to investigate protein distribution within the section. Figures 2A–C show images obtained following histological staining of an MCF7 xenograft section; protein distributions within the tumour and necrotic area were generated. Figure 2D shows the section after matrix coverage. MALDI-MS images were acquired at a spatial resolution set at 250 μm. The protein distributions shown in Figures 2E–I were in good agreement with results from profiling analysis as well as the histological staining. Signals at *m/z* 14 154 and 24 995 were mainly located in the

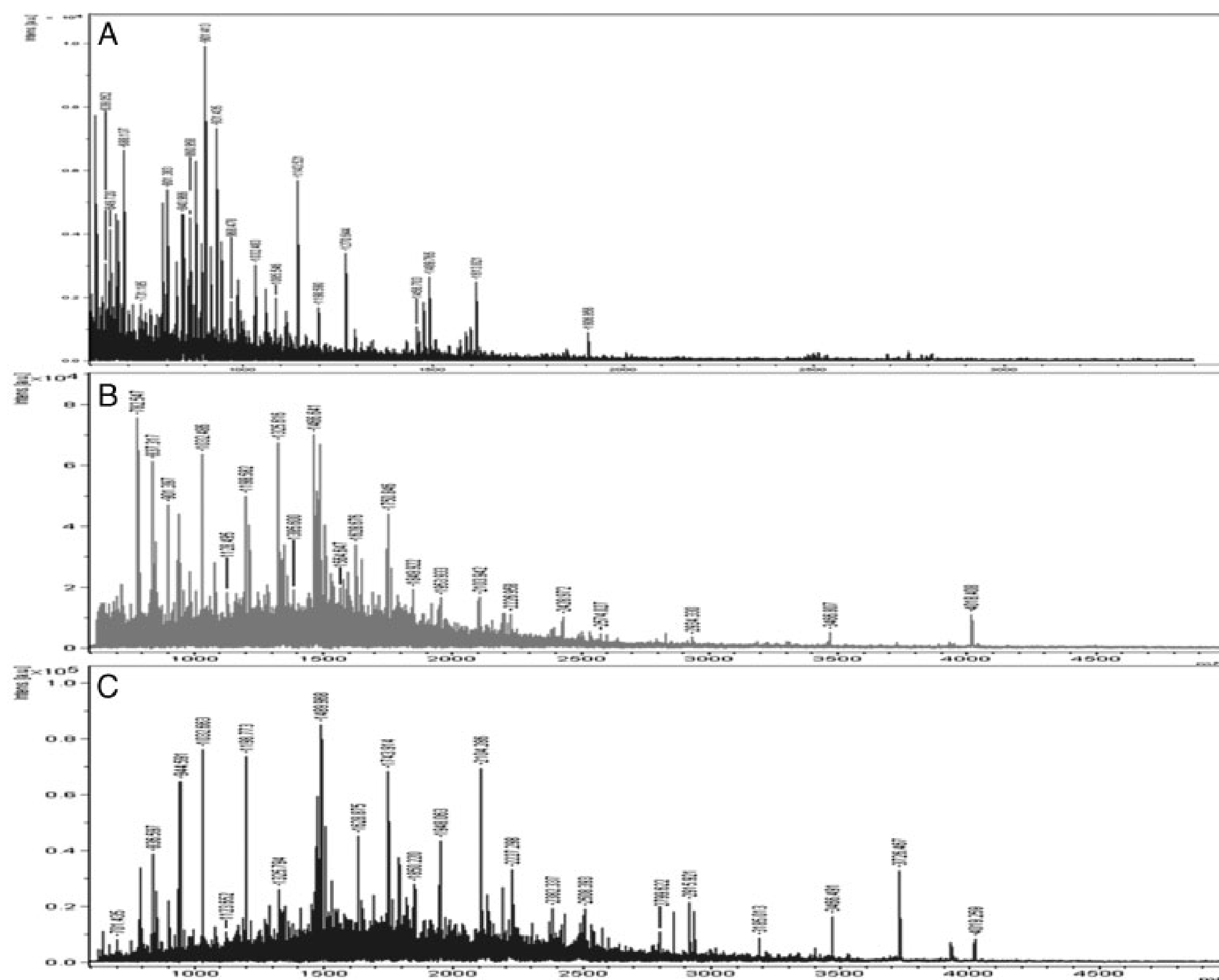

Figure 3. Evaluation of method improvement for *in situ* digestion. Figure 3A displays the observed peptide profiles after an *in situ* digestion performed with trypsin in water at room temperature. Figure 3B shows the observed peptide profiling after *in situ* digestion with a trypsin solution containing 0.1% of OcGlc for 18 h at room temperature. Figure 3C reports the *in situ* digested protein profile when using a trypsin solution containing 0.1% of OcGlc and incubating the section at 37°C for 2 h.

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tumour region as observed in the profiling experiments. Alternatively the protein signal at *m/z* 12 422 was predominantly found in the necrotic region compared with the signal at *m/z* 7762, which was found in both regions. Additional observations can be made from these data. Some protein delocalisation can be observed on the resulting images. This may be due to the matrix coverage method, which was performed by manually spraying the matrix solution onto the section. Several papers have reported the minimisation of protein delocalisation from matrix application by using other devices such as robotic printers, which allow an improved matrix deposition [15]. However, the MALDI images were found to be in general agreement with the results from direct profiling analysis. We have previously reported a study of protein distribution in MCF7 breast tumour xenografts using MALDI-MSI combined with principal component analysis to identify protein signals characteristic of different regions within the tissue sections [25]. The data presented here are in good agreement with our previous work.

