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TAG-MASS: SPECIFIC MOLECULAR IMAGING OF TRANSCRIPTOME AND PROTEOME BY MASS SPECTROMETRY BASED ON PHOTOCLEAVABLE TAG

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ABSTRACT -

MALDI Imaging of tissues has become a promising technique for tracking biomarkers while determining their localization and structural characterization. We have now developed specific targeting probes (oligonucleotides, antibodies), named Tag-Mass. This approach is based on probes modified with a photocleavable linker coupled with a tag cleaved and detected by Mass Spectrometry. Tag-Mass development is key for rapid, highly sensitive and accurate approach to correlate levels of expression of different mRNA or proteins in diseases. This opens the door to histo-proteomics studies involving tissue microarray for diagnostics and patient treatment monitoring.

***Equal contribution**

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INTRODUCTION

MALDI Imaging is a novel and powerful tool for direct detection and localization of biomolecules¹⁻⁶. Developments in this field have led to the study and anatomical localization of drugs, lipids, peptides and proteins in tissue sections³⁻⁸. This method eliminates time consuming and work intensive steps such as extraction, pre-purification or separation. Many successful applications of this technique have been undertaken recently. Of particular interest has been the application of MALDI imaging to the direct analysis of diseased tissues in order to hunt for specific biomarkers^{4, 9}. The analysis of nucleotide sequences is difficult however, and presents a real challenge for mass spectrometry. Moreover, as for traditional MALDI analysis, MALDI Imaging of proteins has a restricted mass range, and low abundance proteins, such as membrane or high molecular weight proteins, are hard to detect^{4-6, 9, 10}.

To overcome these problems, we proposed and developed a new concept of specific MALDI Imaging called "Tag-Mass". This strategy is based on the indirect MALDI imaging of mRNA or proteins using a probe labeled with a photocleavable linker, which is cleaved by the MALDI UV laser, releasing a Tag molecule. The Tag is a known molecule with a characteristic mass that can be detected efficiently by MALDI-MS, thus allowing for molecular images to be obtained more accurately and efficiently. By combining this approach with *in situ* hybridization or immunocytochemical methods, we are able to obtain, in addition to the standard molecular images, specific transcriptomic and peptidomic/proteomic maps. Molecular distributions and correlation of transcriptome and proteome expression could lead to important advances in the field of medical research, allowing for faster diagnosis by correlating the presence of transcripts with the corresponding proteins. Toward these goals, we report here on the feasibility of a specific MALDI Tag-Mass Imaging approach, which opens a new application field of Diagnostic Mass Spectrometry through the characterization of biomolecules present in cells that change their phenotypes during disease development.

MATERIAL AND METHODS

α -cyano-4-hydroxycinnamic acid (HCCA), 3-hydroxypicolinic acid (3-HPA), Angiotensin II, Des-Arg-Bradykinin, Substance P, ACTH 18-39, ACTH 7-38 and bovine Insulin were obtained from Sigma-Aldrich and used without any further purification. Trifluoroacetic acid (TFA) was purchased from Applied Biosystems. Acetonitrile p.a. and methanol p.a. were from J.T. Baker.

For Tag synthesis, solvents (DMF, dichloromethane) purchased from Biosolve were of the peptide synthesis grade and used as they are. The amino acids, the 4-[4-[1-(Fmoc-amino)ethyl]-2-methoxy-5-nitrophenoxy]butanoic acid (photoclivable linker) were purchased from Novabiochem,

the N-methylmorpholine (NMM), the N,N'-Diisopropylcarbodiimide (DIPCD), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDAC), the N,N-Diisopropylethylamine (DIPEA), from Aldrich, the trifluoroacetic acid, the 2-(1H-Benzotriazole 1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/N-Hydroxybenzotriazole (TBTU) from Biosolve, the goat anti-rabbit IgG, the PD 10 (G 25 resin column) from Sigma and the 3-Maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) from Pierce.

Tag synthesis

Photocleavable tagged oligonucleotide

The peptide is synthesized on Symphony (Protein Technologies Inc.) and purified on a Delta-Pak C18 15 μ m 100A column (Waters). The oligonucleotide is synthesized from 3' to 5' on Expedite (Applied BioSystem). The amine function with photocleavable linker is added in 5' before cleavage and deprotection. These steps are performed using a NH₄OH 28% solution during 24 hours in the dark. The amino oligonucleotide is then purified on a Delta-Pak C18 15 μ m 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 hours.

The oligo-peptide conjugate is then purified on a Delta-Pak C18 15 μ m 300A column (Waters) and characterized by MALDI-MS (see Mass Spectrometry section).

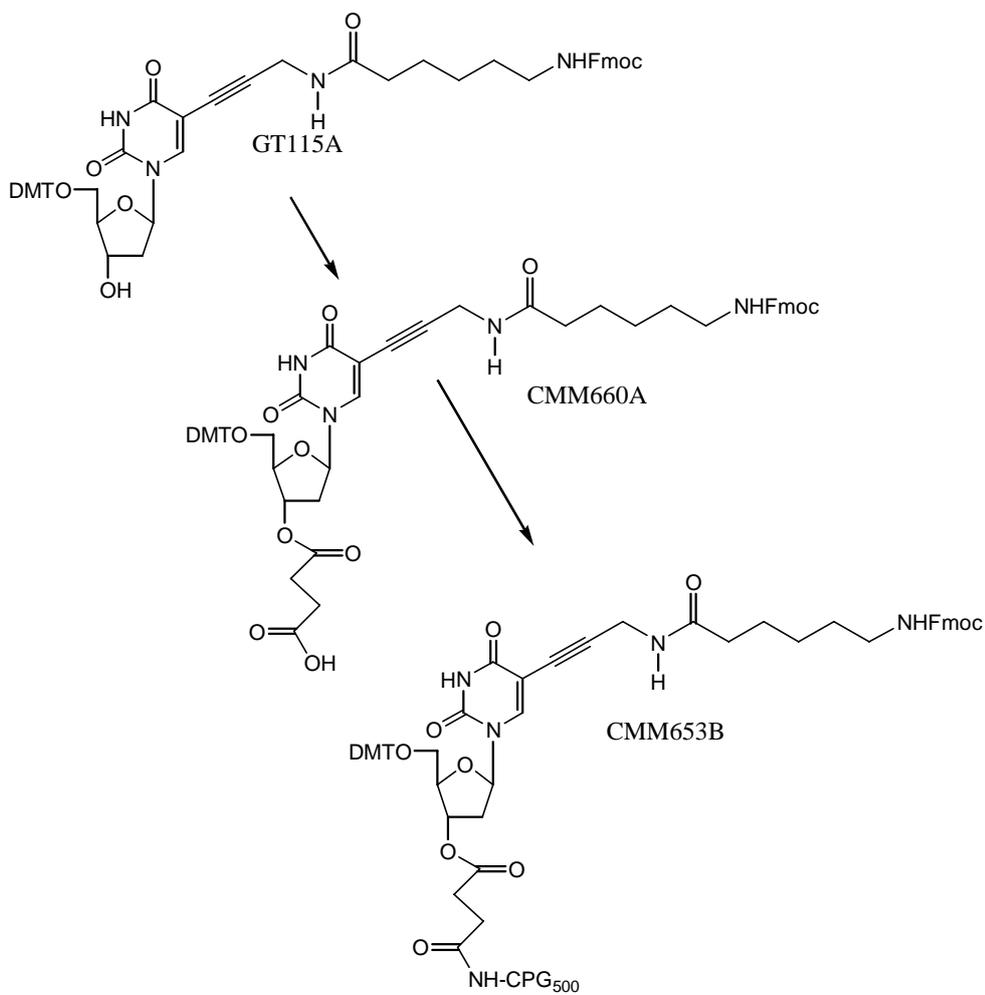
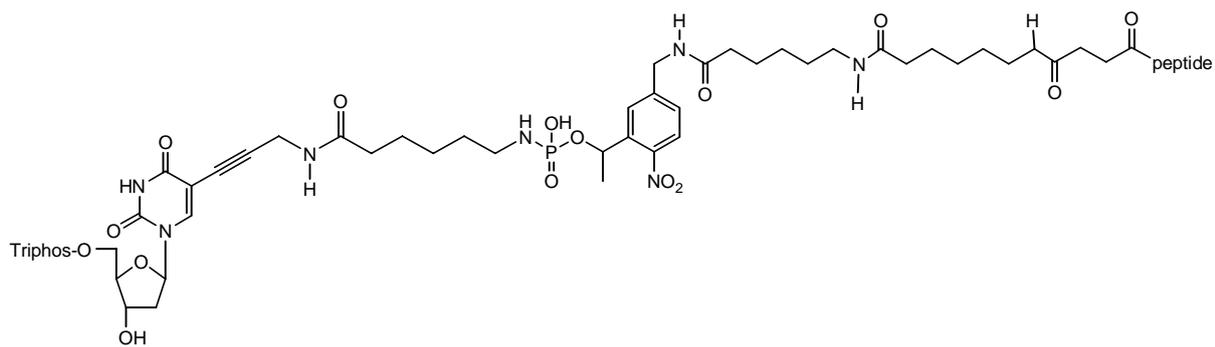
Photocleavable tagged antibody

