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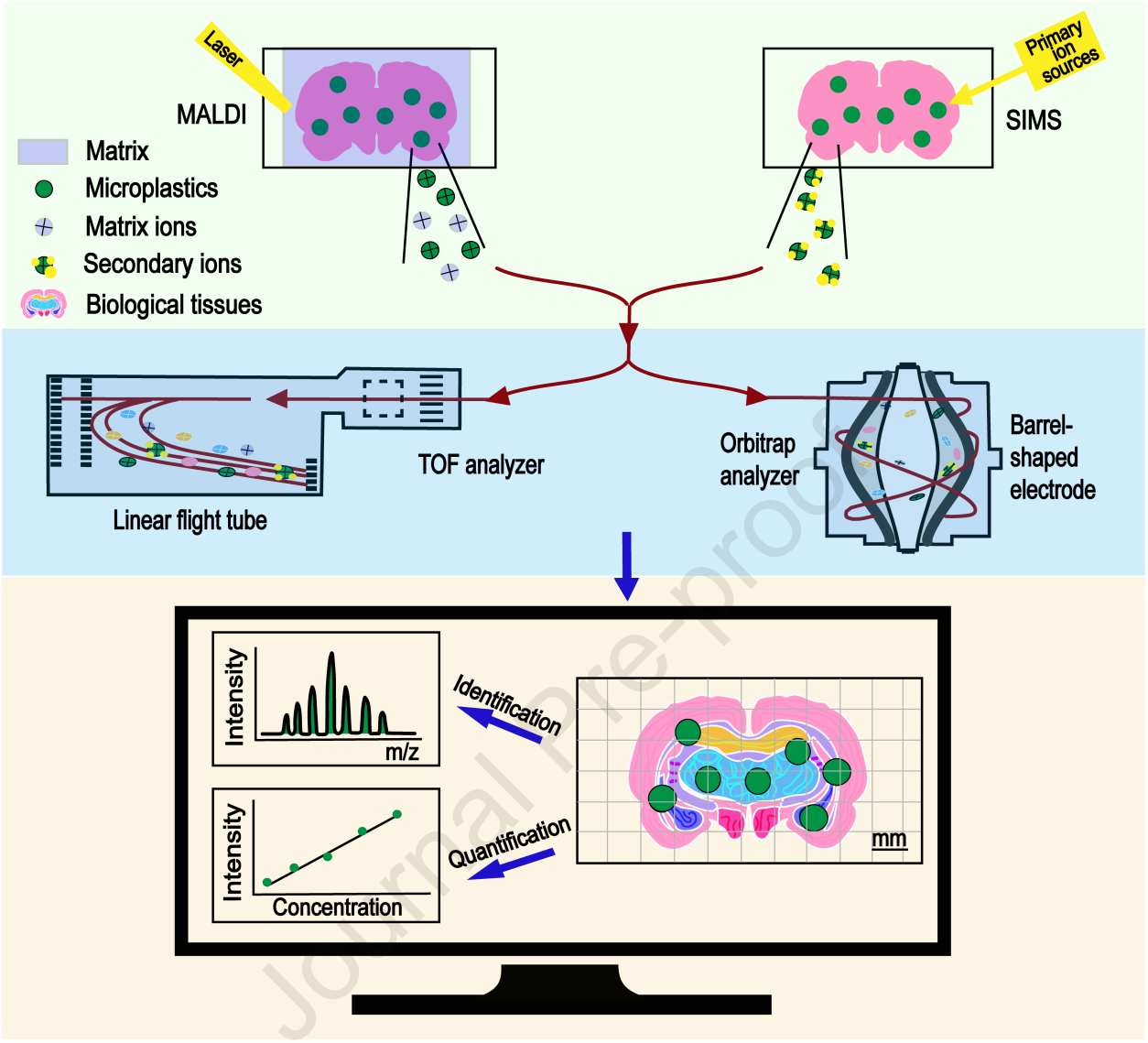

In situ imaging of microplastics in living organisms based on mass spectrometry technology

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Abstract:

