Chapter 20

Specific MALDI-MSI: TAG-MASS

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Abstract

MALDI imaging as a molecular mass spectrometry imaging technique (MSI) can provide accurate information about molecular composition on a surface. The last decade of MSI development has brought the technology to clinical and biomedical applications as a complementary technique of MRI and other molecular imaging. Then, this IMS technique is used for endogenous and exogenous molecule detection in pharmaceutical and biomedical fields. However, some limitations still exist due to physical and chemical aspects, and sensitivity of certain compounds is very low. Thus, we developed a multiplex technique for fast detection of different compound natures. The multiplex MALDI imaging technique uses a photocleavable group that can be detect easily by MALDI instrument. These techniques of targeted imaging using Tag-Mass molecules allow the multiplex detection of compounds like antibodies or oligonucleotides. Here, we describe how we used this technique to detect huge proteins and mRNA by MALDI imaging in rat brain and in a model for regeneration; the leech.

Key words: Matrix-assisted laser desorption/ionization, time-of-fight, mass spectrometry, mass spectrometry imaging, mRNA, antigens, photocleavage.

1. Introduction

As an innovative technique, MALDI-IMS is a powerful tool for direct detection and localization of endogenous and exogenous molecules within biological samples (1, 2). The last developments have led to use this technique to obtain the distribution of various compounds such as lipids, drugs, peptides, and proteins within tissue sections (1–4). Non-targeted aspect of MALDI-IMS is one of the big advantages of the technology compared to other imaging techniques as well as strength of MS for structural elucidation.

S.S. Rubakhin, J.V. Sweedler (eds.), *Mass Spectrometry Imaging*, Methods in Molecular Biology 656,
DOI 10.1007/978-1-60761-746-4_20,

Many successful applications of this technique have been undertaken recently. In particular, MALDI-IMS of lipids, peptides, and proteins for clinical applications by studying pathologies has shown to be a very promising application by providing information on the variations of abundance and localization of markers (3–14). Moreover, biological processes bring into play many different signaling pathways involving various classes of molecules ranging from oligonucleotides, to proteins, peptides, and lipids. In particular, the correlation of mRNA expression with their corresponding protein regulation, or more generally the correlation of transcriptome to proteome, is of special interest for better understanding of biological mechanisms. This is especially an essential aspect when studying pathologies for earlier diagnosis.

However, some specific classes of biomolecules such as oligonucleotides or sugars are still non- or hardly accessible to the direct analysis by MALDI, as are also, very hydrophobic proteins, membrane proteins, high mass proteins (>30 kDa), or lower abundance ones. Ideally, oligonucleotides should be directly detected from tissues, although their large size and low abundance in cells added to analytical difficulties in mass spectrometry (salts adducts and gas phase instability) render their analysis difficult (15). In the same time, multiplex techniques are necessary for diagnosis and prognosis. More and more tissue micro arrays (TMAs) are used today to analyze large number of disease tissues and new, fast, and reproducible multiplex techniques are necessary (9).

We, thus, have proposed a new concept of possible multiplex and specific detection and tracking of biomolecules with a special focus on mRNA and proteins for transcriptome/proteome correlations. This concept relies on affinity detection by using a specific designed probe, called Tag-Mass which can be detected by mass spectrometry (16). Tag-Mass offers more selectivity to MALDI-MSI for selectively and specifically tracking known markers of physiological stages in cohorts of samples with a high sensitivity (3, 16, 17).

1.1. The Tag-Mass Concept: Selective Multiplexed Imaging of Biomolecules from Tissue Sections

The "Tag-Mass" strategy is an affinity-based strategy where a probe is directed against a specific target, using a probe that can be imaged by MALDI-MSI (3, 16, 17). The Tag-Mass is a modified probe bearing a reporter group where the reporter group is used in MALDI-MSI to indirectly obtain the image of the probe. The reporter is designed to be a molecule of known molecular mass that is easily detectable under MALDI conditions taking care to use a molecule that is not corresponding to an endogenous compound. To image a probe indirectly via the direct image of the reporter, the reporter must be linked to the probe and released in the final step just before or during the MALDI sequence.

In the Tag-Mass, the release of the reporter group is obtained by photodissociation under the MALDI laser irradiation using a photocleavable moiety that binds the reporter to the probe (**Fig. 20.1a**). Thus, the reporter is detached from the probe during the MALDI-MSI acquisition. Many different reporters can be used for this purpose, but most of the times peptides were used. The photocleavable linker is chosen to present a specific absorption band in the UV at a wavelength (340 nm) very closed to that of MALDI lasers (i.e., 337–355 nm). Thus, after hybridization of the modified probe to its target, a classical MALDI-MSI sequence is performed. At a specific location of the acquisition, the presence of a probe will be signed by the presence of the reporter released under the MALDI laser irradiation, which traduced by the observation of a peak at the m/z expected for the reporter (**Fig. 20.1b**). Reconstruction of the reporter molecular image gives, then, the image of the probe, i.e., those of the targeted molecule (3, 16, 17). Such a concept is compatible with all types of probes including mRNA probes, antibody probes, or even lectins or aptamers owing to image with high selectivity, respectively, mRNA, antigens, oligosaccharides (including glycosylated proteins), and drugs. Tag-mass workflow, MALDI-MSI, is combined with hybridization techniques including in situ hybridization (ISH) and immunohistochemistry (IHC) (3, 16, 17). Specific MALDI-MSI or Tag-Mass MSI by using reporter moieties that can be distinguished by their change in m/z is a technique that can be used in multiplex conditions. Theoretically, there are no limits in the multiplexing conditions except the hybridization step itself because of kinetic competition during the affinity reactions or steric obstruction problems. Tag-Mass can also be use for semi-quantification in multiplex conditions by using a reporter presenting the same physico-chemicals properties, i.e., same analytical behavior using, for example, isotopically labeled reporter such as differentially deuterated peptides.