Peptides were custom made by Eurogentec S.A. using solid phase peptide synthesis (SPPS) on a 0.25 millimole (mmole) scale using Fmoc (9-fluorenylmethyloxycarbonyl amino-terminus protection) standard synthesis protocols 4 equivalent of Fmoc-AA) with double coupling reactions (twice 40minutes) using TBTU/NMM has activator on a Symphony (Rainin Instrument Co, Woburn, MA, USA) synthesizer. The photocleavable linker (4 equivalents) was introduced manually using DIPCDI/DIPEA (2 hours) as activator. Purifications were performed by RP-HPLC on a Waters (Milford, MA, USA) Delta-Pak C18 [15 μ m-100A-25x100mm] column using a Waters liquid chromatography system consisting of Model 600 solvent delivery pump, a Rheodine injector and a automated gradient controller (Solvent A: H₂O-0.125% TFA; Solvent B: CH₃CN-0.1% TFA, Gradients: 5-15% to 30-60% B in 20minutes). Detection was carried out using Model M2487 variable wavelength UV detector connected to the Waters Millenium software control unit. The Quality Control was performed by analytical RP-HPLC on a Waters Delta-Pak C18 [5 μ m-100A-150x3.9mm] column (Solvent A: H₂O-0.125% TFA; Solvent B: CH₃CN-0.1% TFA. Gradient:

100% A to 60% B in 20minutes) using a Waters Alliance 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector and by MALDI-TOF MS (see mass spectrometry section).

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

Synthesis of a dUTP-peptide conjugates with a photocleavable linker



In order to prepare this triphosphate, a Fmoc protected CPG resin was required. The succinylate was prepared from GT115A (100mg). The sample was relatively pure but contained a small amount (by TLC) of a higher running non-tritylated compound (originates from the Sonogashira reaction 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 2 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 functionalisation. 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(254nm) and the intensity of the DMT cation on treatment with HCl fumes. Since the non-succinylated product will not react, the resin was functionalised using this mixture. The resin was prepared but the loading is very low, $5.4\mu\text{molg}^{-1}$ (180mg).

The resin was detritylated using 2% TCA/DCM washed with DCM and the process repeated until no orange colour due to the DMT cation was observed. This was then dried (suction under argon) and the resin soaked in pyr/DMF 1:3 (0.4ml) for 5 minutes before a solution of 0.1M Eckstein's reagent in dioxane was added (0.1ml). The reaction was allowed to stand for 15 minutes after which time the resin was washed (dioxane, MeCN) and dried (suction under argon). The resin was then soaked in a solution on 0.5M *bis*-(tributylammonium) pyrophosphate in anhydrous DMF and tri-*n*-butylamine for 20 minutes and the resin washed (DMF, MeCN) and dried (suction under argon). The product was oxidised (iodine/water/pyridine/THF for 30 minutes), washed (MeCN) dried (suction under argon). The Fmoc protecting group was removed (20% piperidine/DMF, 0.5ml, 20mins) 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, 0.5ml) was added and the reaction was allowed to stand for 20 minutes. The solution was removed and the resin washed (MeCN) and dried (suction under argon). A mixture of cap A/cap B (1:1, 0.5ml) was added and the resin soaked for 5 minutes before removing the capping reagents and washing and drying the resin as before. The product was oxidized ($\text{I}_2/\text{THF}/\text{pyr}/\text{H}_2\text{O}$, 5mins) 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:0.1M NH_4Cl with 10% acetonitrile; Buffer B: 1M NH_4Cl with 10% acetonitrile; flow rate 2.5 mL/min. using 6Triphos.mth. This gave 3 fractions (A:-7mins, B:-7.9mins and C:-10.3mins). All 3 fractions were lyophilized overnight before being desalted by reverse phase HPLC Buffer A: Water; Buffer B: acetonitrile; flow rate 4 mL/min. The 3 fractions were again lyophilized overnight before being suspended in 200ul of water. M.S. showed that

CMM661A pk 1 was definitely not the triphosphate but it could be either CMM661pk 2 or 3 (very similar M.S. 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 (10ul) and the maleimide NHS ester (50ul) were added to each sample and the reactions agitated overnight. The samples were diluted with milliQ water (500ul) and filtered. The samples were purified by RP-HPLC, buffer A: 0.1M TEAA, buffer B: MeCN, flow rate 4mL/min. using MeCN50.mth and the coupling of the peptide was carried out on these fractions.

Tissue preparation for *in situ* hybridization (ISH)

Formalin Fixed Paraffin Embedded tissues (FFPE) sections of 10 µm were transferred onto a conductive ITO-glass. Sections were stored at 30°C overnight for good adherence. Tissues were then dewaxed using xylene (2x15 minutes), and then hydrated during 5 minutes in 3 steps mixed ethanol/water baths (96°, 70°, 30°). Sections were prepared according to classical ISH protocols. Tissues were incubated in glycine 0,1M/Tris HCl buffer (pH7.4), then treated for 15 minutes with proteinase K for protein digestion (1µg/µL in 1M/Tris HCl, 0.5M EDTA pH 8). After post-fixation with 4% paraformaldehyde (0.1M Phosphate/5mM MgCl₂ buffer pH 7.4) for 15 minutes, a 10 minute bath with triethanolamine (0.1M pH 8) was carried out. Sections were washed with SSC 2X then ultrapure water for 5 minutes. Probes were denaturated at 100°C for 10 minutes, and after a 3 step tissue dehydration (30°, 70°; 96°), hybridization was done for 16H at 55°C dissolving cDNA probes in hybridization buffer (Dextran sulfate 10%, formamide 50%, SSC 20X 20%, Denhardt's 100 X 10%). Tissues were incubated with Rnase (10µg/mL, 37°C 30 minutes) then rinsed 10 minutes with successive SSC/2mercaptoethanol (0.07%) solutions (2X, 1X) and twice 0.5X at 55°C for 30 minutes. After rinsing slices with SSC 0.1X for 5 minutes 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.

Tissue preparation for immunocytochemistry (ICC)

Adult male Wistar rats weighing 250-350 g (animal welfare accreditation by the French ministry of the agriculture N° 04860) maintained under standard care were used. Animals were sacrificed by decapitation and immediately dissected to remove the brain. Frozen sections of 10 µm were performed on a cryostat and immediately transferred onto a conductive Indium-Tin Oxide (ITO) glass (Bruker Daltonics, Wissenbourg, France).

After drying sections for 5 minutes at room temperature, tissues were heated at 80°C for 20 seconds to allow for good adherence on slides. Rat brain sections were incubated at room temperature with 500 µL buffer (0.1M PBS / 1% BSA / 1% normal goat serum / 0.05% triton

X100) for 30 minutes. The same buffer was used to dilute carboxypeptidase D (CPD) antibody (1/400), and incubation was performed overnight at 4°C. After 3 times washing in PBS, sections were incubated with peroxidase-conjugated secondary antibody (anti-rabbit IgG 1/100 developed in goat; Jackson Immunoresearch Inc. Europe LTD) or FITC-conjugated secondary antibody (anti-rabbit IgG 1/100 developed in goat; Jackson Immunoresearch Inc. Europe LTD) or using photocleavable tagged antibody (1/100) for 80 minutes at room temperature. After another 3 washing steps in PBS buffer, the sections for peroxidase ICC were incubated in chloronaphthol with 0.05% H₂O₂ 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 3 times for 5 minutes with ultrapure water to remove salts, and sections were kept drying at room temperature before matrix application. Tissues were then, compared using microscopy.

Mass Spectrometry

Sample preparation for MALDI/MS analysis.