Plastic pollution is widely present in terrestrial and aquatic ecosystems, and microplastics (MPs) can be detected in organisms. *In situ* detection methods for MPs in organisms have attracted widespread attention. Traditional imaging characterization methods of MPs, including stereo microscopes and fluorescence microscopy, are typically used to image artificially added microsphere standards under laboratory conditions. However, they cannot specifically identify MPs in biological samples. Thus, there is a need for a detection technique that can provide spatial distribution information of MPs in biological samples, as well as measure their quality and quantity. In this perspective, to obtain high-resolution images with chemical composition analysis, we compared ion sources for ionizing plastic macromolecules and mass analyzers for analyzing macromolecules. Matrix-assisted laser desorption/ionization (MALDI) is suitable for imaging characterization, while time-of-flight (TOF) and Orbitrap mass spectrometry are suitable for polymer mass spectrometry analysis. Furthermore, we propose a technique that combines MALDI with TOF or Orbitrap, which holds promise for the *in situ* imaging of MPs in biological samples.

Keywords: Microplastics; Organisms; *in situ* imaging; Mass spectrometry imaging

1. Introduction

Microplastics (MPs) are widely present in terrestrial and aquatic environments [1] in the form of fragments, fibers, and films with a diameter less than 5 mm [2, 3]. Studies have shown that MPs also exist in organisms, such as the guts and livers of fish [4, 5], the roots and stems of plants [6-8], and human blood [9, 10]. Besides, submicroplastics (100 nm to 1 μm) and nanoplastics (< 100 nm) have been shown to penetrate the blood-brain barrier of fishes [11, 12], the placental barrier of humans [13, 14], the skin barrier of humans and mice [15, 16], and undergo internalization by bovine oviductal epithelial cells and human colon fibroblasts [17]. Therefore, The MPs in organisms need to be characterized and detected.

The detection methods for MPs are divided into qualitative methods and quantitative methods [18]. Qualitative detection aims to confirm the existence of MPs and characterize the type, morphology, and size of MPs. The coupling of microscopes with vibrational spectroscopy techniques to identify MPs is the most common approach [19]. Quantitative detection of MPs in terms of quantity can also be performed using microscopic imaging. Furthermore, the distribution characteristics of fluorescently stained or radiolabeled MPs in organisms can be characterized by the intensity of fluorescence or radioactivity. Mass spectrometry (MS) detection can provide mass-related information about MPs, but it cannot simultaneously obtain their morphology, size, and aging degree. This perspective aims to seek an *in situ* mass spectrometry imaging (MSI) method for MPs in organisms without labeling, and to quantitatively analyze plastics in imaging. This will deepen our understanding of the metabolic and transport mechanisms of MPs in biological tissues, and help us to evaluate the potential risks of MPs to organisms and even ecosystems.

2. Imaging characterization of MPs

Currently, microscopy imaging techniques are conventional detection methods for characterizing MPs within organisms (Table 1). Imaging techniques can be used to describe the physical properties (shape and size) of MPs and to quantify the observed MPs. In previous studies, laser confocal microscopy (CLSM), scanning electron microscopy (SEM), and atomic force microscopy (AFM) were used to observe

fluorescently labeled polystyrene (PS) MPs with a diameter of 10 μm in the digestive tracts of rotifers [20]. SEM was used to characterize the digestion of polylactic acid (PLA) with a diameter of 25 μm by gastric lipases in mice, and fluorescence microscopy (FM) was used to characterize the migration process of fluorescent PLA plastic polymers in mice [21]. Although these techniques are typically used to image artificially added microsphere standards under laboratory conditions, they cannot specifically identify environmental MPs in biological samples.