This concept can be extended by looking for alternative ways of releasing the reporter moiety, e.g., chemical released or even released by prompt fragmentation pathways (i.e., before the end of the delay time period). The reporter can also be designed to be observable in LDI conditions avoiding, thus, the use of the MALDI matrix (18, 19). Although this latest solution could be less sensitive than using MALDI conditions but would increase spatial resolution of images. Extension to other ion production sources can also be searched. For secondary ion mass spectrometry (20, 21) (SIMS) or laser ablation inductively coupled plasma mass spectrometry (22–24) (LA-ICP), probes bearing directly a monoatomic element easily detectable by these techniques should be used, if the element as a good sensitivity of analysis and is not present naturally in the surface to study. Such techniques induce quite important fragmentation yields and the reporter element

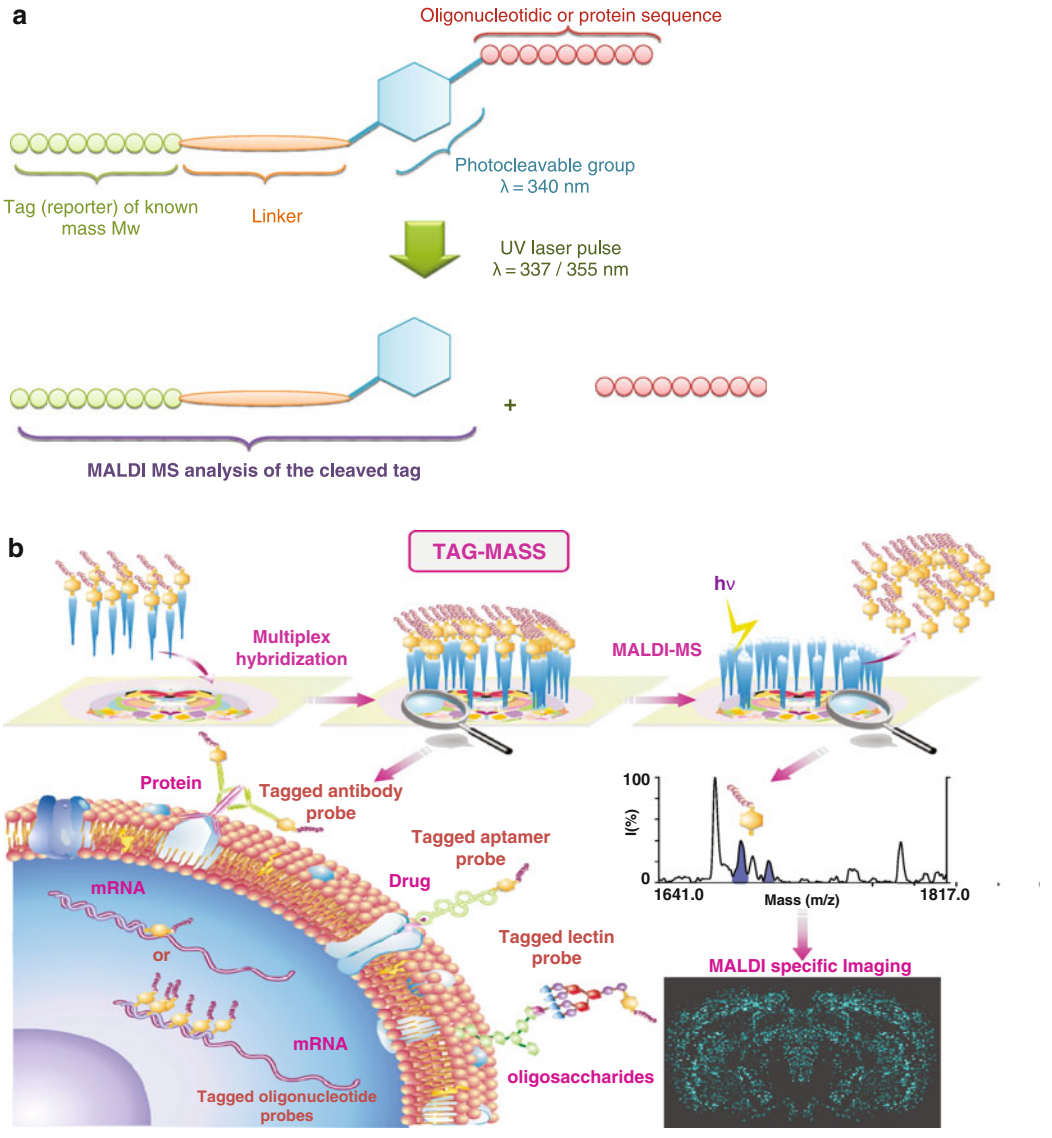


Fig. 20.1. (**a**) Schematic representation of the reporter release under photodissociation by the MALDI laser using a photocleavable-reporter system coupled to the probe. (**b**) Workflow of multiplex specific MALDI-MSI (Tag-Mass).

will appear as a fragmentation product. For example, gold-labeled secondary antibodies are a good solution for imaging antigens in LA-ICP MS at a spatial resolution below 10 μm.

We present, here, the workflows for Tag-Mass of antigens and mRNA using a photocleavable probe bearing a peptide as reporter moiety. For antibodies, preference was given to use indirect IHC with a primary–secondary antibody system. Indeed, indirect IHC is known to present better performances by decreasing steric obstruction problems and increasing detection level, since secondary antibodies will recognize consensus epitope present in the

primary antibody sequence allowing attachment of several secondary antibodies. Moreover, secondary antibodies are easier to produce since they require much less specificity. For mRNA, modified uracile nucleotides were used. This requires a specific synthesis in order to add the photocleavable group and reporter moiety directly on the nucleotide basis for keeping both 3′ and 5′ termini free. In fact, the modified nucleotide is to be used for the probe amplification. In former experiments, modified primers (by the addition of a photocleavable-reporter system) were used. This approach had revealed several disadvantages including lake of sensitivity (only one reporter per probe), high cost (specific synthesis required for each mRNA to be localized), and impossibility to amplify the probe by in vitro translation (only one terminus of the primer free). Development of modified uracile nucleotides was a great advance in this respect. Modified nucleotides are available for all probes construction, the sensitivity is increased by the incorporation of several reporters in the probe sequence (amplification of the signal) and probes can be obtained by in vitro translation. Only tagged Uracile strategy will be presented here. Specific MALDI-MSI can also be performed in multiplexing conditions. An example of duplex imaging of two antigens (Cystatin B/Cathepsin D) from a FPE tissue section of the leech *T. tessulatum* are presented here as an example for multiplexing.

2. Materials

2.1. Preparation of Frozen Tissue Sections

2.1.1. Snap-Frozen Tissues

1. Isopentane cooled at –45°C with dry ice. Vapors may cause drowsiness and dizziness, so work in a hood.

2.1.2. Tissue Cryosection and Thaw Mounted

1. Optimal cutting temperature polymer, OCT.
2. Indium tin oxide (ITO)-coated glass slides or other holder compatible with mass spectrometry analysis.
3. A cryomicrotome, Leica CM150S (Leica Microsystems, Nanterre, France).

2.1.3. Pre-analysis Treatment: Tissue Fixation

1. Ethanol 75% (–20°C): 75 ml of absolute ethanol (≥99.8%) and water (HPLC grade) to 100 ml. Prepare fresh. Store at –20°C.
2. Ethanol 95% (−20°C): 95 ml of absolute ethanol (≥99.8%) and water (HPLC grade) to 100 ml. Prepare fresh. Store at −20°C.

2.1.4. Pre-analysis Treatment: Lipids Removal

1. Chloroform (−20°C): 100 ml of chloroform (≥99.9%). Store at −20°C. Chloroform is harmful by inhalation, so work in the hood.