3.2 *In situ* protein identification by MALDI-MSI

3.2.1 An improved method for direct protein identification in frozen MCF7 breast xenograft tissue sections

Direct protein imaging and profiling analysis from xenograft tissue sections with MALDI-MS enabled the acquisition of protein distribution information. However, to help the understanding of the biological processes occurring during tumour progression, identification of these proteins is required. The possibility of identifying such analytes directly from tissue sections has been assessed. To improve the peptide extraction and direct analysis of digested protein, micro-digestion was performed using a robotic printer. Micro-digestion performed by using an automatic printer has been previously shown to yield good sensitivity and reproducibility [16, 19, 20]. Here *in situ* digestion and matrix deposition on xenograft tissue sections were performed using a SunCollect automatic spotter (see Supporting Information). Mass spectra were obtained from each printed spot. Figure 4c displays a mass spectrum acquired after *in situ* digestion and matrix deposition. Numerous peptide signals were detected up to *m/z* 4000 with an $S/N>3$ (see Supporting Information). MS/MS analyses were also performed and allow the identification of several proteins. Peptide signals resulting from the most abundant proteins such as actin (*m/z* 1198.7), haemoglobin (*m/z* 1529.7) and albumin (*m/z* 1467.8) were readily detected. These signals were used for spectral recalibration purposes. This aimed at improving mass accuracy as well as facilitating database search results.

In order to improve the detection of low-abundant protein and high mass proteins several experimental conditions including different reagents, humidity, digestion time and temperature were evaluated. Figure 3 shows a comparison of results obtained after *in situ* digestion

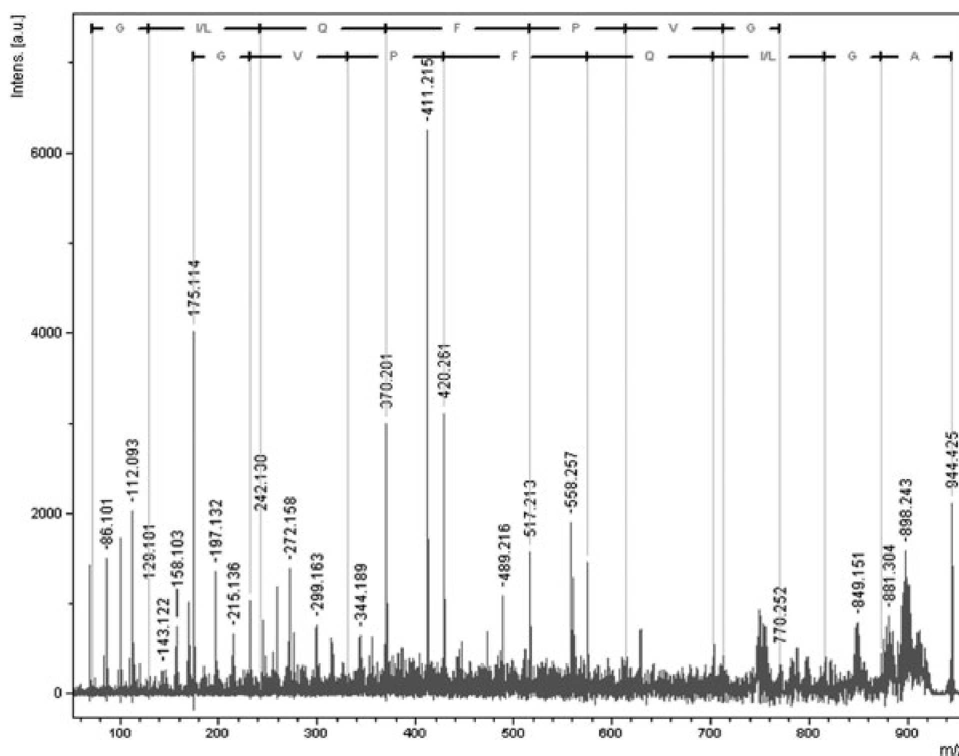

Figure 4. MS/MS spectrum of the ion at *m/z* 944, which has been assigned to histone H2A.

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performed with trypsin in either water (Fig. 3A) or in a solution containing the detergent OcGlc (Figs. 3B and C). Examination of the data obtained from *in situ* digestion with trypsin in water only (Fig. 3A) shows that the majority of the signals detected were from the most abundant proteins present in tissue samples, *i.e.* actin, haemoglobin and albumin as described above. The use of OcGlc has been found to greatly improve the digestion process. OcGlc is a non-ionic detergent and it has been reported to enhance protein solubilisation, thus improving in gel protein digestion [34–36]. Here OcGlc was added to the trypsin buffer for *in situ* digestion. Adding 0.1% of OcGlc to the trypsin solution improved the *in situ* digestion of several proteins and therefore numerous signals in addition to those resulting from the most abundant proteins were detected. The concentration, *i.e.* 0.1%, used has been found to be compatible with direct MALDI-MSI analysis. Figure 3B shows the resulting peptide profile after *in situ* digestion with a trypsin solution containing OcGlc followed by incubation at room temperature for 18 h. These data show that incubating the section in a humid environment after trypsin deposition enhances the activity of the trypsin and improves the digestion of low-abundant proteins. This also increased the number of signals detected and improved protein identification. However, optimal trypsin performance was found to be after a digestion time of 2 h at 37°C with a trypsin solution containing 0.1% of OcGlc in a humid chamber (Fig. 3C).