Table 1 Applications and limitations of conventional imaging techniques for MPs

Imaging techniques	Applications	Limitations	Ref.
Stereo microscope	• Characterizing the size and morphology of MPs. • Counting the quantity of MPs. • Magnification to a certain degree to observe the details of MPs.	• Low magnification. • Unable to provide qualitative analysis, prone to false positives. • Not feasible for automation, time-consuming and labor-intensive. • Unable to perform *in situ* imaging of MPs.	[13, 20]
FM	• Characterizing the size and morphology of MPs. • Characterizing the migration and fragmentation behavior of MPs in environmental and biological samples.	• Fluorescent labeling of plastics is required, which may lead to false positive results due to dye leaching. • Fluorescence quenching can result in missed detection of MPs. • Chemical additives in synthetic plastics may exhibit fluorescence, interfering with the identification of MPs. • Environmental background may obscure the fluorescent signal of plastics.	[6, 7, 20, 21, 43]
SEM/TEM	• Characterizing high-resolution surface morphology of MPs. • Combining spectroscopic analysis techniques for chemical composition analysis of MPs. • Characterizing the distribution of MPs in environmental and biological samples.	• Coupled with energy-dispersive X-ray spectroscopy, the morphology and elemental composition of MPs can be determined. However, the specificity in identifying plastics is limited due to their common composition of C, H, and O. • Plastics cannot be quantitatively detected.	[6, 8, 20, 21]
AFM	• Surface imaging of nanoplastics. • Testing the mechanical properties of MPs. • Application of MP detection in environmental and	• The specificity in identifying plastics is limited. • Sample preparation requires a high level of precision, with the need for smooth and clean surfaces. Flexible or irregular plastic samples	[20]

	biological samples.	are difficult to meet the requirements. • Susceptible to environmental influences, such as temperature and humidity. • Plastics cannot be quantitatively detected.	
Infrared spectroscopy imaging	• Possessing micrometer-level spatial resolution. • No damage to the samples. • Identifying the chemical composition of MPs. • Rapid analysis with short detection time. • No need for additives or fluorescent labeling of MPs.	• High sample preparation requirements, unable to perform in situ imaging of plastics. • Plastics cannot be quantitatively detected.	[44]
Near-infrared hyperspectral imaging	• Possessing nanometer-level spatial resolution. • In situ imaging of MPs in environmental and biological samples without causing damage to the sample. • No need for additives or fluorescent labeling of MPs.	• Weak spectral specificity, prone to false positive results. • Limited ability to differentiate between plastics of different compositions. • Plastics cannot be quantitatively detected.	[20, 43]
Raman spectroscopy imaging	• Possessing nanometer-level spatial resolution. • Imaging of MPs without causing damage to the sample. • No need for additives or fluorescent labeling of MPs.	• Fluorescent substances in the sample (environmental matrix or plastic additives) can interfere with Raman signals. • Plastics cannot be quantitatively detected.	[19, 20]

Compared to the above imaging techniques, vibrational spectroscopy imaging techniques, such as infrared spectroscopy imaging, near-infrared hyperspectral imaging, and Raman spectroscopy imaging, rely on the characteristic absorption peaks of plastics in their spectra for relatively precise identification of MPs. However, they are prone to false positive results due to matrix interference. These techniques require cumbersome matrix purification, and the MPs should be extracted before detection [20, 22]. This makes *in situ* imaging difficult to achieve.

Additionally, the imaging techniques have limitations in quantitative detection. It is challenging to identify particles smaller than the resolution of the instruments or particles firmly bound to biological matrices using imaging techniques. Although FM [22] and ^{14}C isotope tracing techniques [23] can quantify the fluorescently or radioactively labeled plastic particles, their application in natural biological samples is difficult due

to the potential harm of labeled fluorescence and radioactivity to organisms, as well as their degradation and shedding under environmental conditions.

3. MS detection techniques for MPs

MS detection is widely used in the detection of trace organic pollutants in the environment. MS has the advantages of high sensitivity, high selectivity, high resolution, and fast detection speed, and can be used for both qualitative and quantitative analysis. Some MS techniques have been successfully applied in the detection of MPs or have potential applications for MP detection. Currently, plastic macromolecules need to be cracked or depolymerized into small molecules under high temperature, strong acid, or strong alkali conditions before MS detection [24-28]. This process cannot meet the demand for non-destructive imaging. However, there are some MS ionization methods that can achieve the direct ionization of plastic macromolecules.