2.2. Preparation of FFPE Tissue Section

2.2.1. FFPE Tissue Section

1. Indium tin oxide (ITO)-coated glass slides or other holder compatible with mass spectrometry.
2. Water: 100 ml of water (HPLC grade). Prepare fresh.
3. A microtome and an hotplate warm at 50°C.

2.2.2. FFPE Tissue Dewaxing

1. Xylene: 100 ml of xylene (≥99.9%). Xylene is harmful by inhalation, so work in the hood.
2. Ethanol 100%. Prepare fresh.
3. Ethanol 95%: 95 ml of absolute ethanol (≥99.8%) and water to 100 ml. Prepare fresh.
4. Ethanol 75%: 75 ml of absolute ethanol (≥99.8%) and water to 100 ml. Prepare fresh.
5. Ethanol 30%: 30 ml of absolute ethanol (≥99.8%) and water to 100 ml. Prepare fresh.
6. Water: 100 ml of water (HPLC grade). Prepare fresh.

2.3. Hybridization Buffers for In Situ Hybridization (ISH)

1. Buffer solution glycine 0.1 M/Tris HCl buffer (pH 7.4).
2. RNAse inhibitoring activity solution: Proteinase K (1 μg/μl in 1 M/Tris HCl and 0.5 M EDTA, pH 8).
3. Post-fixation buffer: 4% paraformaldehyde (0.1 M Phosphate/ 5 mM MgCl2 buffer, pH 7.4), 15 min then triethanolamine (0.1 M, pH 8), 10 min.
4. Washing solution: 20× SSC buffer: standard sodium citrate solution : for a 20× SSC solution dissolve 701.28 g NaCl and 352.92 g NaCitrate in a recipe to make 4 l (check to have pH to 7.0) and bring final volume to 4 l , then Autoclave the solution in order to be RNAse free).
5. Dehydration by ethanol (30°, 70°, 96°).
6. Probes denaturation (100°C, 10 min).
7. Hybridization buffer: 0.01 M dextran sulfate, 0.2 M formamide, 20× SSC 20%, 100 × Denhardt's 10%). 16 h, 55°C.
8. Non-hybridized probe degradation buffer: RNase (10 μg/ml), 37°C, 30 min.

9. Rinsed steps:
   a. 20 and 10 mM 2-mercaptoethanol solutions, 10 min.
   b. 0.5× and 0.1× SSC.
   c. Ultrapure water.

2.4. Hybridization Buffers for Immunocytochemistry (IHC)

1. Incubation buffer: 0.1 M PBS/1% BSA/1% normal goat serum/0.05% Triton X100.
2. Primary antibody incubation, overnight, 4°C, on rocking.
3. Washing solution: phosphate-buffered saline (PBS). Prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na2HPO4, 18 mM KH2PO4 (adjust to pH 7.4 with HCl if necessary) and autoclave before storage at room temperature. Prepare working solution by dilution of one part with nine parts water.
4. Secondary antibody solution: antibody diluted in incubation buffer at room temperature, on rocking. Antibody is either anti-rabbit IgG 1/100 developed in goat (Jackson Immunoresearch, Inc., Europe LTD); FITC-conjugated secondary antibody anti-rabbit IgG 1/100 developed in goat (Jackson Immunoresearch, Inc., Europe LTD) or photocleaved tagged antibody 1/100 (Imabiotech, France).
5. Revelation
   a. Photocleavable tagged antibody, precleavage under UV 5 min Staining substrate for peroxidase antibody in chloronapthol with 0.05% H_2O_2 for detection.
   b. For FITC ICC, slices were prepared using phenylenediamine in glycerol.

2.5. Matrix Deposition for Proteins Analysis

2.5.1. Using a Microspotter

1. SA/ANI solution: 1.5 equivalent of aniline (ANI) were added to a solution containing 40 mg/ ml of sinapinic acid (SA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v). Aniline and TFA are toxic, so work in the hood.
2. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.5.2. Using an Automatic Sprayer

1. SA/ANI solution: 1.5 equivalent of aniline (ANI) were added to a solution containing 40 mg/ ml of sinapinic acid (SA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v). Aniline and TFA are toxic, so work in the hood.
2. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.6. Matrix Deposition for Peptides Analysis

2.6.1. Using a Microspotter

1. ANI solution: 1.5 equivalent of aniline (ANI) were added to a solution containing 10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v). Aniline and TFA are toxic, so work in the hood.
2. Chemical Inkjet Printer CHIP-1000 (Shimadzu Biotech, Kyoto, Japan).

2.6.2. Using an Automatic Sprayer

1. ANI solution: 1.5 equivalent of aniline (ANI) were added to a solution containing 10 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) in acetonitrile/aqueous TFA 0.1% (6:4, v/v). Aniline and TFA are toxic, so work in the hood.
2. ImagePrep (Bruker Daltonics, Bremen, Germany).

2.7. Mass Spectrometry Analysis

2.7.1. MALDI-MSI Experiment

1. Peptide calibration standard II (Bruker Daltonics, Wissenbourg, France): Angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17, ACTH clip 18–39, Somatostatin 28, Bradykinin Fragment 1–7, Renin Substrate Tetradecapeptide porcine. Covered mass range: ~700–3,200 Da. Store at −20°C.
2. Protein Calibration Standard I (Bruker Daltonics, Wissenbourg, France): Insulin, ubiquitin I, cytochrome C, myoglobin. Covered mass range: ~5,000–17,500 Da. Store at −20°C.
3. An Ultraflex II TOF–TOF equipped with a Smartbeam laser and all the Flex software suite (FlexControl, FlexAnalysis, and FlexImaging) (Bruker Daltonics, Bremen, Germany).

2.7.2. MS/MS Analysis

1. An Ultraflex II TOF–TOF equipped with a Smartbeam laser and all the Flex software suite (FlexControl, FlexAnalysis, and FlexImaging) (Bruker Daltonics, Bremen, Germany).
2. Biotools (Bruker Daltonics, Bremen, Germany).

2.8. MALDI Imaging Analysis

1. MALDI matrix used for antibody detection: α-Cyano-4-hydroxycinnamic acid (HCCA) and 3-hydroxypicolinic acid (3-HPA) (Sigma-Aldrich).

2. Calibrant solutions of angiotensin II, Des-Arg-bradykinin, substance P, ACTH 18–39, ACTH 7–38, and bovine insulin (Sigma-Aldrich).
3. Trifluoroacetic acid (TFA) (Applied Biosystems). Acetonitrile and methanol (J.T. Baker).
4. Sprayer of matrix (ImagePrep, Bruker Daltonics).