For classical analysis 1 µL of sample solution and 1 µL of matrix solution were mixed on the MALDI plate according to the procedure of the dried-droplet preparation.

Classical analysis in linear Mode.

Acquisition parameters were set to: acceleration voltage: 20 kV; 1st grid voltage: 94%; guide-wire voltage: 0.05% ; extraction delay time: 200 ns.

Each recorded mass spectrum results from the average of 100 laser shots.

MALDI-MS Direct Analysis and MALDI imaging

MALDI-TOF mass spectra and imaging were performed on a Voyager-DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA) with delayed extraction (DE) and a 337 nm pulsed nitrogen laser. For routine analysis, HCCA was used at concentration's of 10 mg/mL in ACN /H₂O (2:1, v/v, 0.1% TFA) and 3HPA at 50mg/mL in H₂O/ammonium citrate (100mM). For direct tissue analysis and MALDI imaging, HCCA was used at 10 mg/mL in ACN /H₂O (2:1, v/v). Matrix was applied onto the tissue using a micropipette (typically 20 µL for a whole rat brain slice) and then dried at room temperature. External calibration was performed using a peptide mixture (1.6 µM bradykinin, 1.6 µM Substance P, 1.6 µM ACTH 18-39, 3.2 µM ACTH 7-38, 4.8 µM bovine Insulin and 4.8 µM bovine Ubiquitin in H₂O). Slices were visualized in the mass spectrometer using a color CCD camera (SONY). Each recorded mass spectrum is the result of the average of 100 laser shots on the area of interest. Acquisition parameters were set as follow:

acceleration voltage: 25 kV, 1st grid voltage: 94%, guide-wire voltage: 0.1%, extraction delay time: 200 ns.

Images were reconstructed using MALDI Imaging Tools (MIT, M. Stoeckli, Novartis Inc., Switzerland). 30 000 points covering the whole slice with 100 laser shots per position were scanned. From each position, the software measures an average mass spectrum with its coordinates on the slice.

RESULTS AND DISCUSSION

The Tag-mass concept: from indirect detection of photocleaved tag to images of the tissue distributions of mRNAs and proteins

In situ analysis of tissues by MALDI Imaging does not allow the detection of oligonucleotides because of their large size and low abundance in cells, in addition to analytical difficulties inherent in the identification of oligonucleotides in mass spectrometry^{11, 12}. Somewhat different challenges are encountered in the analysis of high mass (> 30 kDa) and low abundance proteins when probing tissue by MALDI-MS^{2, 4, 5}. To solve these problems and to provide access to the full proteome and transcriptome for MALDI imaging, we have designed a new approach to the specific imaging of these molecules. This approach is based on the indirect detection of mRNA and proteins *via* a “Tag-mass” indicator. The “Tag” is a small molecule of a mass that can be readily detected by MALDI. It is coupled to an appropriate probe by means of a linker that can be cleaved efficiently by the UV-laser irradiation during the MALDI procedure (Figure 1A). The approach can be applied in MALDI imaging by using specific probes that will target either mRNA or proteins in tissue wholemounts or slices. Tagged photocleavable linkers can be chemically attached to DNA, cDNA, singlestranded cRNA, or antibody probes. These probes can then be used with hybridization methods, such as like *In Situ* Hybridization (ISH) for oligonucleotides probes¹³, or with epitope binding ImmunoCytoChemistry (ICC) for antibody probes to target mRNA or proteins, respectively¹⁴.

In MALDI, material ejection is promoted by laser irradiation and restricted to the area where the laser beam impacts the sample surface. The mass spectrum reflects the molecular composition of the tissue in this specific site. If the tagged probe hybridizes to its complementary mRNA sequence, then the laser irradiation will photocleave the linker, inducing tag release, leading to a characteristic signal of known mass. At positions where no target mRNA are present, the characteristic signal for the tag will not be observed. Thus, as in standard MALDI imaging, scanning the tissue section, images of mRNAs can be obtained indirectly by reconstructing the molecular image of the tag molecule on the basis of its mass signal mass data. This approach brings mass spectrometry’s sensitivity and the possibility of multiple imaging at once using multiple tags (Figure 1B).

The same strategy can be adapted for mapping target proteins using tagged antibodies in combination with ICC experiments. A specific tagged antibody can be used for indirect experiments. However, in our opinion, addition of the linker and tag on a secondary antibody recognizing the C-terminus of a primary antibody is easier to use and allows for signal amplification. Thus, as for ICC experiments, a primary antibody will specifically bind its target antigen. The tagged secondary antibody will then specifically recognize the first one. Then, as for mRNA, the scanning of the tissue sections by MALDI MS will allow indirect detection of the protein by monitoring the tag in the mass spectra. As for oligonucleotides, multiple different protein specific images can be obtained by MALDI in one experiment by using directly tagged primary with different peptides or secondary antibodies developed in different animal species.

Inherent in this approach to specific molecular imaging of mRNA and proteins are the sensitivity and multiplex possibilities of mass spectrometry. It should be of great interest for transcriptome/proteome co-localization mapping, and will find application when and where co-localizing a protein and its corresponding mRNA are important. In particular, it may permit mapping in tissues and slices the heterogeneous distribution of localized transcriptional regulation by monitoring the relative levels of a transcript and the corresponding protein.

Photocleavable tagged oligonucleotide probes: mRNA MALDI imaging

As a first step¹⁵, the performance of photocleavage dissociation by MALDI UV laser was evaluated for oligonucleotides. A photocleavable linker was bound to the 5' oligonucleotide end and peptides were chosen as tags and photodissociation was assumed to induce cleavage directly between the phosphate group and the photoactivable linker (Figure 3). As shown in Figure 4, photocleavage by laser irradiation is efficient and results in the characteristic signal expected for the tag (m/z 1626.37 noted P-PC). However, the MALDI mass spectrum clearly shows that 100% photocleavage yield is not achieved, since signals corresponding to the whole intact structure are still observed (m/z 6970.50 for MH^+ ion and 3489.92 for MH_2^{2+} ion). Signals at m/z 5351.03 and 2678.79 correspond respectively to the MH^+ and MH_2^{2+} of the oligophosphate after photocleavage. Peak broadening from salt adducts and metastable fragmentations decreases mass accuracy, a common occurrence in MALDI oligonucleotide measurements. At m/z 1163.9, the low abundance peak was attributed to a fast cleavage of the linker disulfide bridge liberating the peptide. To study the possibility of using photocleavage under multiplex analysis conditions, a mixture of three oligopeptides corresponding to three different 20 mers oligonucleotides and three different peptides was investigated. As presented in Figure 3, expected characteristic signals of the photodissociation (P-PC) were observed as proved by the presence of peaks at m/z 1346.95, 1603.74 and 1891.72. As expected from previous data fast fragmentation ions were also observed (m/z 1161.87, 1449.88) and

were especially intense for one of the peptides. For subsequent experiments, bradykinin was selected because it gives the best sensitivity (100 fmoles) and ease of detection in all analytical conditions (P3).

To increase sensitivity by suppressing fast fragmentation signals a new photocleavable linker/tag system was designed replacing the disulfide bridge by a maleimide group for binding the tag peptide to the photocleavable linker (Figure 5). Studies performed on this structure clearly demonstrate the suppression of fast fragmentation signal and increase of the expected signal for photocleavage. This photocleavable linker/tag system was used to construct double-stranded cDNA oligonucleotidic probes. Proenkephalin was chosen as the model target mRNA because previous studies gave low background for this molecule's mRNA distribution in the rat brain¹⁶. Standard untagged and tagged primers bearing the photocleavable linker/tag system were added to the proenkephalin cDNA sequence. Both types of primers were used for RT-PCR amplification and have given the same results in terms of product and yield of amplification. RT-PCR was used to make a 400 bp double-stranded cDNA oligonucleotidic probe (data not shown). The probe was tested in ISH experiments and followed by MALDI analysis of hybridized rat brain tissue sections for tag detection. The mass spectra obtained clearly demonstrated the possibility of detecting the characteristic ion signal for the tag in some specific region of the section hybridized with the tagged probes. This signal was never found for control tissue sections where the untagged proenkephalin probes were used. However, signal intensity for the tag was weak and somewhat lacking in sensitivity, although the feasibility of the proposed strategy was unambiguously demonstrated. Therefore, the strategy was slightly modified in order to improve sensitivity, increase specificity and facilitate usage.

