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Application Note

# Multiplexed Targeted Imaging of Intact Proteins in Tissue by MALDI-IHC, on the SELECT™ SERIES MRT

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## Abstract

Here we demonstrate full compatibility of MALDI-IHC analysis with a SELECT SERIES MRT MALDI mass spectrometer and highlight potential advantages over a standard OA-MALDI-Tof. The high mass accuracy (>500 ppb) allows for unambiguous identification of released tags, easily identified from endogenous signals. The high mass resolution of the MRT (>200,000 FWHM) significantly reduces occurrence of tag signals overlapping with endogenous signals of similar mass. This technique provides higher confidence in protein localization information compared to traditional immunofluorescence, where tissue auto-fluorescence (background fluorescence of molecules in the tissue) and signal overlap can cause high interfering signals, resulting in non-specific detection. MALDI-IHC provides the added advantage of enabling the analysis of significantly higher numbers of markers (Hiplexed) within a single image.

## Benefits

- Visualization of targeted protein directly on tissue without requiring digestion
- High sensitivity localization information for intact endogenous proteins
- The high resolution and mass accuracy of the SELECT SERIES MRT reduces back ground signals and

increases confidence in probe tag identification

## Introduction

Protein analysis with mass spectrometry imaging (MSI) techniques can be very difficult. This is caused by poor extraction into the MALDI matrix due to their size and low solvent solubility. Proteins are also typically of high molecular weight, this leads to low ionization efficiency, and a molecule outside of the instrument transmission mass range (as ions produced by MALDI are predominately singly charged). This would therefore, traditionally, have required on-tissue protein digestion prior to MSI analysis.

Unfortunately, on-tissue digestion can reduce localization information, impacting feature clarity in the final image. Digestion typically requires prolonged exposure of the tissue to a wet environment; this has the potential to allow the movement of molecules within the tissue section. Specificity information can also be lost with digestion, as many proteins contain similar peptide sequences, without LC separation $MS^E$ fragmentation and sequencing identification is unfeasible. This leaves only a targeted MS/MS approach for analysis which requires high signal intensity and a highly targeted methodology, resulting in, typically, the observation of only the most abundant proteins. As such, traditional fluorescence immunohistochemistry techniques are still heavily relied upon for protein imaging. These, however, are typically limited to a maximum of five tags per tissue section and suffer greatly from auto-fluorescence (natural back-ground fluorescence from endogenous molecules present in the tissue).

A promising mass spectrometry imaging technique has recently become available known as multiplexed MALDI-immunohistochemistry (IHC), whereby photo-cleavable peptide tags - attached to antibodies - are bound to tissue and released using UV prior to MALDI imaging analysis. These tags are small enough to easily be ionized by MALDI and transition through the mass spectrometer, and their analysis provides localization information of the original protein.

MALDI-IHC is shown to yield results similar to traditional fluorescence immunohistochemistry, but with the advantage of allowing superior multiplexing on a single sample image. The technique can be highly multiplexed, being limited only by the number of available masses, unlike the fluorescence spectrum which avoiding spectral overlap in excitation and emission, typically can visualize only two to five markers. At present there are studies demonstrating the ability to analyze 17+ individual proteins within a single analysis of MALDI Hiplex-IHC with a

reported theoretical capability of 100+ plex.[1,2] Other benefits of these tags are that the protocol is rapid and localization information of the original proteins is retained better than on-tissue digestion techniques. Unlike traditional fluorescence immunohistochemistry background noise can also be very low with MSI, as it does not suffer from tissue autofluorescence causing non-specific detection. The tags have been designed with a higher molecular weight to avoid potential endogenous small molecule signal interference from the tissue and MALDI matrix peak interference.

Here we demonstrate the compatibility and advantages of this technique on a SELECT SERIES™ MRT mass spectrometer, an orthogonal time of flight mass spectrometer comprising of a series of ion guides, a quadrupole, and a collision cell, coupled to a multi reflecting time of flight mass detector. The instrument delivers a high mass resolution of >200,000 FWHM, by utilizing multiple reflections of the ion beam between two gridless electrostatic mirrors in the mass analyzer. Extending the ions' flight path to 49 meters, spatially refocusing the ion packets through a series of periodic lenses after each reflection maintains sensitivity. In addition to high mass resolution, this mass spectrometer provides routine part-per-billion mass accuracy, independent of acquisition speed. The MALDI source coupled to the SELECT SERIS MRT mass spectrometer employs a variable beam focusing from 130-10 µm on target beam width. A two-part attenuation system allows for dynamic range adjustment of the on-target laser energy, giving the option of scalable image resolution to suit the desired application.