3. Methods

The methods are described according to their sequential used in experiments. One part is dedicated to the protocols or hybridization themselves, i.e., ISH and IHC using the photocleavable modified probe for both cryostat sections (from frozen samples or fixed and frozen samples) and microtome sections (for fixed and paraffin-embedded [FPE] samples). It must be noticed that the protocols are tissue and target dependent. This is usual in hybridization. Thus, proposed protocols are given for rat brain tissue sections and shall be slightly modified and optimized for other applications according to the tissue and the probe (specificity, selectivity). **Figures 20.2** and **20.3** give examples of MS spectra and molecular images obtained, respectively, for Proenkephalin mRNA imaging and Carboxypeptidase D (CPD) protein imaging, respectively, using the modified dU for the oligonucléotides probe construction and a rabbit secondary modified antibody for CPD in conjunction with a primary antibody directed against rat CPD and raise in rabbits as antibody. The images are reconstructed based on the reporter signal with a peptide close the to bradykinin sequence. **Figure 20.2** gives the molecular distribution of Proenkephalin mRNA as obtained by specific MALDI-MSI compared to the localization obtained by classical ISH. A good correlation is observed in between ISH and MALDI-MSI images. **Figure 20.3** compared the specific MALDI-MSI localization of CPD with its distribution obtained for classical colorimetric reaction or fluorescence. Specific MALDI-MSI presents higher sensitivity than classical revelation and is not so far from fluorescence detection. Specific MALDI-MSI enables multiplex detection of different epitopes. An example of duplex experiments for proteins is given **Fig. 20.4** where Cystatin B/Cathepsin D system is studied in FPE tissue sections of the leech *Theromyzon tessulatum*. Experiments were performed using two photocleavable secondary antibodies bearing different reporter peptides represented by different m/z. In such an experiment, one primary antibody is raised in rabbit

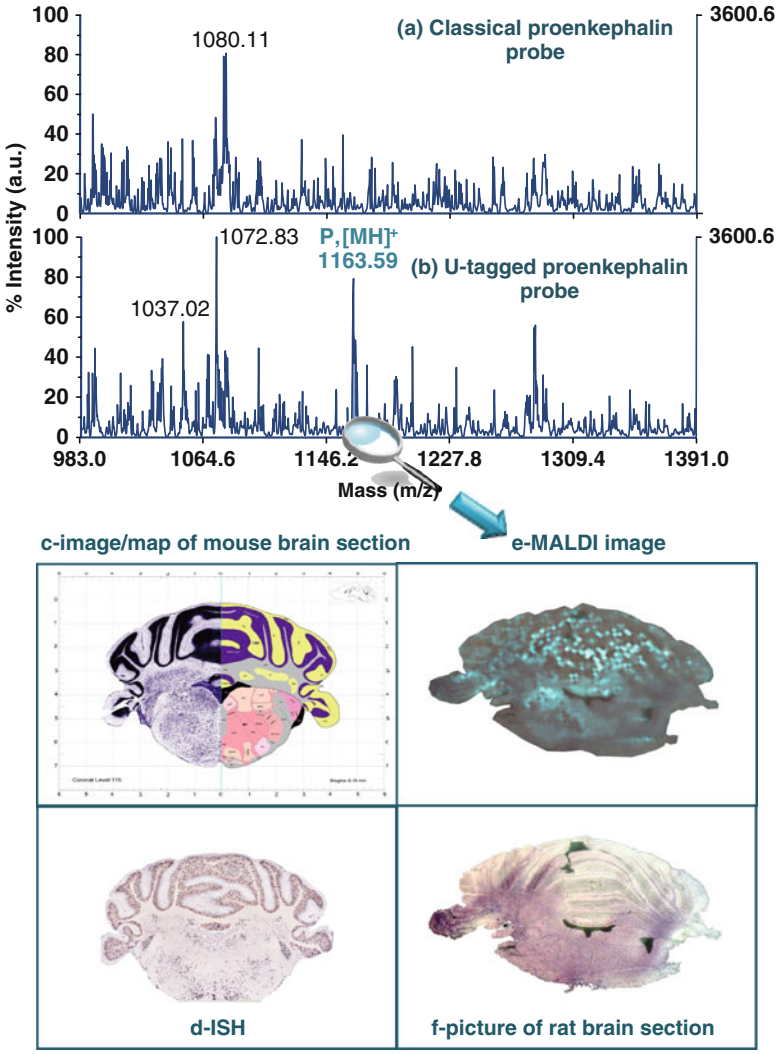


Fig. 20.2. Compared MALDI mass spectra in the linear positive mode recorded on two adjacent rat brain sections in the same region of the brain after ISH of double strand oligonucleotide cDNA probe corresponding to proenkephalin for classical untagged proenkephalin probe (**a**) and the U-tagged proenkephalin probe (**b**). (**f**) Corresponding reconstructed MALDI image on the basis of the tag signal obtained by scanning the tissue section after ISH experiment (7,000 spots separated each of 100 μm) compared (**e**) to proenkephalin mRNA localization in 8-weeks old male C57BL/6 J mouse brain using digoxigenin ISH technique by the Allen Institute (http://www.brainatlas.org/aba). For this experiment, colorimetric detection of bound probe is generated by the alkaline phosphatase substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) that produce a vivid blue/purple particulate reaction product. Figures (**a**) presents the map/picture representation of the mouse brain and figure (**f**) the picture of the rat brain section prior to ISH –MALDI imaging experiment. Reprinted from (14) with permission.

with an anti-rabbit photocleavable secondary antibody, whereas the second one is raised in mouse with an anti-mouse photocleavable secondary antibody. The experiment-exemplified multiplex conditions for proteins, although multiplexing, can also be achieved for mRNA.