Table 1. List of some observed peptides after *in situ* digestion and direct MALDI-MSI analysis

Protein assignment	Accession number	Protein mass (Da)	Observed *m/z* with MALDI-MSI	Sequence	Score	Molecular function
Actin, aortic smooth muscle	P62736	41 982	1198.7	AVFPSIVGRPR	25	Cell mobility
			1790.9	SYELPDGQVITIGNER	82	
Actin, cytoplasmic1	P60709	41 710	1954.1	VAPEEHPVLLTEAPLNPK	48	Cell mobility
Albumin	P02768	69 367	1467.8	RHPDYSVVLLLR	60	
Estrogen receptor beta	Q92731	59 178	837.4	RSGGHAPR	15+*de novo*	Nuclear hormone
Fibroblast growth factor 13	Q92913	27 564	844.5	SGKVTKPK	67, PMF	Nervous system development
			958.6	VTKPKEEK		
			1361.7	MSGKVTKPKEEK		
Haemoglobin subunit alpha	P01942	15 085	1529.7	VGAHAGEYGAEALER	115	
Grp75, HSP 70 kDa, mitochondrial	P38646	73 635	715.4	LVGMPAK	18	Molecular chaperone, control of cell proliferation and call aging
Histone H2A	P0C0S5	13 545	944.5	AGLQFPVGR	26	Gene regulation
			2104.2	HLQLAIRNDEELNKLLGK	24	
			2915.6	VGAGAPVYLAAVLEYLTAEILELAGNAAR	44	
Histone H2B	P33778	13 819	901.5	LAHYNKR	25	
			1743.8	AMGIMNSFVNDIFER	28	
Histone H3	Q6NXT2	15 204	788.5	KLPFQR	28	
			831.5	STELLIR	26	
			1032.6	YRPGTVALR	18	
			1489.9	RSAPATGGVRKPHR	55, Blast	
Histone H4	P62805	11 360	1325.7	DNIQGITKPAIR	35	
			1466.8	TVTAMDVVYALKR	29	
High mobility group protein B1	P09429	24 878	1944.9	RPPSAFFLFCSEYRPK	35	DNA binding
Metastasis-associated protein 2	O94776	75 023	2428.2	DISSSLNSLADSNAREFEEE SK	50, PMF	Regulation of gene expression as repressor and activator.

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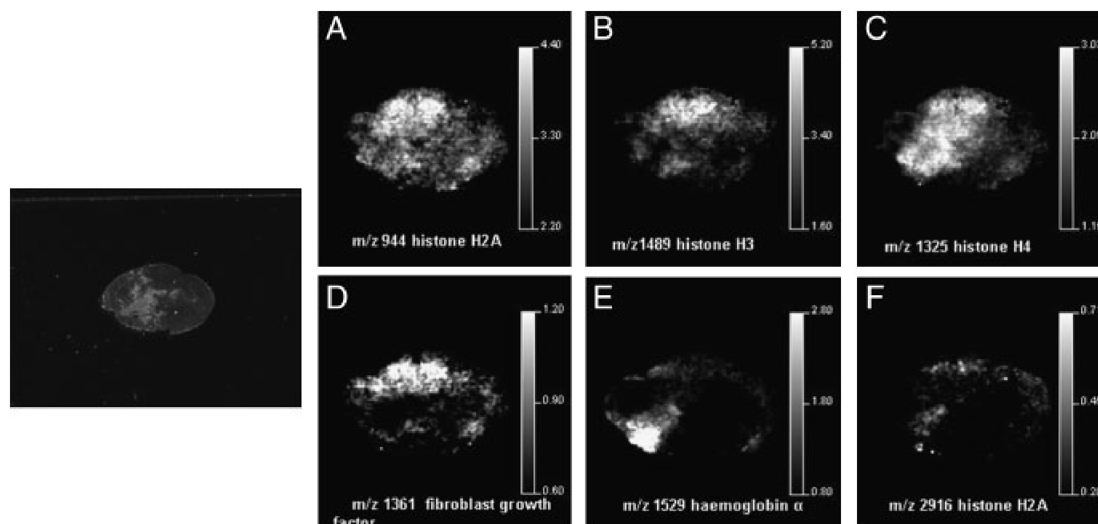

Figure 5. MALDI-MS images of peptide distribution within an MCF7 xenograft tissue section.

In addition to peptides resulting from the most abundant proteins including actin, haemoglobin and albumin, signals from histone species and stress proteins such as Grp75 (a member of the HSP 70 family, resident within the mitochondria [37]) were identified. Direct protein analysis (profiling and imaging) results were found to be data rich in the histone mass range. These results obtained from direct protein identification were in good agreement with those from direct protein analysis as numerous signals were assigned to histone species, including histone H2A, H2B, H3 and H4. Figure 4 shows the MS/MS spectrum of *m/z* 944, which was assigned to histone H2A. Table 1 gives a list of some observed and identified signals in both tumour and necrotic regions: observed *m/z* values with direct MALDI-MS/MS analysis and protein assignments are reported. The signal at *m/z* 715 has been assigned to Grp75, an HSP 70 kDa (Hsp70) using direct MALDI-MS/MS. Hsp70 exists as several isoforms ranging from 66 to 78 kDa and these are known to be molecular chaperones. Hsp 70 has been reported previously to be highly expressed in tumour tissues, in a study carried out using immunohistochemistry methods [2]. Here the obtained MALDI-MS data were in good agreement with those results.