The combination of MALDI IHC and the use of the SELECT SERIS MRT mass spectrometer allows for high confidence tag identification and a reduced background/endogenous peak interference, combined with high image resolution.

## Experimental

### Immunohistochemistry

Sections of human tonsil were purchased pre-tagged directly from Ambergen (Ambergen) and human kidney (ccRCC ISUP grade 3) formalin fixed paraffin embedded (FFPE) tissues were prepared in-house in accordance with the AmberGen MALDI HiPLEX-IHC Miralys™ Imaging laboratory Workflow user guide (Control number v173(J)) protocol, this is based on the protocols of G. Yagnik et al (2021) and BSR. Claes et al (2023), comprising of: deparaffinization, rehydration, antigen retrieval, tissue blocking, and then tagging.[1,3]

The pre stained tonsil tissue sections were stained with the probes for CD3ε, CD6, vimentin (VIM), collagen-1A1,

cytokeratin (PanCK), and Ki67. The kidney tissue sections were stained with the probes for VIM, smooth muscle actin (Actin-aSM), PanCK, and Ki67 (Table 1).

Prior to matrix coating the PC-MTs present in the tissue were photocleaved by UV illumination for ten minutes using an AmberGen light box.

| Protein target | Tissue | Reporter mass $(M+H)^+$ (Da) |
|---|---|---|
| CD3ε | Tonsil | 1,161.65 |
| CD68 | Tonsil | 1,216.75 |
| VIM | Tonsil | 1,230.84 |
| Collagen-1A1 | Tonsil | 1,234.87 |
| PanCK | Tonsil | 1,288.72 |
| Ki67 | Tonsil | 1,320.76 |
| VIM | Kidney | 1,230.84 |
| Actin-aSM | Kidney | 1,251.68 |
| PanCK | Kidney | 1,288.72 |
| Ki67 | Kidney | 1,320.76 |

*Table 1. A list of the tags used within this study indicating which tissue type was labelled with each tag and the corresponding reporter mass.*

## MALDI Sample Preparation

Matrix coating was performed using the method described in the AmberGen MALDI HiPLEX-IHC Miralys Imaging laboratory Workflow user guide (Control number v173(J)): A matrix solution of Ultra-pure (>99.9%) α-cyannohydroxycinnmic acid (Merck, Poole) was prepared in 70:30 Acetonitrile:0.1% Triflouroacetic acid in water at a concentration of 10 mg/mL. The matrix solution was vortexed until visually dissolved, then sonicated for 10 minutes. The HTX™ M5 matrix sprayer applied an even matrix coating to the slides, with the settings outlined in Table 2.

| Nozzle temp (°C) | Flow rate (ml/min) | Linear velocity (mm/min) | Number of passes | Row spacing (mm) |
|---|---|---|---|---|
| 60 | 0.1 | 1350 | 8 | 3 |
| **Nozzle height (mm)** | **Pattern** | **Gas pressure (PSI)** | **Drying time (s)** | **Tray temp** |
| 40 | CC | 10 | 10 | ambient |

*Table 2. Details of the HTX M5 sprayer conditions for matrix application.*

Once matrix application was complete the samples were recrystallized, in a recrystallization chamber consisting of a sealed petri dish containing 1 mL of isopropyl alcohol absorbed onto filter paper, per sample. The samples were suspended from the lid of the petri dish by means of magnets and incubated at 55 ºC for two minutes. Once recrystallized the samples were allowed to air dry prior to MALDI analysis.

## Mass Spectrometry

The samples were analyzed using a SELECT SERIES MRT MALDI. Data were acquired in positive ionization mode, with a mass range of 50–2400 Da. As the mass of the tags used are between 1000–1350 Da, a fixed quad setting was used, this was set to 1000 Da. The laser repetition rate was 2 khz and data acquired at 10 s/s. The 50 µm images were acquired with a laser focus setting of 4.0 mm, and the 20 µm images with 5.8 mm.