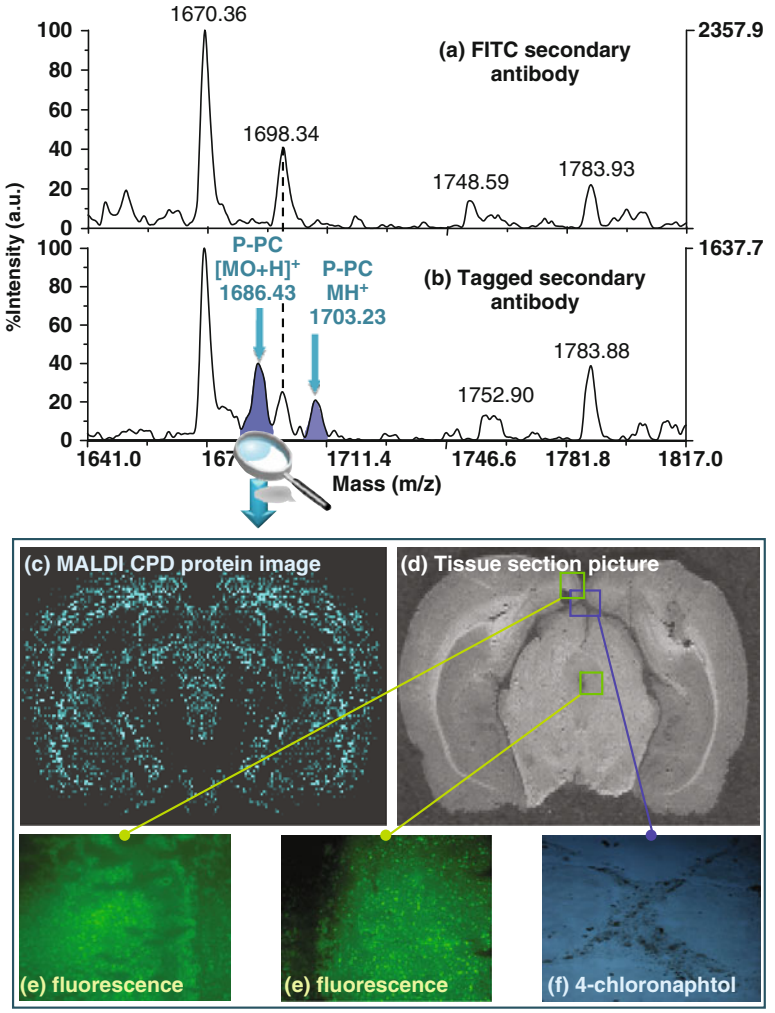


Fig. 20.3. Specific MALDI-MSI of CPD protein from a rat brain tissue section performed using a rabbit anti-rat primary antibody directed against CPD and the modified photocleavable goat anti-rabbit secondary antibody. (**a–b**) MALDI-MS spectra recorder for serial rat brain tissue sections after the IHC experiments for the tagged secondary antibody (**b**) compared to a FITC secondary antibody (**a**) for the same IHC conditions. (**c**) Molecular MALDI images reconstructed using the signal at *m/z* 1,686.43 of the reporter moiety (**d**) tissue section image before IHC experiments. (**e** and **f**) Comparison with fluorescence and 4-cholonaphaol detection using, respectively, a FITC or preroxydase tagged secondary antibody. Reprinted from (14) with permission.

3.1. Tissue Treatment

3.1.1. Tissue Snap-Frozen

1. The organ is dissected and rinsed with a saline solution suited for the considered tissue to remove blood and other tissue fragment of the surface. Alternative: prior to sacrifice, the animal can be perfused with the saline solution to remove blood inside the organ.

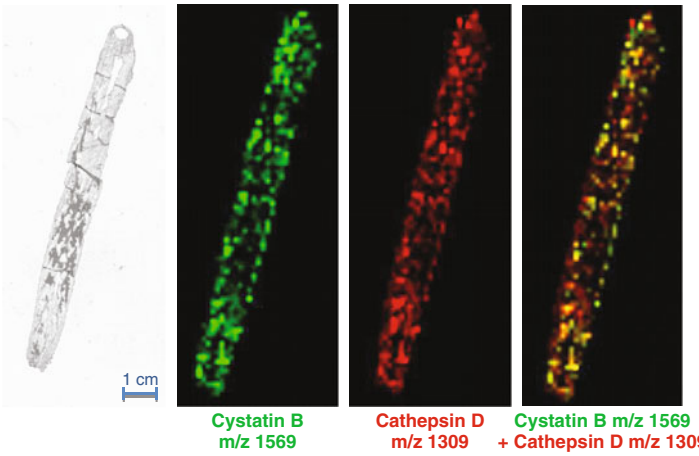


Fig. 20.4. Duplex specific MALDI-MSI of Cystatin B/Cathepsin D from a FPE tissue section of the leech *T. tessulatum* performed using rabbit anti-leech Cathepsin D primary antibody/photocleavable goat anti-rabbit secondary antibody and mouse anti-leech Cystatin B primary antibody/photocleavable goat anti-mouse secondary antibody. Molecular images have been reconstructed on the signals of the two reporter peptides, i.e., *m/z* 1,309 for Cathepsin D and *m/z* 15.

2. Morphology of the organ needs to be carefully maintained. Thus, the tissue should not be placed in a tube or wrap in an aluminum foil to avoid deformation of the organ (adaptation to the outlines of the container).
3. Snap-freezing procedure is applied for tissue conservation to maintain tissue morphology and to prevent ice crystals formation and cell explosion. In fact, different rate cooling of parts of the organ or direct dipping of the organ into liquid nitrogen leads to the formation of cracks and fragmentation of the tissue. Therefore the use of isopentane cooled at −45°C with dry ice is recommended. Freezing time is dependent on the size of the organ. It is preferable not to use an embedding media. For very small organs or surgical pieces, cutting without embedding material increases deformations and damages of the tissue sections. In such a case, a solution containing non-polymeric compounds, namely 10% gelatin solutions, helps to obtain high-quality tissue section. Tissue is embedded in 10% gelatin directly after dissection and frozen as previously described.
4. After snap freezing, tissue is removed from isopentane and stored at −80°C. We heartily recommend not overpassing a storage period of 6 months. Over 6 months storage, variation in the molecular profiles could be observed if no sample stabilization procedure is performed. Preferentially tissues should be analyzed a few days or weeks after snap freezing.

3.1.2. Tissue Cryosection and Thaw Mounted

1. The use of cryopreservative solutions containing organic polymers such as optimal cutting temperature (OCT) polymer should be restricted to the attachment of the tissue to the sample holder and not used for complete embedding of the tissue. Moreover, all parts of the cryostat in contact with the tissue must be cleaned to prevent any contamination between two different samples or with the polymer containing solution. In the case of contact between the tissue and cryopreservative solutions containing polymers, MS spectra will be dominated by polymer signals such as PEG signal distribution.
2. Tissue is placed in the cryostat during sufficient time before sectioning for slow warming of the sample to the cryostat temperature. If the tissue is too cold, poor-quality sections are obtained.
3. 10 μm thickness tissue sections are cut using cryomicrotome at −20°C. Different tissue types may need other temperature settings. 10 μm thickness is optimal. Smaller sections have not enough molecules for extraction and thicker sections may cause problems of conductivity (due to the insulating nature of tissues) and charge effects by charge accumulation at the sample surface during MALDI analysis. Charge effects will decrease spectral quality in axial TOF configuration instruments resulting in a progressive peak shifting toward the high m/z ratio.
4. Collect the tissue sections onto ITO glass slides pre-cooled at −20°C. Transfer is performed by applying the cooled ITO slide onto the section. The cuts are thus stick on the cold slide. Adhesion of the frozen sections to the glass slides is obtained by heating putting fingers under the slide or by placing the slide at room temperature. This transfer procedure, contrarily to classical thaw mounting, prevents formation of ice crystals at the surface of the cryostat microtome cutting plate.
5. Care must be taken of air bubbles formation at the surface of the tissue section that may leads to artifacts during MS analysis.
6. Mounted sections are stored in a sealed container at −80°C until their use.