An alternative approach using dUTP-TAG was developed, based on the labeling of uracil with the same photocleavable linker/tag system described above. This new design keeps both the 5' and 3' ends of the nucleotide free, allowing the synthesis of riboprobes containing the modified base by *in vitro* translation. This first step increases the specificity of the method, thus preventing non specific binding to genomic DNA. Moreover, use of a tagged nucleotide leads to the incorporation of one tag for each T nucleotide (Figure 6), increasing sensitivity. Finally, the use of tagged nucleotides has the very practical consequence, that these nucleotides can be incorporated using standard procedures, regardless of the probe being amplified.

The dU-tagged nucleotides were tested for photocleavage, as shown in Figure 7. Again, the characteristic signal of the tag peptide was observed (m/z 1163.23), with very good S/N ratio and signal intensity, demonstrating that the maleimide group incorporation prevents fast fragmentation. The dU-tagged system was used for amplifying the proenkephalin probe by RT-PCR. As expected from the structure (Figure 8) and previously described for dUTP-DIG systems¹⁷ (Ree et al., 1992),

dNTP ratios had to be optimized, with best results obtained with dU-TAG and dTPP in a ratio of 1:3. ISH was performed with the multiple tagged proenkephalin amplified probes. MALDI analysis of the hybridized rat brain tissue sections was then carried out and compared to the analysis of adjacent tissue sections hybridized with untagged proenkephalin probe amplified in the same conditions but with unmodified dNTP (Figure 9). The characteristic signal of the tag peptide was observed in rat brain tissue for the multiply-tagged proenkephalin probe, whereas this signal never appeared when the untagged probe was used. As expected, mass spectra reveal a clear increase in detection of the ion corresponding to the tag peptide. MALDI imaging was subsequently performed on this tissue section by scanning the tissue and recording corresponding mass spectra (7000 spots, 100 μm steps) (Figure 10). Based on the characteristic tag signal (Figure 9b), an image of the molecular distribution of proenkephalin mRNA was reconstructed, as presented in Figure 10d. The image fits the tissue morphology well (Figure 10c) and matches the proenkephalin mRNA distribution obtained by ISH with autoradiography¹⁸ or the one obtained in mouse brain with digoxigenin probe¹⁹ (Figure 10b). These experiments clearly demonstrate the feasibility of specific mRNA imaging by MALDI mass spectrometry.

Photocleavable tagged antibody probes for antigen MALDI imaging

The same concept was applied in obtaining specific images of proteins using tagged secondary antibodies. As previously explained, in order to gain sensibility and ease of use, secondary antibodies were tagged on the C-terminal part of their Fc chain (Figure 8). Immunocytochemistry (ICC) experiments were performed with a standard primary antibody directed against the protein of interest and peroxidase- or FITC-tagged secondary antibodies were replaced by our specifically modified antibody for MALDI imaging (Figure 11). The tagged antibody was first tested for evaluating the yield of photocleavage and checking out the presence of expected tag signals (Figures 11a,b). Examination of the mass spectra leads to the observation of two characteristic signals for the tag (m/z 1686.43 and 1703.23) (Figure 11b). The higher m/z signal corresponds to the P-PC moiety observed with the oligonucleotide probes. The m/z 1686.43 signal corresponds to the same moiety, minus one oxygen. Such signals have already been described for photocleavable systems, but no mechanism was proposed. Two unexpected, less abundant signals were also observed and were attributed to some of the maleimide reaction intermediates remaining in the solution after purification. As for oligonucleotides, photocleavage with antibodies was highly efficient, resulting in the observation of characteristic signals. For imaging experiments after ICC (Figure 12a), Carboxypeptidase D (CPD) was chosen as the target protein for several reasons, in particular its presence in some rat brain regions as known from previous studies and the impossibility of its direct detection by MALDI imaging because of its large mass and nature

(membrane protein of 180 kDa)²⁰. ICC experiments were carried out in parallel on two adjacent rat brain sections using the same primary antibody and comparing our tagged antibody to the same but FITC-labeled antibody (Figures 12 c,d). As shown in Figures 12c,d, several signals were observed from the direct analysis of the tissue section with the FITC antibody corresponding to peptides present in the tissue section. The mass spectrum obtained in the same region with the tagged antibody also displays these signals (Figure 12a). However, in this case two more signals corresponding to the expected signals for the tag peptide are also observed. This tissue section was then kept for MALDI imaging scanning of 30,000 spots, in 100 μm steps. On the basis of signal intensity for the characteristic ion at m/z 1686.43, which is the most abundant one, the image was reconstructed, giving indirectly the image of the protein (Figure 10B). As observed by the figure, very good image resolution was obtained. The protein distribution in the section is perfectly correlated to tissue structure, as observed when comparing to a picture of the section before ICC experiments (Figure 12b). In parallel, the same experiments were performed on adjacent sections with anti rabbit antibody coupled with a peroxydase enzyme for specific reaction with colorimetric detection using either DAB or 4-chloronaphtol (Figure 12d). Comparison pictures of T-chloronaphtol or DAB labeling to MALDI images based on the tag detection shows that CPD protein is detected in the same brain area using both methods, thus validating the methodology. However, in some specific areas, such as the hippocampus, CPD was detected with the tag-mass system while no signal was detected with the colorimetric reaction, indicating that the indirect detection by mass spectrometry is much more sensitive than classic techniques (Figure 12a). Comparing the MALDI image of CPD to the fluorescence image obtained from the section on which FITC antibody was used, MALDI appears to be as sensitive as fluorescence (Figure 12c,d). It is worth noting that the MALDI molecular strategy gives a more localized signal compared to fluorescence, which is diffuse and presents a significant amount of background noise due to the natural fluorescence of the tissue. Moreover, at low magnification, signals are found in the same areas by fluorescence and MALDI imaging. However, at the present time MALDI imaging does not allow for sub-cellular localization such as what can be achieved by fluorescence. Moreover, no quenching, or auto fluorescence, or quick degradation problems are observed with Tag-Mass tagged-antibodies.

CONCLUSION

We have demonstrated here the application of a new concept for specific imaging of mRNA and proteins, using MALDI mass spectrometry to image target molecules in sectioned tissue. This strategy relies on targeting specific biomolecules with probes that contain a photocleavable linker and a tag moiety that can be photodissociated by MALDI laser irradiation. We have successfully

tested this strategy with peptide tags on oligonucleotide and antibody probes. With this method, we were able to image a 180 kDa membrane protein and proenkephalin mRNA. For oligonucleotides, U-tagged nucleotide probes were found to be the best choice for increased sensitivity and flexibility. Mass spectrometric detection was shown to be very sensitive, yielding distributions of the molecules of interest on whole tissue sections with more sensitivity than most of the classical methods using peroxidase as a substrate, and giving highly resolved images of molecules that could not be observed directly by MALDI imaging. However, method sensitivity could still be increased by using multiplex experiments with several different tags. Image resolution could also be improved and is currently under development. This strategy can also be extended to other types of probes, including lectins or aptamers, which will also allow for the imaging of polysaccharides, peptides, proteins or drugs (Figure 13). It will also be possible to develop different families of tags that will give better signal detection by MALDI. Thus, MALDI matrices or compounds absorbing at the laser wavelength and possibly detectable by Laser Desorption Ionization (LDI) will be investigated in order to acquire images without the need for the matrix. Finally, photocleavage is a very elegant strategy but linkers that would dissociate by prompt fragmentation during the desorption/ionization process could be a good alternative. Linker dissociation by fragmentation could perhaps lead to a higher tag release rate. Moreover, using such linkers could extend the concept from UV- to IR-MALDI, or even to SIMS mass spectrometry for specifically designed linkers and tags, depending on the ion production method used. SIMS applications would also be very attractive, as SIMS imaging allows subcellular probing (classically 1 μm resolution), however, currently SIMS is limited to monoatomic elements or small organic compounds such as lipids. Finally, Tag-mass could open a great opportunity for mass spectrometry to image all biomolecules present in tissues, with a very high sensitivity and without being restricted by low abundance or size. In addition, specific imaging could be used for relative quantification of target molecules. The use of tags that differ from each other only in isotopic composition (e.g. deuterated molecules) and hence behave similarly when analyzed by MALDI would open the way for quantification by MALDI imaging. Finally, the concept could also be transferred to microarrays for more quantitative and specific detection.