MALDI-MS imaging experiments were also performed. MALDI-MS images were acquired at a spatial resolution of 150 μm using an Ultraflex^{TM}II MALDI-TOF/TOF instrument. Figure 5 shows MALDI images of the distribution of some of the identified peptides within an MCF7 tissue section. Difference in intensities can be observed between the necrotic and tumour areas. The signal at *m/z* 1489 corresponding to histone H3 was mainly located in the tumour area while the signal at *m/z* 944 corresponding to histone H2A was found in both the necrotic and the tumour regions. The *in situ* digestion method described here enables the identification and localisation of proteins that are hardly detected by direct MALDI-MSI protein analysis due their high masses or low abundances in tissue samples.

It has been demonstrated that direct protein analysis and identification through *in situ* digestion of frozen MCF7 breast tumour xenograft sections has been achieved using MALDI-MSI. However, the method still requires further development in order to improve direct MS/MS analysis. Several other biomolecules such as lipids and also matrix adducts can interfere with the MS/MS fragmentation of the analyte of interest.

3.2.2 *In situ* protein identification in human FFPE breast cancer tissue sections

FFPE tissue samples are the commonest type of preserved tissue in clinical practice. FFPE is by far the most convenient method to conserve tissue samples. These tissue samples are widely used by histopathologists for evaluating the diagnosis and prognosis of cancers and other diseases. The ability to analyse proteins directly from archival tumour tissue samples that have known outcomes is of tremendous clinical interest. However, direct protein analysis by MALDI-MSI analysis of FFPE tissue sections remains challenging, because the presence of protein cross-linking *via* methylene bridge formation makes the analysis difficult to perform. An alternative approach to get as much information as possible from these tissues is to perform *in situ* enzymatic digestion and then to analyse the resulting peptides for protein identification process. This method has been developed and first described by Lemaire *et al.* [17].

Here direct protein identification has been performed on FFPE breast tumour tissue section using the method

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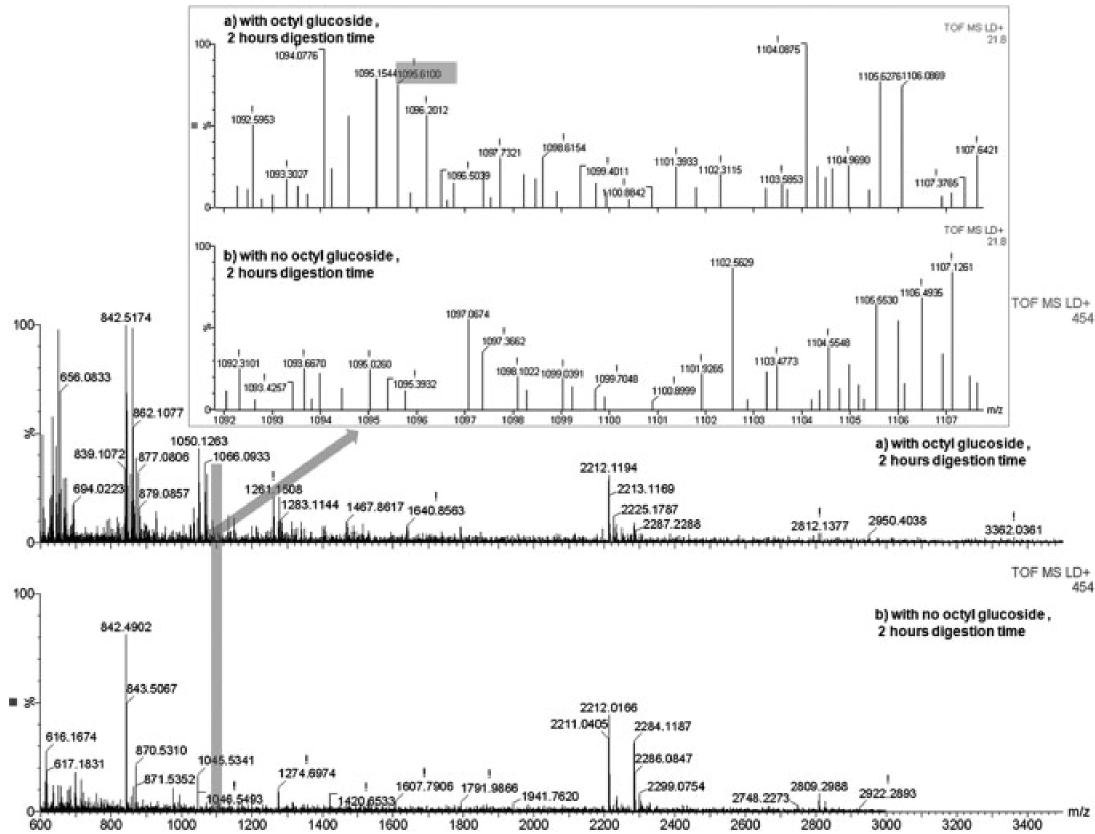

Figure 6. Evaluation of the use of OcGlc in the trypsin buffer for *in situ* digestion of FFPE breast tumour tissue sections. Figure 6A shows the direct peptide profile after on-tissue digestion with a trypsin solution containing 0.1% of OcGlc. Figure 6B displays the direct peptide profile with no OcGlc in the trypsin buffer. Both spectra were acquired after the same digestion time.