3.1 Ionization of MP molecules

The ionization of target analyte molecules is a prerequisite for MS detection. Gentle ionization methods, such as ambient ionization (e.g., Desorption Electrospray Ionization (DESI)), may struggle to ionize the plastic polymers with a high molecular weight of 10^4 Da. Therefore, vacuum ionization methods with higher ionization efficiency, such as matrix-assisted laser desorption/ionization (MALDI) MS and secondary ion mass spectrometry (SIMS), hold greater potential for the detection of plastic polymers [29-31].

SIMS is a hard-ionization technique that can bombard macromolecules into complex fragments, making it suitable for detecting plastic polymers [32]. SIMS bombards the sample surface with high-energy particles (nitrogen or argon), causing the atoms and molecules on the sample surface to become ionized. After bombardment, secondary ions are produced from atoms and molecules, which then enter the mass analyzer for analysis [32]. The spatial resolution of this technique reaches the nanometer level. However, the extensive ion fragmentation and low sensitivity limit the application of SIMS in trace pollutants imaging (Table S1).

MALDI is an ion source that utilizes laser energy to vaporize the matrix with the aid of ionization reagents, resulting in the ionization of target analyte molecules. The

matrix is crucial because it should possess the property of absorbing laser energy and converting it into thermal energy. Ionization reagents serve to enhance the ionization efficiency of the sample and increase the intensity of the mass spectrum signal. After mixing the sample, matrix, and ionization reagents together, a thin film forms on the surface of the sample. Subsequently, the laser irradiates the surface of the sample matrix, causing the matrix molecules to evaporate while absorbing the laser energy. As a result, the sample is released and ionized. The ions of the molecular fragmentation products then enter the mass analyzer for analysis and detection. After absorbing the laser energy, the matrix undergoes dissociation or fragmentation processes, resulting in the generation of charged ions. During this process, interactions occur between matrix ions and analyte molecules, facilitating proton transfer to protonate the analyte. The assistance of matrix and ionization reagents significantly improves the ionization efficiency of the sample, addressing the issue of ionizing non-volatile and high-molecular-weight analytes in MS. In contrast to the ESI techniques such as DESI, which are suitable for ionizing water-soluble compounds with molecular weights below 2000 Da, MALDI ionization is performed under vacuum conditions. This makes MALDI ionization more appropriate for ionizing lipophilic macromolecular polymers with molecular weights of tens of thousands of Da. MALDI-time-of-flight mass spectrometry (MALDI-TOF-MS) has been successfully applied in the detection of MPs. Professor Cai's team quantified PS and polyethylene terephthalate (PET) in sediments and aviation plastic cups using MALDI-TOF-MS [33]. The spatial resolution of MALDI-TOF can reach up to 1-5 μm, which offers great potential advantages in imaging detection (Table S1).

3.2 Mass analysis of MP molecules

The MS detection of polymers also imposes requirements on the mass analyzer. Mass analyzers [24] such as TOF, Orbitrap, and magnetic sector MS can be used for the detection of biomacromolecules, and theoretically, they can also be applied to the detection of MPs.

TOF-MS is renowned for its capability in analyzing macromolecules. Ionized molecules are accelerated by an electric field and fly at a constant velocity in the flight

tube, unaffected by external forces. Due to the variance in ion mass, their flight velocities differ, leading to varied arrival times at the detector. The mass can be determined based on the flight time. Therefore, TOF mass analyzers have m/z-independent trapping conditions [24]. They also have other advantages such as the highest detection speed and high resolution. However, the susceptibility to environmental influences during the detection process, to a certain extent, limits the application of TOF in terms of mass accuracy, resolution, and sensitivity (Table S1).

Orbitrap is a modified ion trap mass analyzer. After entering the orbitrap, ions undergo radial motion under the influence of an electric field and axial oscillation. Due to variance in ion mass, ions exhibit different frequencies in the orbit. The accumulation of charge within the orbit generates a current signal outside the orbitrap, which is detected and converted into a mass spectrum [34, 35]. The orbitrap mass analyzer has high resolving power, high mass accuracy, and short acquisition times. Theoretically, orbitrap has an unlimited mass range [36] and can be used for the analysis of macromolecules. Compared with the orbitrap, Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has higher trapping efficiencies for large ions, increased isotopic fidelity, and more precise resolution (Table S1). However, the high accuracy of FTICR MS comes at the cost of longer signal acquisition times. Mass imaging detection requires frame-by-frame information acquisition for a sample, and the precision of a frame is usually 20-100 microns. Thus, an excessively long acquisition time of tens of hours will be needed for one sample. The relative standard deviation (RSD) of both TOF and Orbitrap in environmental samples can generally be achieved to be less than 3% (Table S1). In general, TOF or Orbitrap is sufficient for analyzing high-molecular-weight compounds.