Tag-mass strategies provide a unique opportunity to obtain transcriptome/proteome co-localization maps, providing very useful information on biological processes, .e.g., for diagnostic screening of the transcript before the synthesis of the corresponding protein. Extending MALDI imaging to specific aptamer probes, for example, will also allow for the co-localization of a drug and its assumed receptor target.

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FIGURE LEGENDS

FIGURE 1:

- A. Schematic representation of the concept of MALDI imaging of mRNA using tagged oligonucleotide probes for detection by photocleavage.
- B. Scheme of the photocleavable linker/tag system for indirect detection after photodissociation under the MALDI UV laser wavelength.

FIGURE 2:

Developed structure of the oligonucleotide tagged by the photocleavable linker tag/system intact and after photodissociation under the MALDI laser.

FIGURE 3:

MALDI mass spectrum in the linear positive mode of an oligopeptide including a 20 mer oligonucleotide sequence and the photocleavable tag/system with 3-HPA as matrix.

FIGURE 4:

MALDI mass spectrum in the reflectron positive mode of three oligopeptides including three different 20 mer oligonucleotides and the photocleavable linker/tag system with three different peptides as tags with HCCA as matrix.

FIGURE 5:

Developed structure of the oligonucleotide tagged by the photocleavable linker/tag system with a maleimide group for bonding to the tag peptide structure.

FIGURE 6:

A. Scheme of the incorporation of the tagged U nucleotide in a single strand RNA probe.

B. Developed structure of the U-tagged nucleotide

FIGURE 7:

MALDI mass spectrum in the reflectron positive mode of the U-tagged nucleotide in solution with HCCA as matrix.

FIGURE 8:

Developed structure of the photocleavable linker/tag system modified secondary antibody

FIGURE 9:

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).

FIGURE 10:

a- Image and map of in 8-weeks old male C57BL/6J mouse brain from the Allen Institute (<http://www.brainatlas.org/aba/>).

b- of Pro-enkephalin mRNA localization in 8-weeks old male C57BL/6J mouse brain using digoxigenin ISH technique the Allen Institute (<http://www.brainatlas.org/aba/>). 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

c- Picture of the rat brain slice before ISH with the U-tagged proenkephalin probe

d- MALDI molecular image reconstructed by scanning the tissue section after ISH experiment with the U-tagged proenkephalin probe (7000 spots separated each of 100 μ m).

FIGURE 11 :

Compared MALDI mass spectra in the linear positive mode recorded on two adjacent rat brain sections in the same region of the brain after ICC experiment with a primary antibody directed

against carboxypeptidase D protein and a rabbit FITC polyclonal secondary antibody labeled with a FITC (a) or bearing the photocleavable linker/tag system (b).

FIGURE 12 :

Rat brain tissue section picture before the ICC experiment and corresponding MALDI molecular image reconstructed on the tag peptide characteristic ion screening after ICC experiment with the tagged secondary antibody (30000 spots separated each of 100 μm) (a,b) and comparison to pictures of specific region of the adjacent rat brain sections after ICC experiment with FITC (c,d) or peroxydase (e) secondary antibody.

FIGURE 13:

Schematic representation of the concept of specific imaging for different target biomolecules and probes.