3.1.3. Pre-analysis Treatment: Tissue Fixation

1. A closed container store at −80°C is warmed at room temperature in a vacuum desiccator to prevent water condensation at the surface of the frozen slide.
2. After complete drying, the ITO slide is washed. Washing steps are optional and dependent on the molecules to be

analyzed. Careful washing is crucial for conserving spatial localization of molecules.

3. For analysis of small molecules like lipids or drugs, no washing steps are used. For macromolecules analysis like peptides or proteins washing procedures are generally used. Washing is performed by immersing the glass slide softly in ice-cold 75% ethanol during 30 s. No agitation or shake is needed. This step washes out salts, cells fragments, or residual fluids.
4. Take the slide out and remove the excess of liquid around the section. A stream of nitrogen over the surface could help to remove excess of ethanol.
5. The ITO glass slide is then placed in a vacuum desiccator to completely dry of the tissue. The time of drying is dependent to the size of the section.
6. Optional: a second bath of fresh ice-cold 75% ethanol during 30 s followed by a complete drying under vacuum desiccator can be achieved.
7. After complete drying, the sample is dipped into cold 95% ethanol during 30 s. No agitation or shake is needed. This step prevents degradation of proteome by dehydration and fixation of the tissue.
8. The slide is completely dried like in steps 4 and 5.

3.1.4. Pre-analysis Treatment: Lipids Removal

1. After complete drying, immerse the glass slide softly in ice-cold chloroform (30 s). No agitation or shake is needed. This step removes lipids (especially phospoholipids) present in high concentration in the tissue (components of cell membranes) and may cause signal suppression in MS spectra.
2. Take the slide out and place it in the vacuum desiccator for complete drying of the tissue.

3.2. Preparation of FFPE Tissue Section

3.2.1. FFPE Tissue Section

1. 10 μm thickness FFPE tissue sections are cut using a microtome at room temperature. Paraffin block can be cooled down −20°C prior sectioning to facilitate tissue sectioning.
2. Sections are transferred onto a conductive ITO glass slide on top of a water droplet.
3. Glass slide is warmed up on a hotplate to leave the cuts unfolds.
4. Excess of water is removed and glass slide is stored in an incubator at 30°C during 20 min for good adherence.

Subsequently obtained glass slides with FFPE tissue sections can be stored during over months at room temperature.

3.2.2. FFPE Tissue Dewaxing

1. After complete drying, the glass slide is gently dipped into a bath of xylene during 5 min. This procedure is repeated to fold. No agitation or shake is needed.
2. The slide is then washed in stepwise immersion, 5 min duration each, into 100% ethanol twice, 95% ethanol, 75% ethanol, and 30% ethanol for rehydration of tissue sections.
3. The ITO glass slide is placed in the vacuum desiccator for complete drying of the sections.

3.3. Tissue Preparation for In Situ Hybridization (ISH)

1. 10 μm thickness FFPE tissue sections obtained as previously described are used for ISH.
2. Paraffin is removed by using xylene baths (two times, 15 min), and then tissue is hydrated during 5 min in three steps of different mixed ethanol/water baths (96°, 70°, 30°).
3. Sections were prepared according to classical ISH protocols. Tissues were incubated in glycine buffer, and then treated for 15 min with proteinase K for protein digestion.
4. After post-fixation with 4% paraformaldehyde for 15 min, then a bath with triethanolamine (0.1 M, pH 8) was carried out for 10 min.
5. Sections were washed with 2× SSC, then ultrapure water for 5 min. Probes were denaturized at 100°C for 10 min, and after a 3 step tissue dehydration (30°, 70°, 96°), hybridization was done for 16 h at 55°C dissolving cDNA probes in hybridization buffer (Dextran sulfate 10%, formamide 50%, 20× SSC 20%, 100× Denhardt's 10%).
6. Tissues were incubated with RNase, then rinsed 10 min with successive SSC solutions and twice 0.5×SSC solutions at 55°C for 30 min. After rinsing slices with 0.1×SSC for 5 min at room temperature, one bath of ultrapure water was carried out to remove the excess of polymers. Tissues were kept drying at room temperature before MALDI matrix application.

3.4. Tissue Preparation for Immunocyto-chemistry (ICC)

1. Frozen sections of rat obtained as previously described are used for ICC.
2. They were incubated at room temperature with 500 μl of incubation buffer for 30 min. The same buffer was used to dilute anti-rat Carboxypeptidase D (CPD) antibody (1:400), and incubation was performed overnight at 4°C.

3. For the leech, Cystatin B and Cathepsin D primary antibodies are used at different concentrations (1/500 Cystatin B and 1/400 Cathepsin D).
4. After washing three times in PBS, sections were incubated with peroxidase conjugated secondary antibody or FITC-conjugated secondary antibody or using photocleavable tagged antibody for 80 min at room temperature.
5. After another three washing steps in PBS buffer, the sections for peroxydase ICC were incubated in chloronapthol with 0.05% H_2O_2 for detection. Reaction was stopped with several PBS and ultrapure water baths. For FITC ICC, slices were prepared using phenylenediamine in glycerol. For photocleavable tagged antibody, tissues were rinsed three times for 5 min with ultrapure water to remove salts, and sections were kept drying at room temperature in dark before matrix application. Tissues were then compared using microscopy.

3.5. Peptide Reporter Analysis

3.5.1. Peptide Reporter Analysis Using Dry Droplet

For classical analysis, 1 μl of sample solution and 1 μl of matrix solution (HCCA/ANI) were mixed on the MALDI plate using the dried-droplet technique as a standard control for the different Tag-Mass molecules before imaging.

3.5.2. Peptide Reporter Analysis Using Microspotter

1. An ITO slide after washing step for frozen tissues or digestion for FFPE or frozen tissues is used.
2. On each defined spot, 20 nl of HCCA/ANI solution is applied. 5 droplets of 100 pl are deposited at each spot per cycle, then 40 iterations are necessary to obtain the total volume. For slides after digestion, the matrix is deposited with the same array than the one used for trypsin deposition. In this case matrix is deposited exactly at the same position than the trypsin.
3. Check matrix coverage using an optical microscope.
4. A rapid MS analysis on one spot is recommended to verify that a sufficient amount of matrix is deposited. Increase of iteration number may improve MSI when signal intensity appears to be low.