## Data Analysis

Data were processed and visualized using the Waters™ High Definition Imaging Software (HDI™).

# Results and Discussion

Initial experiments were performed using the pre-prepared Ambergen validation sample: a six plex human tonsil section tagged to visualise Ki67, Pan cyto keratin (PanCK), Collagen-1A1, Vimentin (VIM), CD68, and CD3ε proteins. The observed results from an acquisition with 50 µm pixels (Figure 1) matched the Ambergen supplied validation sample documentation and those from previously published literature.[1]

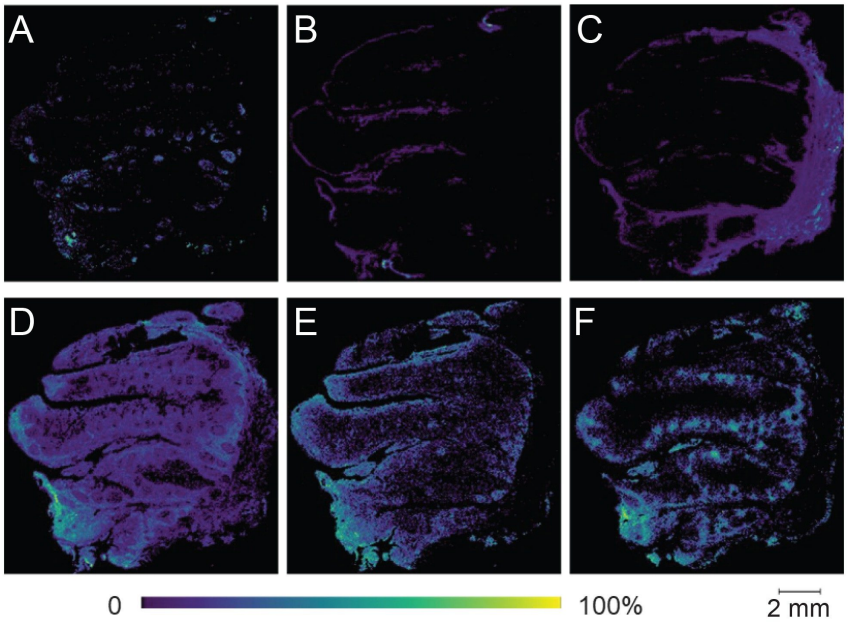



*Figure 1. Results of MALDI analysis of pre-stained human tonsil FFPE section at 50 µm pixel size. A) m/z 1320.7 Ki67, B) m/z 1288.7 PanCk, C) m/z 1234.8 Collagen-1A1, D) m/z 1230.8 VIM, E) m/z 1216.7 CD68, F) m/z 1161.65 CD3ε.*

After running the tonsil validation sample, a human kidney clear cell renal carcinoma (ccRCC) IUSP (internation society for urological pathology) grade 3 was analysed as a 4-plex experiment. The 4 four probe tags were Actin alpha smooth muscle (Actin-aSM), Vimentin (Vim), Pan cytokeratin (PanCK) and Ki67.

A histochemistry microscope image of a consecutive section can be compared to the MALDI-IHC generated images for the four tagged proteins detailed above (Figure 2). On the microscope image of the tissue (F) the carcinoma is marked with a black border and healthy epidermis marked with a blue border. An overlay of all four marker peptides can be seen, showing separate localisation of each of the four proteins investigated (E).

Actin alpha smooth muscle antibodies (Actin-aSM) bind smooth muscle proteins typically localized to vascular smooth muscle cells, but the protein is also seen to be expressed by myofibroblast cells, contributing towards wound healing and matrix remodelling.[4] The tag associated with this protein is visualised (A) and highlights the smooth muscle of the blood vessels as the strongest signals (yellow regions), there also appears to be an

increase in the Actin-aSM on the tumor border this could potentially indicate a healing attempt surrounding the carcinoma.

Vimentin has many roles within the body regulating cell mechanics, it has been shown to have roles in signalling pathways, is expressed by mesenchymal cells as an intermediate filament protein, and play a key role in regulating inflammatory responses.[5] Through this inflammatory response vimentin has been shown to act as an immunosuppressant, localised expression by epithelial cells has been shown as a potential marker for high grade carcinomas.[6] The tag associated with the vimentin protein highlights particularly around the border region of the tumor (B), this is suggestive of a higher grade tumor utilizing vimentin to regulate the patients inflammatory response to the carcinoma.