FIGURE 1

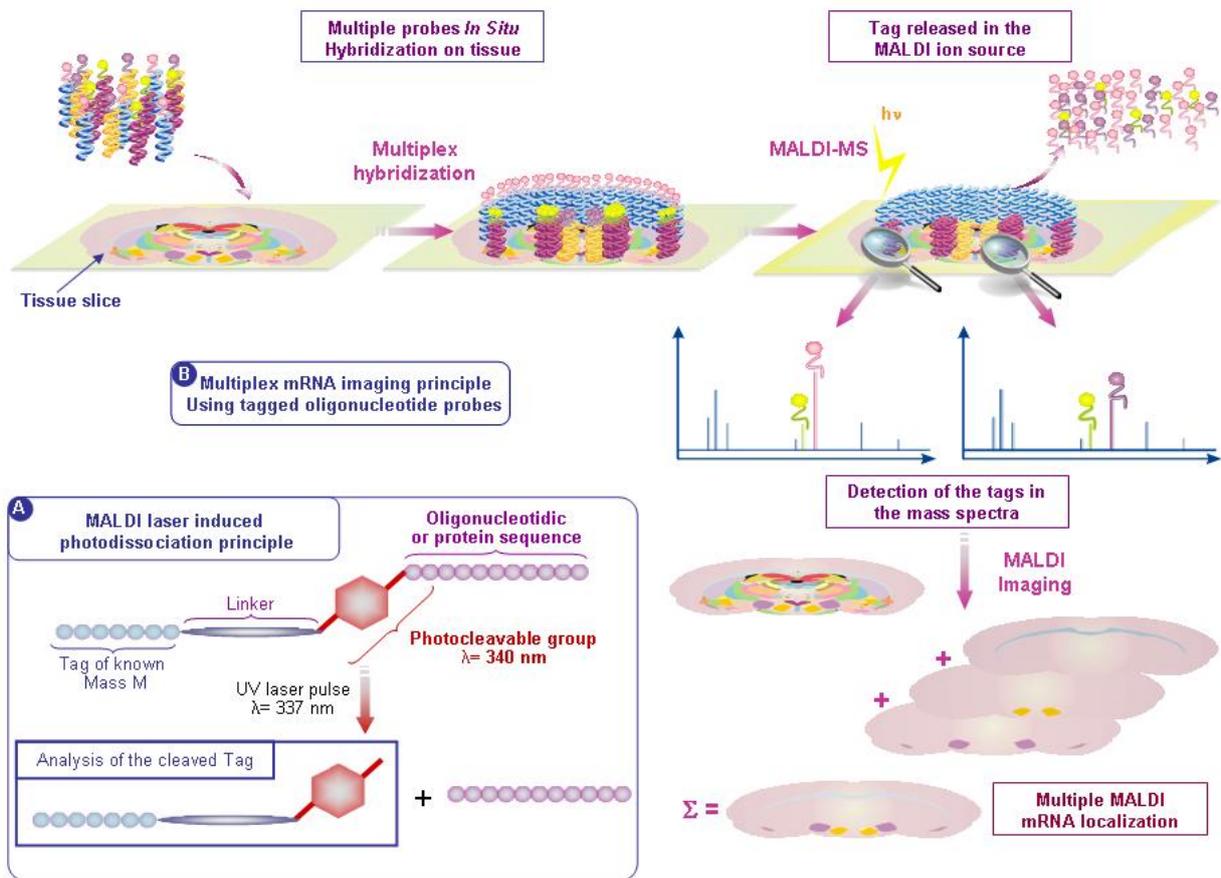


FIGURE 2

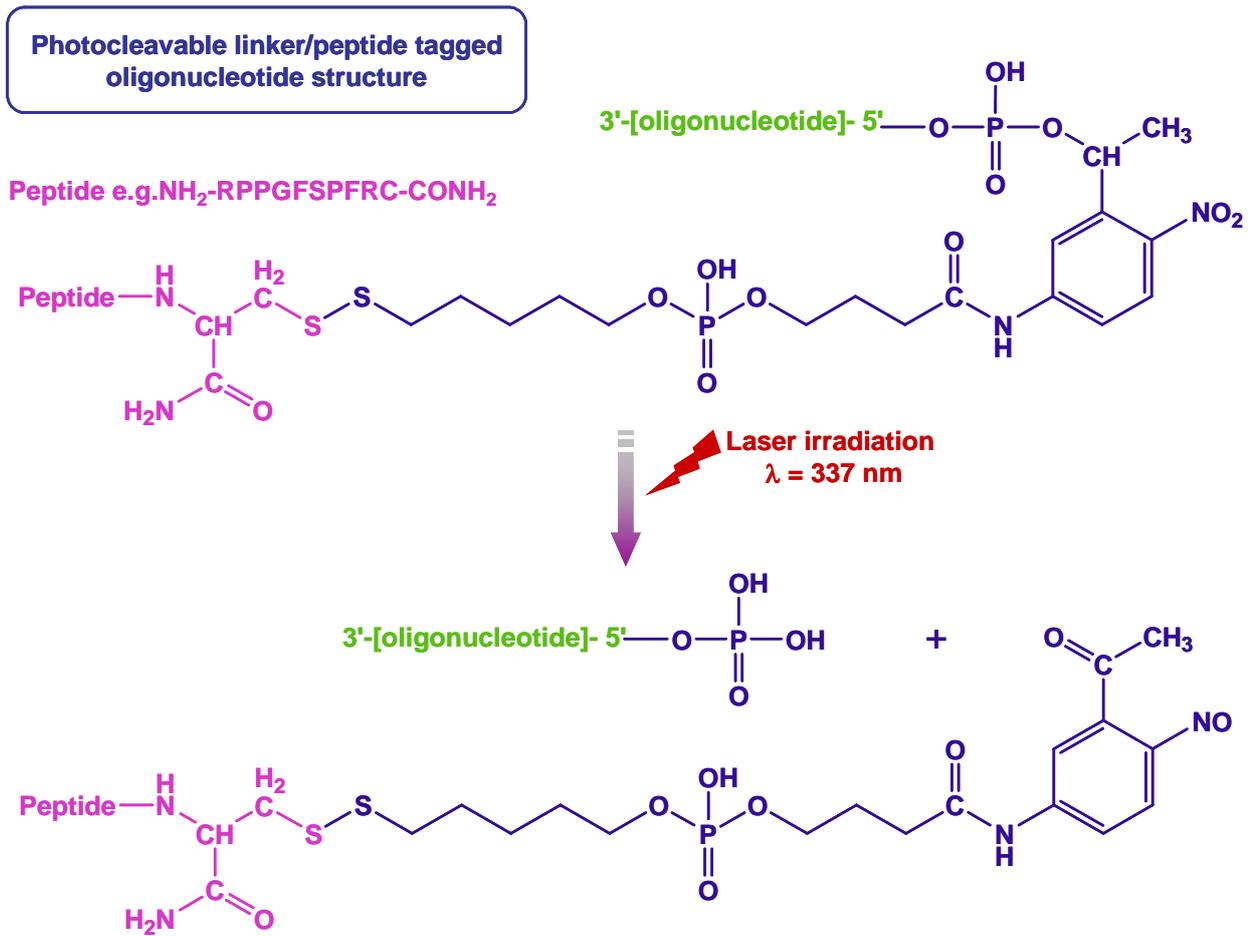


FIGURE 3

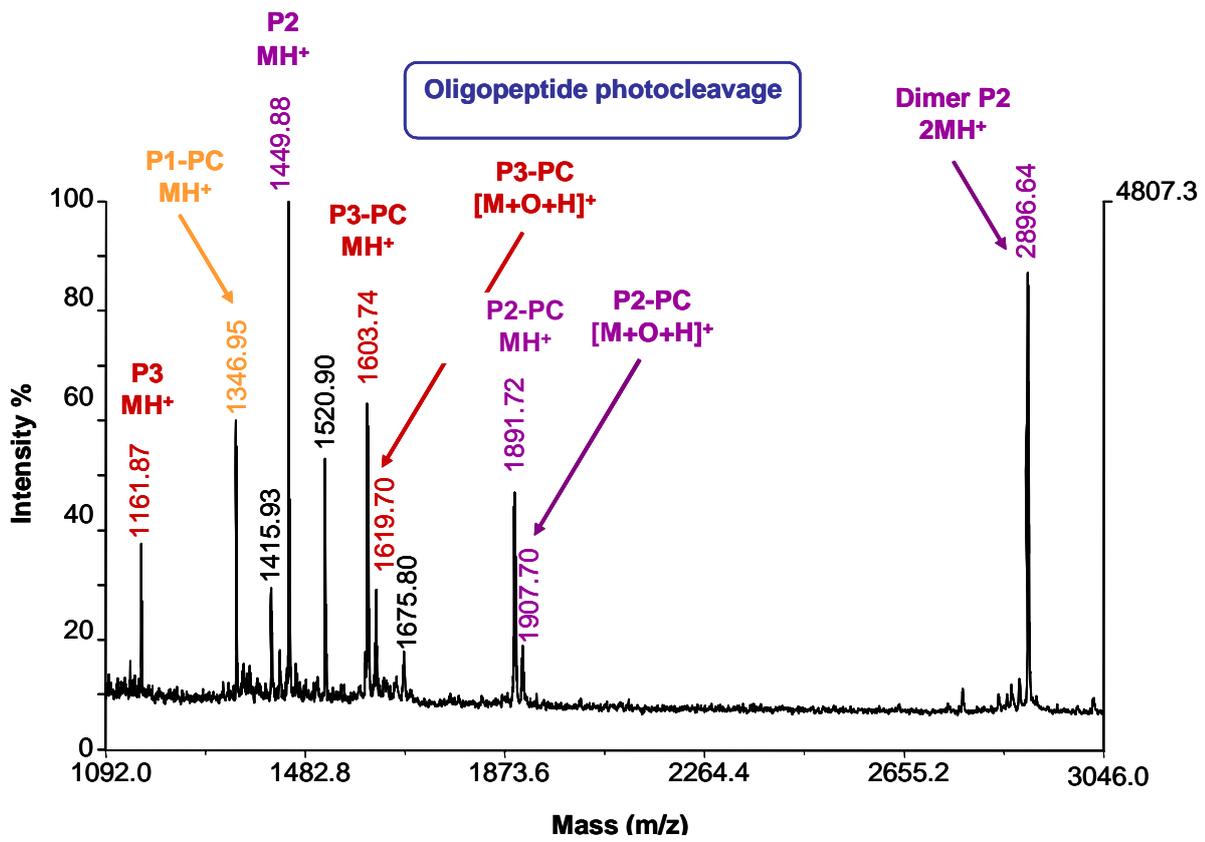


FIGURE 4

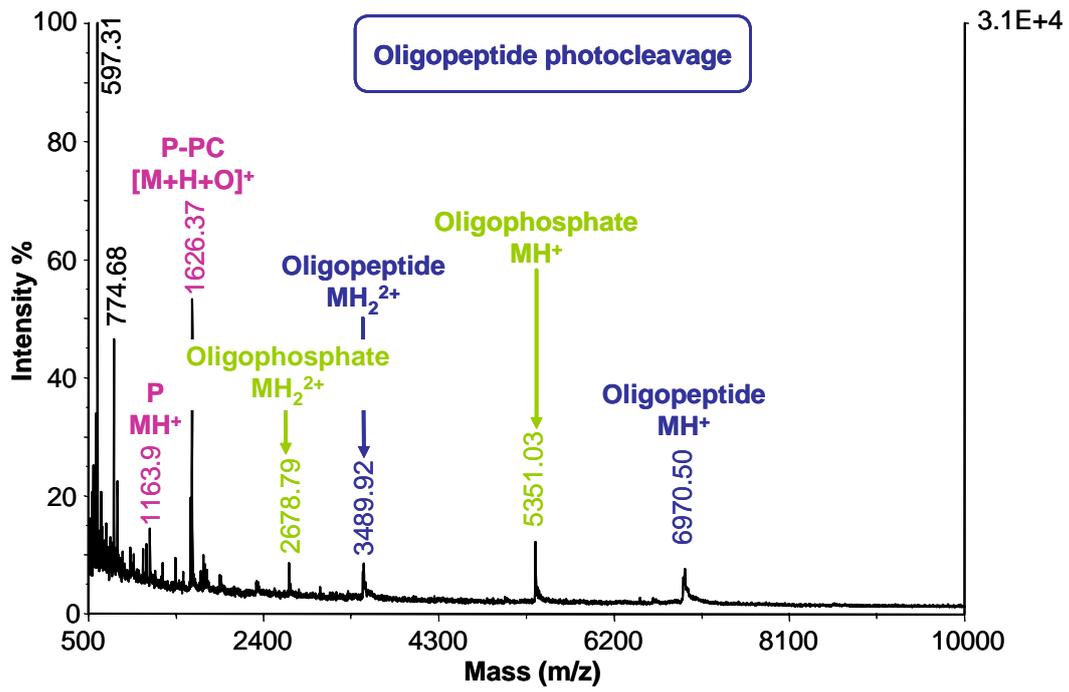