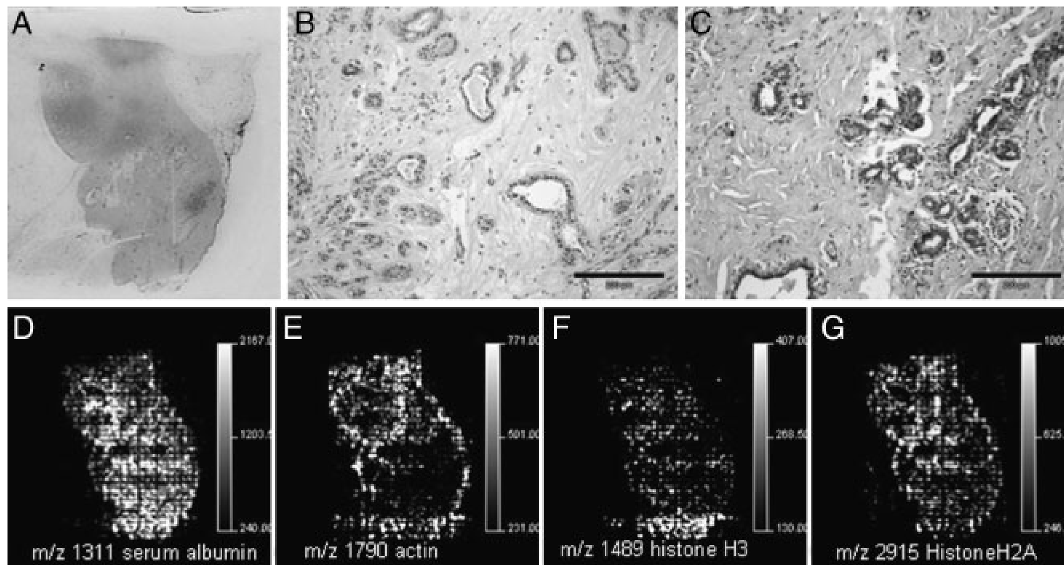

Figure 7. MALDI-MS images of protein localisation within an FFPE breast tumour tissue section. (A) A scan of a histological section (FFPE) from a breast tumour. (B and C) Stained histological images of the section. (D–G) The distribution of serum albumin, actin, histone H3 and histone H2A, respectively, within the tissue section.

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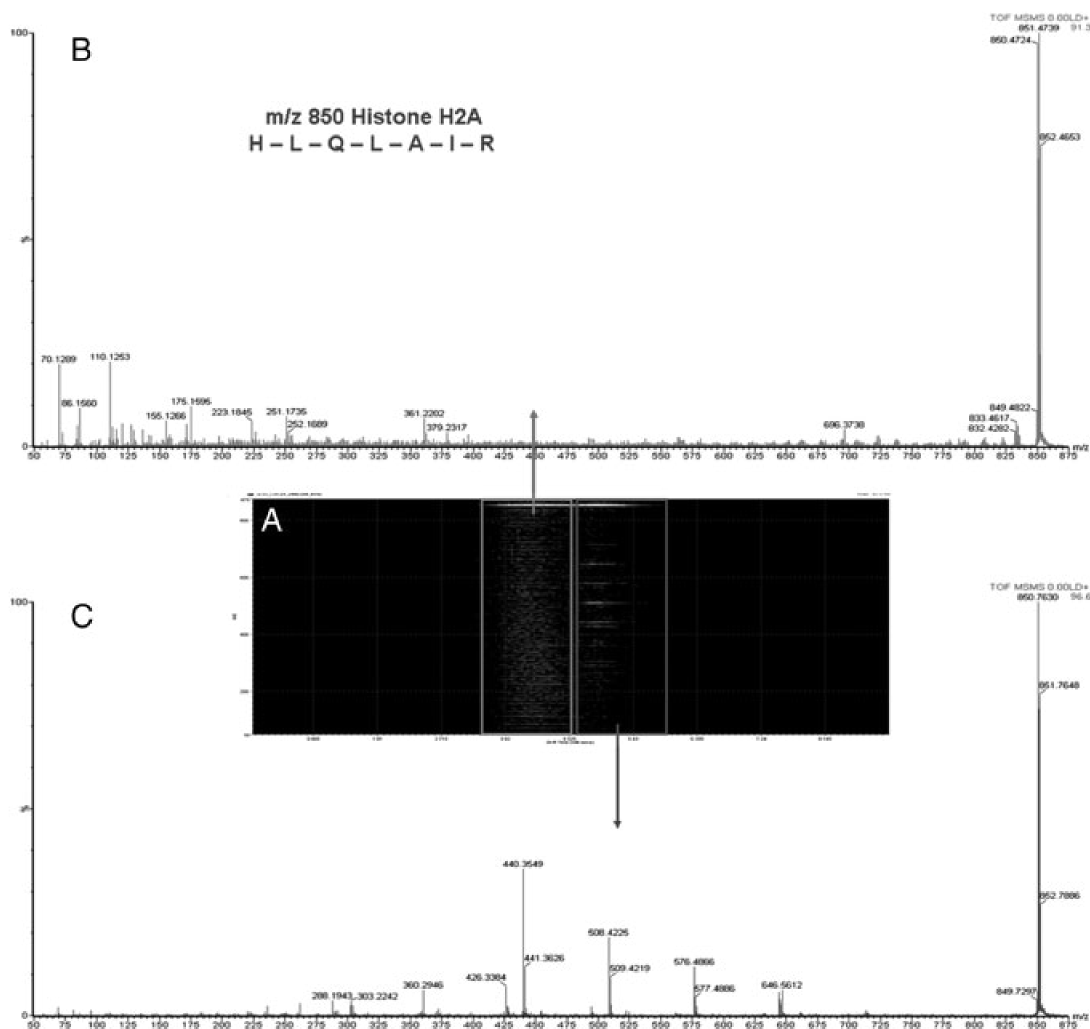