3.5.3. Peptide Reporter Analysis Using an Automatic Sprayer

1. An ITO slide after washing step for frozen tissue or digestion for FFPE or frozen tissues is used.

2. A method with different step of spraying, incubation, and drying phase is needed. The ImagePrep method for HCCA/ANI deposition is based on the normal HCCA method included in the ImagePrep. Optimization is required for each type of tissue. Briefly, the spray time is around 2 s (depending the surface of tissue section). An incubation time of 20 s (except for initialization phase: 10 s) allows an effective extraction of proteins. A particular attention is drawn to correctly set the drying time for complete crystallization on the tissue section. If the time is too short, the section will be too wet and a delocalization of molecules will be observed. The minimum drying time is around 120 s.
3. Check matrix coverage using an optical microscope.
4. A rapid MS analysis at one position can be performed to check out that a sufficient amount of matrix has been deposited. If not, some cycles of the last phase of deposition can be done again and may improve MSI when signals intensity is too low.

3.6. Mass Spectrometry Analysis

3.6.1. MALDI-MSI Experiment: In Linear Mode

Acquisition parameters were set to acceleration voltage, 20 kV; first grid voltage, 94%; guide-wire voltage, 0.05%; extraction delay time, 200 ns. Each spectrum was an average of 500 laser shots at 100 Hz.

3.6.2. Mass Spectrometry Analysis for Proteins MSI (For Frozen Tissue Analysis Exclusively)

1. 0.5 μl of protein calibration solution is deposited near to the tissue section and mix with 0.5 μl of HCCA /ANI solution.
2. The mass spectrometer is calibrated with the calibration spot.
3. Using FlexImaging an area of interest is selected on the tissue after definition of the teaching points.
4. The distance between each measurement point is set. Distance between measurement points is, depending on the method, used for matrix deposition:
   With Chip-1000 deposition, the spots are generally spaced by 250 μm center to center. It is possible to define the same raster than the one defined during matrix deposition. Due to the size of the spot it is possible to accumulate spectra at different position in the same spot. This increase statistics and reduce spot-to-spot variability.

With ImagePrep deposition, distance between two measurements can be chosen by the user. Generally the resolution is around 100 μm.

5. In FlexControl, the adequate methods for proteins analysis is set in positive linear mode and a total of 500 spectra are acquired at each position at a laser frequency of 100 Hz.
6. The images are saved and reconstructed using FlexImaging 2.1.

3.6.3. Mass Spectrometry Analysis for Peptides MSI

1. 0.5 μl of peptide calibration solution is deposited near to the tissue section and mixed with 0.5 μl of ANI solution.
2. The mass spectrometer is calibrated with the calibration spot.
3. Using FlexImaging an area of interest is selected on the tissue after definition of the teaching points.
4. The distance between each measurement point is set. Distance between measurement points is dependent of the method used for matrix deposition.
   4.1. With Chip-1000, deposition spots are generally spaced by 250 μm center to center. It is possible to define the same raster than for matrix deposition. Due to the size of the spots spectra can be accumulated at different positions in the same spot.
   4.2 With ImagePrep deposition, distance between two measurements is chosen by users. Generally the resolution is around 100 μm.
5. In FlexControl, the adequate methods for peptides analysis is set in positive reflector mode and a total of 500 spectra are acquired at each position at a laser frequency of 100 Hz. Although negative reflectron mode can also be used for specific class of peptides.
6. The images are saved and reconstructed using FlexImaging 2.1.

4. Notes

4.1. Photocleavable Tagged Oligonucleotide

The peptide is synthesized on Symphony (Protein Technologies Inc.) and purified on a Delta-Pak C18 15 μm 100A column (Waters). The oligonucleotide is synthesized from 3′ to 5′ on Expedite (Applied BioSystems). The amine function with photocleavable linker is added in 5′ before cleavage and deprotection. These steps are performed using a NH_4OH 28% solution during 24 h in the dark. The amino oligonucleotide is then purified

on a Delta-Pak C18 15 μm 300A column (Waters). The amino function of the oligonucleotide is coupled with a heterobifunctional reagent comprising a maleimide function. The maleimido oligonucleotide is solubilized in water and added to a 1.2 equivalent of peptide in solution. The mixture is stirred for 16 h.

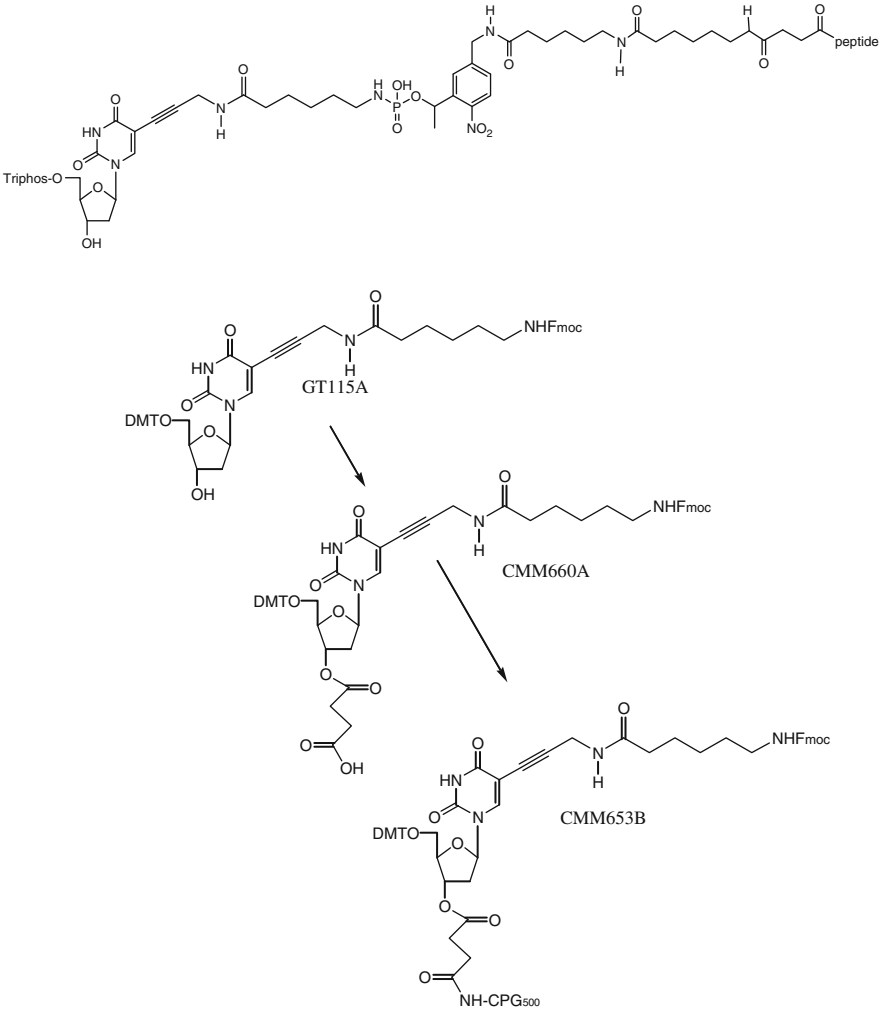
The oligo-peptide conjugate is then purified on a Delta-Pak C18 15 μm 300A column (Waters) and characterized by MALDI-MS (Voyager STR, Applied BioSystems).