FIGURE 5

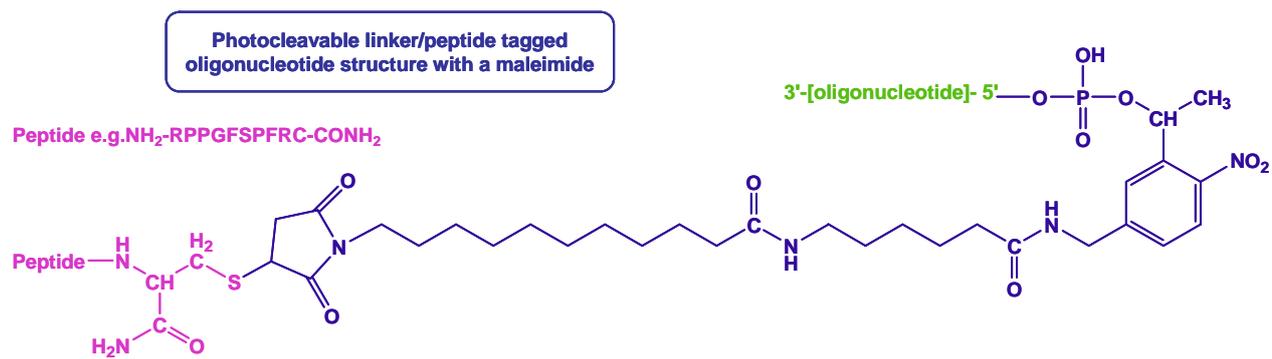


FIGURE 6

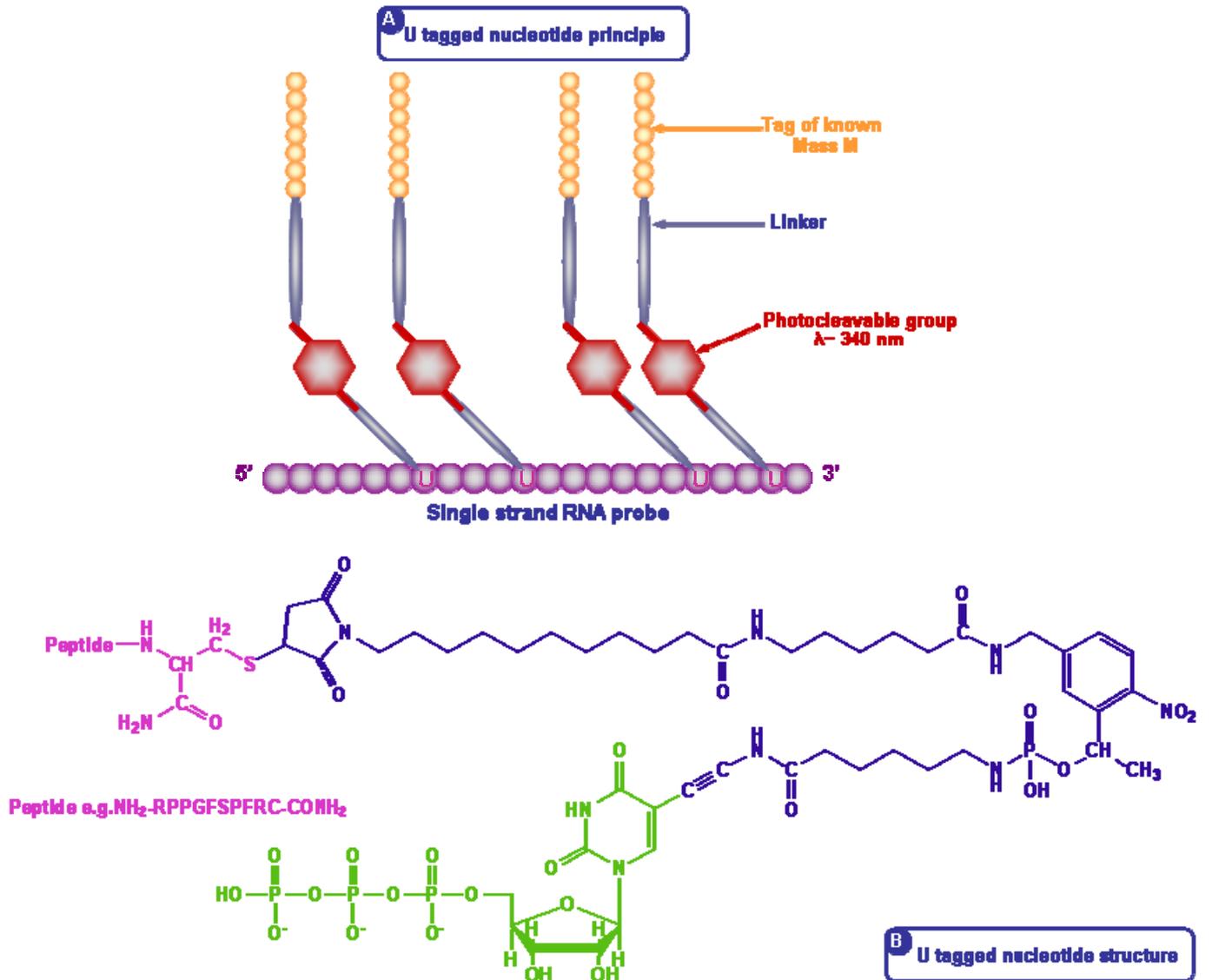


FIGURE 7

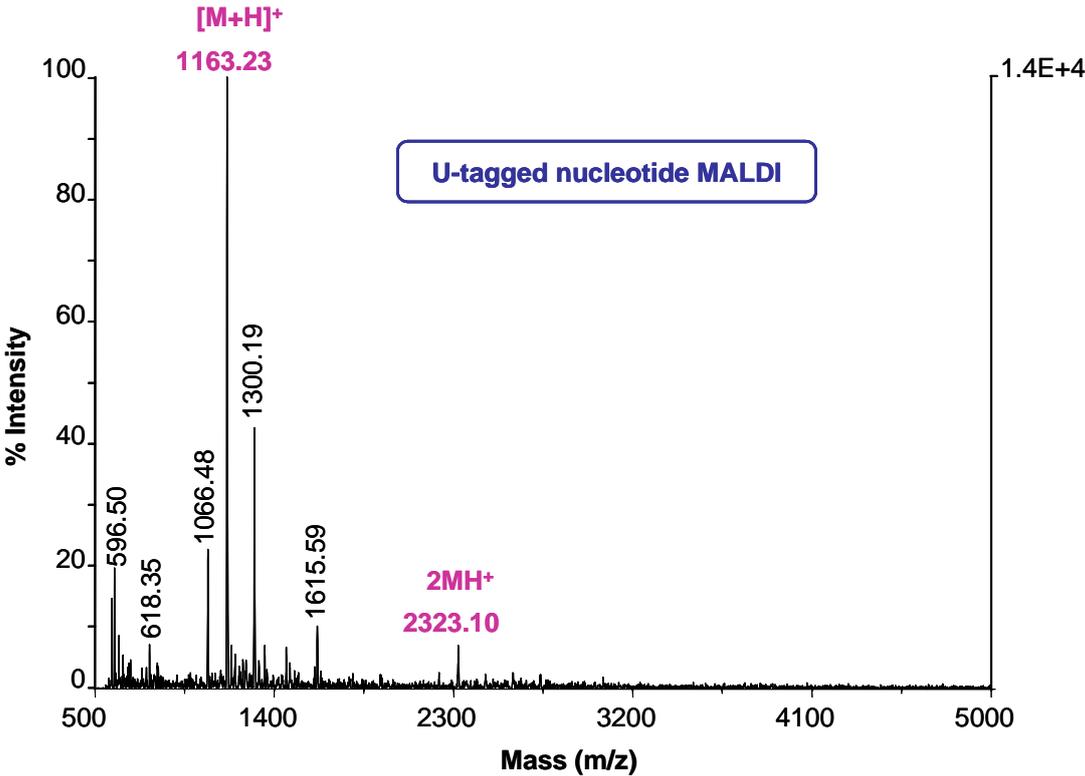


FIGURE 8

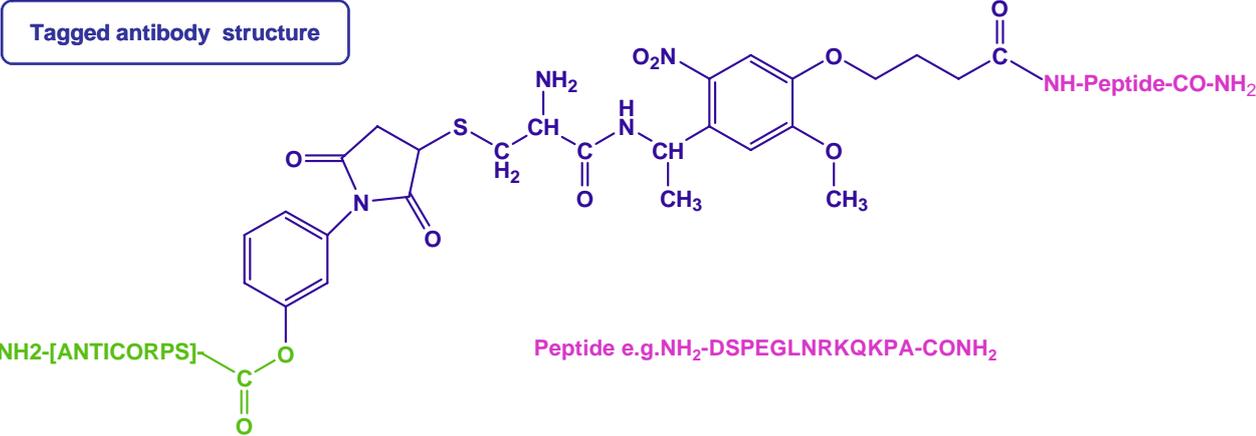


FIGURE 9