Figure 8. IMS of the signal at 850. The observed ''Driftscope'' plot (A) displays interference between species in MS/MS analysis mode. These species can be separated using their mobility. Different MS/MS spectra (B and C) can be extracted using the IMS

described above. The use of a detergent was also assessed for *in situ* digestion of FFPE tumour tissue sections. Adding 0.1% of OcGlc in the trypsin solution increased the number of peptide signals detected as well as improved signal intensities. Figure 6 shows a comparison between direct peptide MALDI-MS profiling obtained after 2 h *in situ* digestion from FFPE breast tumour tissue sections either with a trypsin solution containing OcGlc (Fig. 6A) or with no OcGlc (Fig. 6B). Spectra were acquired using a MALDI SYNAPT^{TM}HDMS system. It can be clearly seen (Figs. 6A and b) that more peptide signals are observed after on-tissue digestion with a trypsin solution containing 0.1% of OcGlc. The signal at *m*/*z* 1790.9 is increased when adding OcGlc to the trypsin buffer for *in situ* digestion. In addition to the most abundant proteins, several peptide signals assigned to other proteins including membrane-associated proteins have been identified and their distribution within the tissue section studied after *in situ* digestion using detergent addition to the tryptic buffer. This can be seen in the zoomed-in spectra. The detection of the signal at *m*/*z* 1095.6, which was assigned to retinoic acid early transcript 1G protein (a single-pass type I membrane protein), was improved. Figure 7 shows MALDI images of the localisation of proteins within an FFPE breast tumour section. Images were acquired at a spatial resolution set at 200 μm using the MALDI SYNAPT^{TM}HDMS.

In situ digestion has been found useful for proteomic analysis by MALDI-MSI as it provides direct protein identification and localisation from the tissue section without any need for sample fractionation or extraction of proteins from the tissue section. As numerous peptides are generated this results in a complex mixture of signals from the individual components. As a consequence signal overlap from peptides and other biomolecules is observed, thus

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Table 2. List of some identified peptides after *in situ* digestion and direct MALDI-MSI analysis on FFPE breast tumour tissue section by using a MALDI SYNAPT^{TM} instrument

Protein assignment	Accession number	Protein mass (Da)	Observed *m/z* with MALDI-MSI	Sequence	Score	Molecular function
Actin, aortic smooth muscle	P62736	41 982	1198.7	AVFPSIVGRPR	25	Cell mobility
			1790.9	SYELPDGQVITIGNER	82	
Actin, cytoplasmic1	P60709	41 710	2215.1	DLYANTVLSGGTTMYPGIADR	83	Cell mobility
Albumin	P02768	69 367	1311.7	HPDYSVVLLLR	75	
			1467.8	RHPDYSVVLLLR	60	
Haemoglobin subunit alpha	P01942	15 085	1087.6	MFLSFPTTK+Oxidation (M)	26	
			1529.7	VGAHAGEYGAEALER	115	
Haemoglobin subunit beta	P68871	15 866.8	1274.7	LLVVYPWTQR	27	
Histone H2A	P0C0S5	13 545	850.5	HLQLAIR	18	Gene regulation
			944.5	AGLQFPVGR	26	
			2915.6	VGAGAPVYLAAVLEYLTAEILELAGNAAR	44	
Histone H2B	P33778	13 819	901.5	LAHYNKR	28	
			1775.8	AMGIMNSFVNDIFER+2 Oxidation (M)	36	
Histone H3	Q6NXT2	15 204	831	STELLIR	26	
Histone H4	P62805	11 360	1466.8	TVTAMDVVYALKR	35	
Lumican [Precursor]	P51884	38 405	1024.6	FNALQYLR	21	Found in the extracellular matrix of human cartilages
Na+/K+-ATPase alpha 3 subunit variant [Fragment]	Q53ES0	111 779	1002.5	RDLDDLKK	*de novo*	ATP binding, inorganic cation transmembrane transporter
2-oxoglutarate dehydrogenase E1 component-like, mitochondrial [Precursor]	Q9ULD0	114 409	952.5	FMTILRR+Oxidation (M)	21	Mitochondrial matrix, oxidation reduction
Retinoic acid early transcript 1G protein [Precursor]	Q6H3X3	37 082	1095.6	RPLSGGHVTR+Oxidation (HW)	20	Single-pass type I membrane protein
28S ribosomal protein S18b, mitochondrial [Precursor]	Q9Y676	29 377	1105.6	NHKGGVPPQR+Oxidation (HW)	20	Structural constituent of ribosome
Uncharacterized protein C2orf34	Q7Z624	36 105	1094.6	GPVVSAPLGAAR	23	
Tumour protein 63	Q9H3D4	76 736	1127.6	RCPNHELSR+Oxidation (HW)	15+*de novo*	Acts as e sequence specific DNA binding transcriptional activator or repressor

making direct MS/MS analysis difficult to achieve in some cases. Previous methods reported the coupling of nanoLC-nanoESI to MALDI-MSI analysis in order to achieve a better identification of proteins. Here IMS has been found to be a powerful technique for direct analysis of peptides generated after *in situ* digestion. Coupling IMS to MALDI-MSI

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aims at improving the selectivity and facilitate database searching. Figure 8A shows the IMS of two isobaric species of *m/z* 850 arising from the direct MS/MS analysis. When a MASCOT search was performed on the entire MS/MS spectrum without taking into account the mobility separation, no significant results or potential identification were obtained. However the IMS data can be processed in order to extract individual MS/MS spectra corresponding to each analyte. Figures 8B and C display the obtained MS/MS spectra after IMS data processing of the isobaric ions at a "driftime" of 3.9 and 4.9 ms, respectively. These data were imported into MASCOT search resulting in a better search for protein assignment. The MS/MS spectra displayed in Figure 8B allowed the identification of the ion signal at *m/z* 850 as histone H2A. Using a combination of both IMS and MALDI-MSI has been found to be a valuable tool for the *in situ* identification of proteins in FFPE breast tumour tissue sections. Numerous proteins were identified. Table 2 displays a list of some identified proteins from FFPE breast tumour tissue sections. However, further method development is still required in order to improve the detection and identification of stress proteins directly from FFPE tissue sections after *in situ* digestion.