Pan cytokeratin is an antibody typically used in immunohistochemistry to bind type I and II subfamilies of cytokeratin,cytokeratin; cytokeratin is found in all healthy epithelial cells however they have also been shown as a marker to distinguish human epithelial carcinomas from non-epithelial originating carcinomas.[7] The tag associated with the PanCK antibody localizes exclusively within the healthy epithelium and cannot be seen within the tumor tissue, this indicates that the carcinoma is non-epithelial (C).

Lastly, Ki67 is a marker for cell proliferation believed to have an important function supporting cell division nuclei assembly and regulates gene expression during mitosis, therefore is present in all active phases of the cell cycle. [8,9] Being linked to cell division it is absent in the resting cell phase. The Ki67 tag therefore should show the location of nuclei in cells undergoing proliferation, it can be seen as highly increased within the tumor suggesting rapid growth (D).

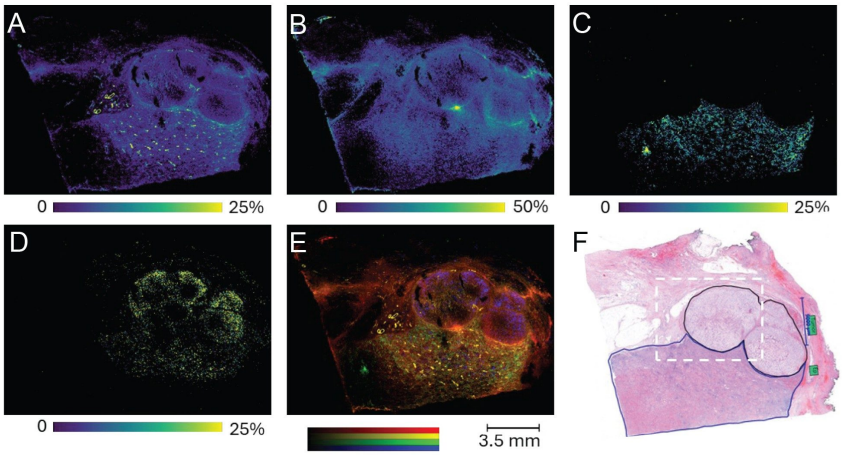
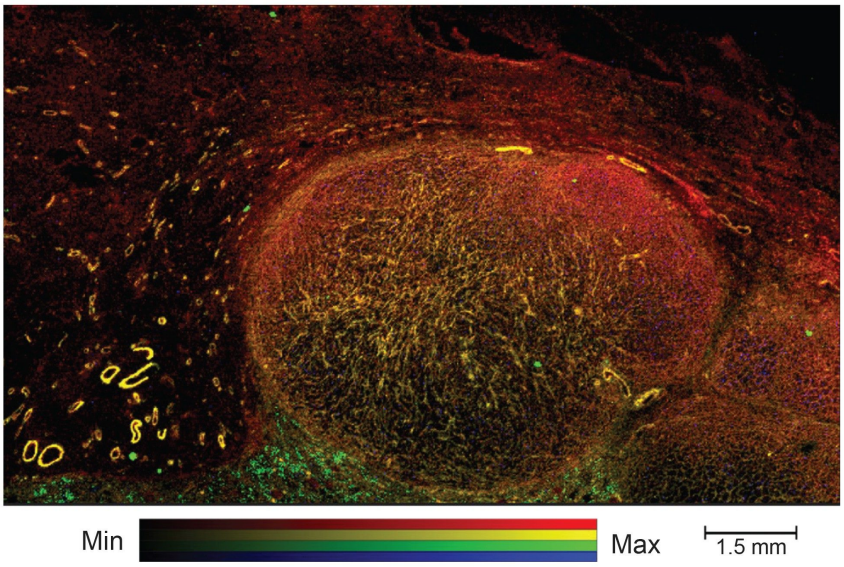