4.2. Photocleavable Tagged Antibody

Peptides were custom made by Eurogentec S.A. using solid phase peptide synthesis (SPPS) on a 0.25 mmol (millimole) scale using Fmoc (9-fluorenylmethyloxycarbonyl amino-terminus protection) standard synthesis protocols (4 equivalents of Fmoc-AA) with double-coupling reactions (twice 40 min) using TBTU/NMM as activator on a Symphony (Rainin Instrument Co, Woburn, MA, USA) synthesizer. The photocleavable linker (4 equivalents) was introduced manually using DIPCDI/DIPEA (2 h) as activator. Purifications were performed by RP-HPLC on a Waters (Milford, MA, USA) Delta-Pak C18 (15 μm−100A−25×100 mm) column using a Waters liquid chromatography system consisting of Model 600 solvent delivery pump, a Rheodine injector, and a automated gradient controller (solvent A: H_2O-0.125% TFA; solvent B: CH3CN-0.1% TFA, gradients: 5–15 to 30−60% B in 20 min). Detection was carried out using Model M2487 variable wavelength UV detector connected to the Waters Millennium software control unit. The Quality Control was performed by analytical RP-HPLC on a Waters Delta-Pak C18 (5 μm−100A−150×3.9 mm) column (solvent A: H_2O-0.125% TFA; solvent B: CH3CN-0.1% TFA, gradient: 100% A−60% B in 20 min) using a Waters Alliance 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector and by MALDI-TOF MS (Voyager STR, Applied BioSystems).

The Functionalization with the photolinker derivatized peptide A was done as follow: a solution of 0.5 mg of MBS in 300 μl of DMF is added to a solution of 4 mg of goat anti-rabbit IgG in 2 ml of PBS and mixed for 30 min. The solution is then desalted on a PD 10 column using 50 mM phosphate buffer at pH =6. To this desalted activated IgG, a solution of 1 mg of the photocleavable derivatized peptide in 300 μl of DMF and 1 ml of PBS is added and stirred for 3 h at room temperature. Afterward, the reaction mixture is dialyzed overnight against PBS (membrane cut-off 12–14,000).

In order to prepare this triphosphate, a Fmoc-protected CPG resin was required. The succinylate was prepared from GT115A (100 mg) (Scheme **20.1**). The sample was relatively pure but contained a small amount (by TLC) of a higher running non-tritylated compound (originates from the Sonogashira reaction



Scheme 20.1. Synthesis of a dUTP-peptide conjugates with a photocleavable linker (see text for details).

and does not interfere with subsequent reactions and was not visible in the NMR spectra of the sample). Since it was not possible to purify the succinate, the reaction was modified slightly. It is normal to add two equivalents of succinic anhydride to the reaction to get quantitative yield but if this is not removed completely, the amino residues of the cpg resin can become blocked during functionalization. Therefore, 1.5 equivalents were used since the exact purity of the product is undetermined. The reaction did not go to completion (from TLC this was more than 50%) by comparing the intensity of the components on the TLC by UV (254 nm) and the intensity of the DMT cation on treatment with HCl fumes. Since the non-succinylated product will not react, the resin was functionalized using this mixture. The resin was prepared but the loading is very low, 5.4 μ mol g^{-1} (180 mg).

The resin was detritylated using 2% TCA/DCM washed with DCM and the process repeated until no orange color due to the DMT cation was observed. This was then dried (suction under argon) and the resin soaked in pyr/DMF 1:3 (0.4 ml) for 5 min before a solution of 0.1 M Eckstein's reagent in dioxane was added (0.1 ml). The reaction was allowed to stand for 15 min after which time the resin was washed (dioxane, MeCN) and dried (suction under argon). The resin was then soaked in a solution on 0.5 M *bis*-(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine for 20 min and the resin washed (DMF, MeCN) and dried (suction under argon). The product was oxidized (iodine/water/pyridine/THF for 30 min), washed (MeCN), and dried (suction under argon). The Fmoc protecting group was removed (20% piperidine/DMF, 0.5 ml, 20 min) and the resin washed thoroughly (DMF, MeCN) and dried (suction under argon). This was then washed with DCI and a solution of DCI/photolabile amino linker CEP (1:1, 0.5 ml) was added and the reaction was allowed to stand for 20 min. The solution was removed and the resin washed (MeCN) and dried (suction under argon). A mixture of cap A/cap B (1:1, 0.5 ml) was added and the resin soaked for 5 min before removing the capping reagents and washing and drying the resin as before. The product was oxidized (I_2/THF/pyr/H_2O, 5 min) and the resin washed and dried as before. This was cleaved from the resin with cNH_4OH at room temperature for 30 min, then purified by anion exchange HPLC on a Dionex NucleoPac100 HPLC column using the following solvent system Buffer A:0.1 M NH_4Cl with 10% acetonitrile; Buffer B: 1 M NH_4Cl with 10% acetonitrile; flow rate 2.5 ml /min. using 6Triphos.mth. This gave three fractions (A:–7 min, B:–7.9 min, and C:–10.3 min). All three fractions were lyophilized overnight before being desalted by reverse phase HPLC Buffer A: Water; Buffer B: acetonitrile; flow rate 4 ml/min. The three fractions were again lyophilized overnight before being suspended in 200 μl of water. MS showed that CMM661A pk 1 was definitely not the triphosphate but it could be either CMM661pk 2 or 3 (very similar MS profiles). (CMM662A was formed from CMM661A pk 2 and CMM663A was formed from CMM661A pk 3). Both samples were then used in the subsequent reaction. Bicarbonate buffer (10 μl) and the maleimide NHS ester (50 μl) were added to each sample and the reactions agitated overnight. The samples were diluted with milliQ water (500 μl) and filtered. The samples were purified by RP-HPLC, buffer A: 0.1 M TEAA, buffer B: MeCN, flow rate 4 ml /min. using MeCN50.mth and the coupling of the peptide was carried out on these fractions.

The use of a cryopreservative solution containing polymer compounds such as a solution with an optimal cutting temperature (OCT) polymer should be restricted to the attachment of

the tissue to the sample holder and not for wholly embedded the tissue. Moreover, all parts of the cryostat in contact with the tissue need to be cleaned to prevent any contamination between two different samples or with a polymer contain solution. In the case of contact between the tissue and a polymer containing cryopreservative solution, MS spectra will be dominated by polymer signals.

Acknowledgments

This study was supported by grants from the Centre National de la Recherche Scientifique (DPI), Ministère de L'Enseignement Supérieur et de la Recherche, the Agence Nationale de la Recherche PCV (To IF)

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