4 Concluding remarks

MALDI-MSI is continuing to show its potential for the investigation of protein distribution directly from tissue sections. It has been shown here that numerous protein signals can be detected within a tumour tissue section and moreover that the spatial distribution of proteins can be studied, thus allowing the discrimination between regions. The use of on-tissue digestion demonstrated the ability of the technique to directly identify proteins. The strategy described here highlights an improved method for *in situ* protein investigation and identification within frozen and FFPE tumour tissue sections. Using a detergent in the trypsin buffer enabled the enhanced detection and localisation of low-abundant proteins and tumour-related proteins within frozen and FFPE tissue sections. Additionally, the use of IMS has been shown to improve the sensitivity as well as the database search results. However, further improvement of the method and protocol refinements may lead to the identification of more proteins.

The identification of proteins directly from tissue with MALDI-MSI following *in situ* enzymatic cleavage is a new proteomic approach and has the potential to deliver localisation and identification of tumour protein targets such as angiogenesis factors or drug-resistant biomolecules.

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5 References

[1] Scriven, P., Brown, N. J., Pockley, A. G., Wyld, L., The unfolded protein response and cancer: a brighter future unfolding? *J. Mol. Med. (Berlin, Germany)* 2007, *85*, 331–341.

[2] Aghdassi, A., Phillips, P., Dudeja, V., Dhaulakhandi, D. *et al.*, Heat shock protein 70 increases tumorigenicity and inhibits apoptosis in pancreatic adenocarcinoma. *Cancer Res.* 2007, *67*, 616–625.

[3] Lee, E., Nichols, P., Spicer, D., Groshen, S. *et al.*, GRP78 as a novel predictor of responsiveness to chemotherapy in breast cancer. *Cancer Res.* 2006, *66*, 7849–7853.

[4] Matsumoto, A., Hanawalt, P. C., Histone H3 and heat shock protein GRP78 are selectively cross-linked to DNA by photoactivated gilvocarcin V in human fibroblasts. *Cancer Res.* 2000, *60*, 3921–3926.

[5] Caprioli, R. M., Farmer, T. B., Gile, J., Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. *Anal. Chem.* 1997, *69*, 4751–4760.

[6] Chaurand, P., Norris, J. L., Cornett, D. S., Mobley, J. A., Caprioli, R. M., New developments in profiling and imaging of proteins from tissue sections by MALDI mass spectrometry. *J. Proteome Res.* 2006, *5*, 2889–2900.

[7] Trim, P. J., Atkinson, S. J., Princivalle, A. P., Marshall, P. S. *et al.*, Matrix-assisted laser desorption/ionisation mass spectrometry imaging of lipids in rat brain tissue with integrated unsupervised and supervised multivariant statistical analysis. *Rapid Commun. Mass Spectrom.* 2008, *22*, 1503–1509.

[8] Atkinson, S. J., Loadman, P. M., Sutton, C., Patterson, L. H., Clench, M. R., Examination of the distribution of the bioreductive drug AQ4N and its active metabolite AQ4 in solid tumours by imaging matrix-assisted laser desorption/ionisation mass spectrometry. *Rapid Commun. Mass Spectrom.* 2007, *21*, 1271–1276.

[9] Hsieh, Y., Casale, R., Fukuda, E., Chen, J. *et al.*, Matrix-assisted laser desorption/ionization imaging mass spectrometry for direct measurement of clozapine in rat brain tissue. *Rapid Commun. Mass Spectrom.* 2006, *20*, 965–972.

[10] Chaurand, P., DaGue, B. B., Pearsall, R. S., Threadgill, D. W., Caprioli, R. M., Profiling proteins from azoxymethane-induced colon tumors at the molecular level by matrix-assisted laser desorption/ionization mass spectrometry. *Proteomics* 2001, *1*, 1320–1326.

[11] Schwamborn, K., Krieg, R. C., Reska, M., Jakse, G. *et al.*, Identifying prostate carcinoma by MALDI-Imaging. *Int. J. Mol. Med.* 2007, *20*, 155–159.

[12] Lemaire, R., Menguellet, S. A., Stauber, J., Marchaudon, V. *et al.*, Specific MALDI imaging and profiling for biomarker

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hunting and validation: fragment of the 11S proteasome activator complex, Reg alpha fragment, is a new potential ovary cancer biomarker. *J. Proteome Res.* 2007, *6*, 4127–4134.

[13] Stoeckli, M., Chaurand, P., Hallahan, D. E., Caprioli, R. M., Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. *Nat. Med.* 2001, *7*, 493–496.

[14] Reyzer, M. L., Caldwell, R. L., Dugger, T. C., Forbes, J. T. *et al.*, Early changes in protein expression detected by mass spectrometry predict tumor response to molecular therapeutics. *Cancer Res.* 2004, *64*, 9093–9100.