**Image intensity ranges have been adjusted to improve visualisation only, no lower cutoff was applied.*

*Figure 2. Results of MALDI analysis of stained human ccRCC ISUP grade 3) FFPE section with a 50 µm pixel size. A) m/z 1251.7 Actin-aSM, B) m/z 1230.8 VIM, C) 1288.72 PanCK, D) m/z 1320.7 Ki67, E) H&E stained consecutive section blue border region—epidermis, black bordered region—Tumor. F) 4 Color overlay of A-D, yellow A, red B, green C, blue B. White outlined region acquired at 20 µm figure 2 Image intensity ranges have been adjusted to improve visualisation only, no lower cutoff is applied.*

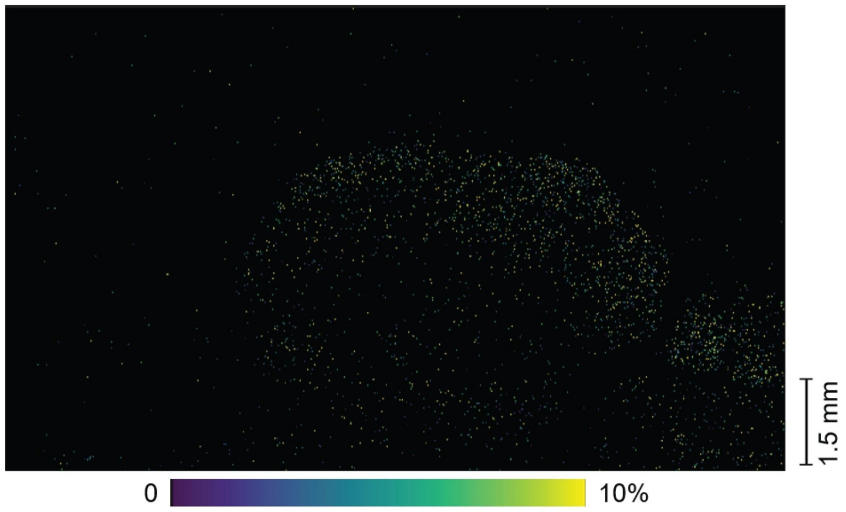
A subsection of the kidney section was selected and analysed at a higher special resolution of 20 µm pixel size this image demonstrated a good signal strength and high image quality (Figure 3).




**Maximum intensity has been scaled for visualization no minimum cut off has been applied.*

*Figure 3. 20 µm pixel size image 4 color overlay of the left side tumor lobule, maximum intensity has been scaled for visualization no minimum cut off has been applied, yellow; Actin-aSM, max intensity 25%, red; Vim, max intensity 50%, green; PanCK, max intensity 25%, blue; Ki67, max intensity 10%.*

Reducing the pixel size enabled finer image resolution of features of interest than with the 50 µm pixel size images. Notably refining the Ki67 probe tag image, corresponding to a protein indicative of nuclear proliferation. The minimal background offered by the MRT has allowed localisation of the Ki67 signal to individual pixels, potentially indicating the possibility of cellular resolution (Figure 4).