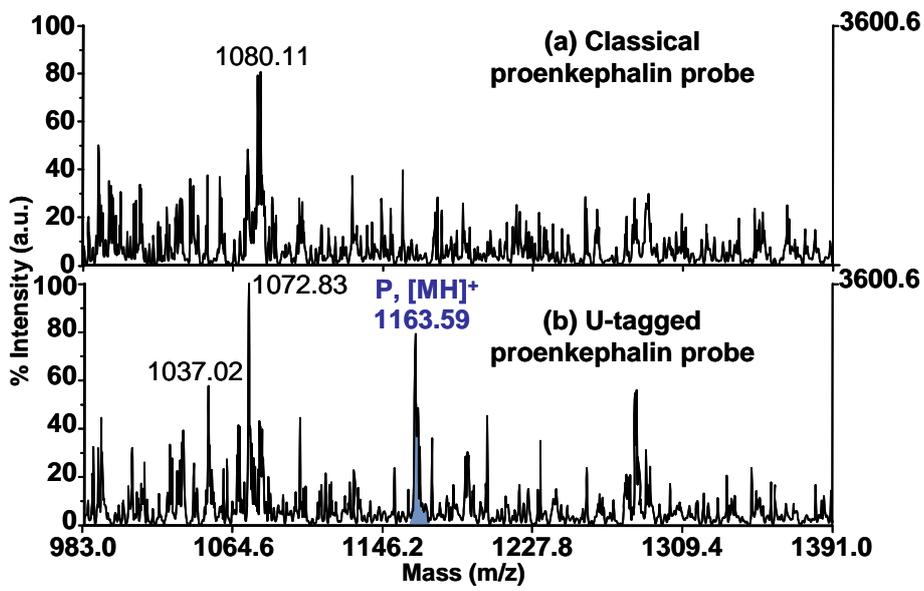


FIGURE 10

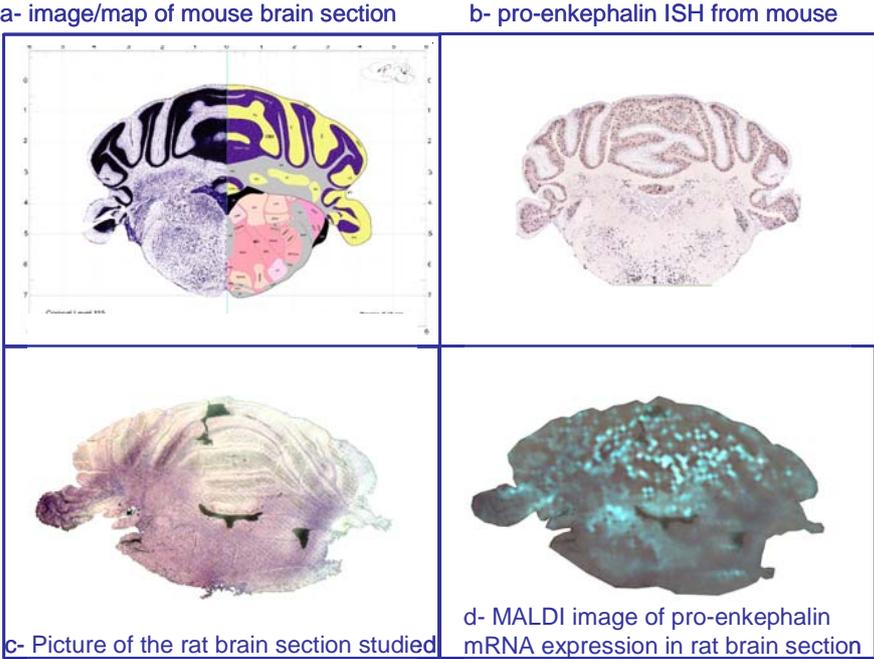


Figure 11

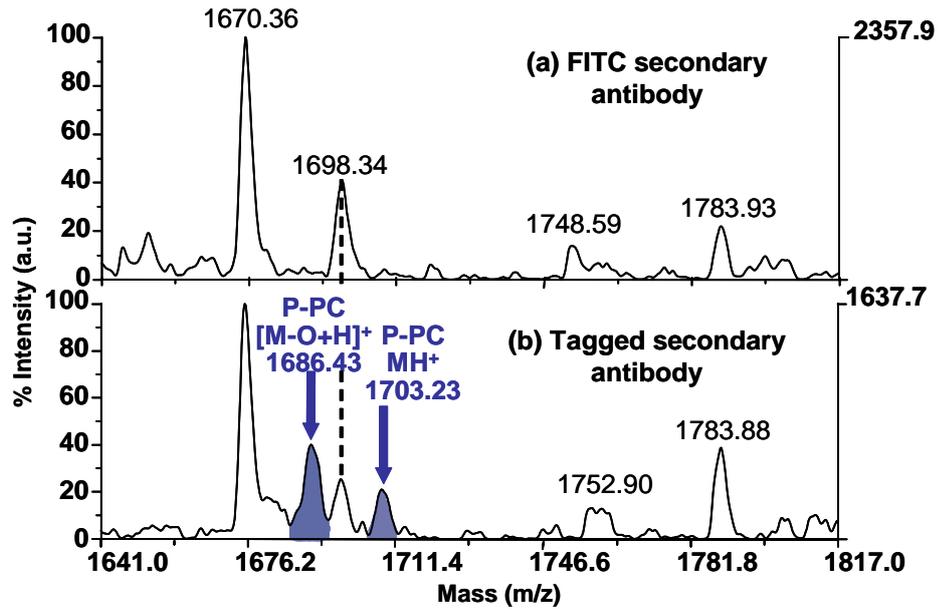


Figure 12

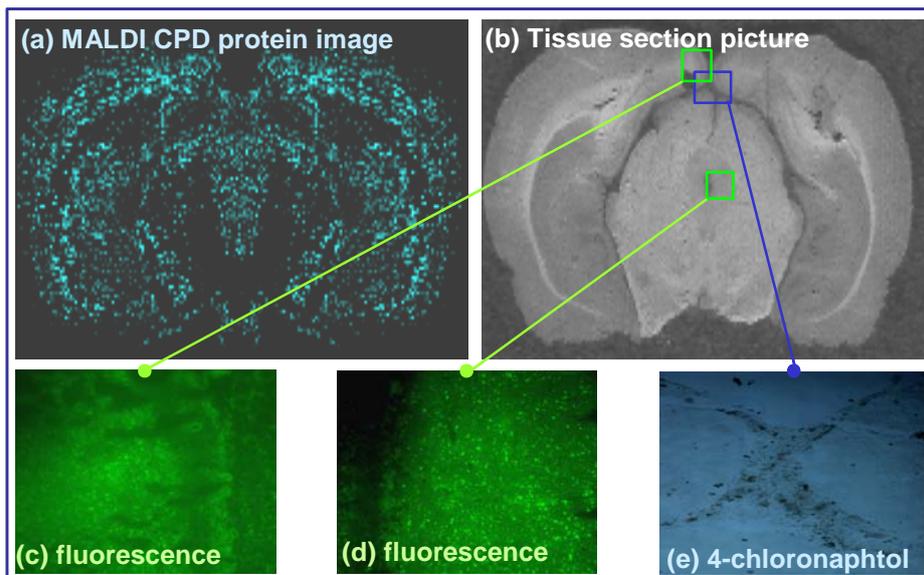


FIGURE 13