[15] Aerni, H., Cornett, D. S., Caprioli, R. M., Automated acoustic matrix deposition for MALDI sample preparation. *Anal. Chem.* 2006, *78*, 827–834.

[16] Shimma, S., Furuta, M., Ichimura, K., Yoshida, Y., Setou, M., Direct MS/MS analysis in mammalian tissue sections using MALDI-QIT-TOFMS and chemical inkjet technology. *Surf. Interface Anal.* 2006, *38*, 1712–1714.

[17] Lemaire, R., Desmons, A., Tabet, J. C., Day, R. *et al.*, Direct analysis and MALDI imaging of formalin-fixed, paraffin-embedded tissue sections. *J. Proteome Res.* 2007, *6*, 1295–1305.

[18] Stauber, J., Lemaire, R., Franck, J., Bonnel, D. *et al.*, MALDI imaging of formalin-fixed paraffin-embedded tissues: application to model animals of Parkinson disease for biomarker hunting. *J. Proteome Res.* 2008, *7*, 969–978.

[19] Shimma, S., Furuta, M., Ichimura, K., Yoshida, Y., Setou, M., A novel approach to *in situ* proteome analysis using chemical inkjet printing technology and MALDI-QIT-TOF tandem mass spectrometer. *J. Mass Spectrom. Soc. Jpn.* 2006, *54*, 133–140.

[20] Groseclose, M. R., Andersson, M., Hardesty, W. M., Caprioli, R. M., Identification of proteins directly from tissue: *in situ* tryptic digestions coupled with imaging mass spectrometry. *J. Mass Spectrom.* 2007, *42*, 254–262.

[21] Day, R., Salzet, M., Fournier, I., Anonymous United Kingdom Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (Second Edition). *Br. J. Cancer.* 1998, *77*, 1–10.

[22] Lemaire, R., Wisztorski, M., Desmons, A., Tabet, J. C. *et al.*, MALDI-MS direct tissue analysis of proteins: improving signal sensitivity using organic treatments. *Anal. Chem.* 2006, *78*, 7145–7153.

[23] Hood, B. L., Darfler, M. M., Guiel, T. G., Furusato, B. *et al.*, Proteomic analysis of formalin-fixed prostate cancer tissue. *Mol. Cell. Proteomics* 2005, *4*, 1741–1753.

[24] Wong, J. W., Cagney, G., Cartwright, H. M., SpecAlign–processing and alignment of mass spectra datasets. *Bioinformatics* 2005, *21*, 2088–2090.

[25] Djidja, M., Carolan, V., Loadman, P. M., Clench, M. R., Method development for protein profiling in biological tissues by matrix-assisted laser desorption/ionisation mass spectrometry imaging. *Rapid Commun. Mass Spectrom.* 2008, *22*, 1615–1618.

[26] Armstrong, D. W., Zhang, L. K., He, L., Gross, M. L., Ionic liquids as matrixes for matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* 2001, *73*, 3679–3686.

[27] Tholey, A., Heinzle, E., Ionic (liquid) matrices for matrix-assisted laser desorption/ionization mass spectrometry-applications and perspectives. *Anal. Bioanal. Chem.* 2006, *386*, 24–37.

[28] Lemaire, R., Tabet, J. C., Ducoroy, P., Hendra, J. B. *et al.*, Solid ionic matrixes for direct tissue analysis and MALDI imaging. *Anal. Chem.* 2006, *78*, 809–819.

[29] Pringle, S. D., Giles, K., Wildgoose, J. L., Williams, J. P. *et al.*, An investigation of the mobility separation of some peptide and protein ions using a new hybrid quadrupole/travelling wave IMS/oa-ToF instrument. *Int. J. Mass Spectrom.* 2007, *261*, 1–12.

[30] Riba-Garcia, I., Giles, K., Bateman, R. H., Gaskell, S. J., Evidence for Structural Variants of a- and b-Type Peptide Fragment Ions Using Combined Ion Mobility/Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* 2008, *19*, 609–613.

[31] Xia, Q., Wang, H., Wang, J., Liu, B. *et al.*, [Nano-ESI-MS/MS identification on differentiation-associated proteins in M1 mouse myeloid leukemia cells induced by IL-6]. *Acta Academiae Medicinae Sinicae* 2004, *26*, 483–487.

[32] Sausville, E. A., Burger, A. M., Contributions of human tumor xenografts to anticancer drug development. *Cancer Res.* 2006, *66*, 3351–3354.

[33] Chaurand, P., Schwartz, S. A., Caprioli, R. M., Assessing protein patterns in disease using imaging mass spectrometry. *J. Proteome Res.* 2004, *3*, 245–252.

[34] Sutton, C. W., Pemberton, K. S., Cottrell, J. S., Corbett, J. M. *et al.*, Identification of myocardial proteins from two-dimensional gels by peptide mass fingerprinting. *Electrophoresis* 1995, *16*, 308–316.

[35] Cohen, S. L., Chait, B. T., Influence of matrix solution conditions on the MALDI-MS analysis of peptides and proteins. *Anal. Chem.* 1996, *68*, 31–37.

[36] Katayama, H., Nagasu, T., Oda, Y., Improvement of in-gel digestion protocol for peptide mass fingerprinting by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 2001, *15*, 1416–1421.

[37] Wadhwa, R., Taira, K., Kaul, S. C., An Hsp70 family chaperone, mortalin/mthsp70/PBP74/Grp75: what, when, and where? *Cell Stress Chaperones* 2002, *7*, 309–316.

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