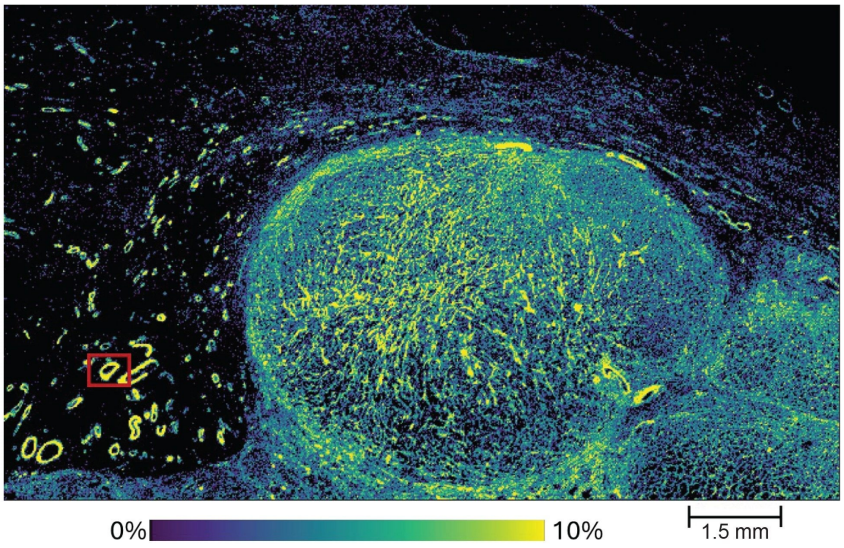


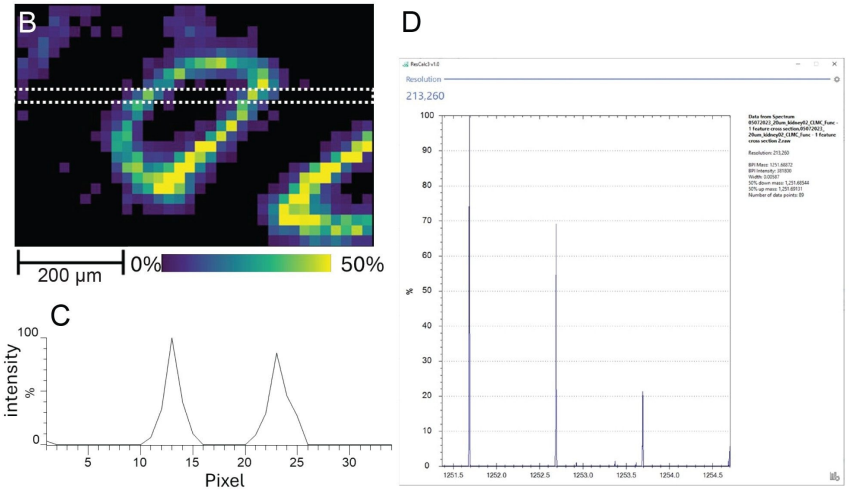

** Maximum intensity has been scaled for visualization no minimum cut off has been applied.*

*Figure 4. Ki67 located to individual 20 µm pixels, increased localization is seen within the tumor tissue suggesting rapid cellular proliferation.*

One of the advantages of the SELECT SERIES MRT is that it produces data allowing for very low/ absent background signal, as the mass accuracy and mass resolution delivered by the mass spectrometer are high. Potential interfering signals with similar masses to the *m/z* of interest are easily distinguished due to the high mass resolution of the mass spectrometer. Thus, reducing the possibility of signal overlap with endogenous signals or exogenous interference from the sample preparation steps. This combined with the high mass accuracy (<500 ppb RMS) adds confidence that the signal observed, and image generated relates only to the protein of interest.

In Figure 5 we demonstrate this using the Actin-aSM probe tag *m/z*, the red box indicates the region of tissue selected for this extraction (A), which is a cross-section of a blood vessel. This area has been magnified (B) and dotted lines drawn to indicate the pixel numbers shown in the extracted ion chromatogram (C), this clearly demonstrates that there is an absence of signal within the center of the blood vessel and in the surrounding tissue, that only the actin-aSM signal is observed within the blood vessel walls. The mass resolution (D) for the extracted actin-aSM peak has been calculated to be >200,000 (213,260 FWHM).




** Maximum intensity has been scaled for visualization no minimum cut off has been applied.*

*Figure 5. Shows the image generated from visualization of the actin-aSM tag (A), the red box indicates a blood vessel viewed cross-sectionally. This area has been magnified and a band indicated with dotted lines a single pixel in width and 35 pixels in length (B). An extracted ion chromatogram (XIC) can be seen showing the signal intensity over these 35 pixels (C), and the mass resolution for the tag has been calculated at >200,000 FWHM (D).*

## Conclusion

A section of a human kidney clear cell renal carcinoma (ccRCC) IUSP (internation society for urological pathology) grade 3 was analyzed to visualize the localization of four intact proteins. This was achieved using MALDI IHC analysis on a SELECT SERIES MRT MALDI mass spectrometer. Images were acquired at both a 50 µm and 20 µm pixel size, demonstrating excellent image quality and signal strength of the probe tags with both image resolutions. The data generated shows low to non-existent background noise and a mass resolution >200,00 FWHM, giving confidence in signal identification and clear features. MALDI-IHC paves the way for multiOMIC spatially resolved experiments on the same mass spectrometer. The analysis shows the ability to clearly differentiate and visualize proteins to benign and cancerous portions of tissue sections. As well as this it can provide valuable localized information about individual molecules giving insight as to their function at specific sites within the tissue itself.

## References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