The magnetic sector MS is also a mass analyzer capable of analyzing macromolecules. The ionized sample, under the influence of a magnetic field, experiences Lorentz force and deflects along a trajectory. Due to variance in ion mass, ions are separated in mass-to-charge ratio orbits. Then, the separated ions are recorded by the detector. Isotope ratio mass spectrometry (IRMS), as a type of magnetic sector MS, has been used to trace the sources of MPs rather than their abundance [24]. However,

IRMS requires complete decomposition of organic compounds at temperatures exceeding 1,000 °C to ensure accurate isotope testing results [37]. Due to the breakdown of plastic macromolecules, this type of MS is unsuitable for the analysis of MPs.

4. Prospect of *in situ* imaging of MPs in biological samples using MSI

Scientists are seeking a detection technology that can provide *in situ* spatial distribution information of samples, as well as the quality and quantity of MPs. MSI combines microscopic imaging and MS, enabling the acquisition of both surface morphology in samples and mass spectra information of various chemical compounds. By matching and overlaying these two types of information, high-resolution images with chemical composition analysis can be obtained.

MSI is an MS technique that evolved from measuring the spatial distribution of endogenous compound molecules in biological tissues [38]. In 1998, the distribution characteristics of phospholipids on cell membranes were studied using TOF-SIMS [39]. Recently, MSI has been used to characterize the distribution of exogenous environmental pollutants within organisms. For example, the distribution of imipramine and chloroquine in the kidneys and brains of mice was visualized using atmospheric pressure-MALDI-TOF [40].

The development of *in situ* imaging and quantitative methods for MPs will help understand the migration and transformation processes of MPs in the environment, as well as the transport mechanisms in biological tissues. MALDI and SIMS coupled with TOF or Orbitrap mass analyzers are proficient in analyzing and identifying macromolecules such as plastic polymers. The reported concentration of PET MPs in mussels from the market in Tianjin is 75.4 ng/g, while 12 MP particles with sizes of 5 to 10 μm were detected in four human placentas [13]. According to Hermabessiere's calculation method [25], the mass concentration of polypropylene (PP) plastic is estimated to be 10^3 ng/g from the quantity concentration. This is significantly higher than the Instrumental Detection Limits (IDLs) for MALDI-TOF-MS detection, which are 5.2 ng [27,32]. By combining *in situ* ionization techniques such as MALDI and SIMS with MS like TOF or Orbitrap (which are proficient in macromolecular analysis and identification), this technology will perform excellently in the analysis and

identification of plastic MPs. By combining the collected MS data with the spatial information of optical images, the spatial images of chemical compositions can be generated, theoretically enabling the characterization of plastic polymers through MSI (Fig. 1). Attempts to perform plastic polymer *in situ* imaging using MALDI-TOF-MS[41] and TOF-SIMS [42] have begun (Fig. 2).

Fig. 1 Schematic diagram of an ideal MSI technique.

Fig. 2 Optical image (a), surface layer-MALDI-MS images (b-d) of a bilayer film prepared with PMMA (green) and PS (purple), intensity profile (e) along the red line in (d) [41]. Optical image (f-j) and surface layer-MALDI-MS images (g-k) of logo patterns scribed on the surface of PMMA film [41]. Simultaneous visualization of three different types of MPs (PMMA, GMA, and PVC) from a paramecium (l-p) [42].

Due to the hard ionization principle of TOF-SIMS, plastic macromolecules can be fragmented into complex fragments upon bombardment. The resulting mass spectral signals may not be sufficiently clear, leading to inaccurate quantification [38]. In contrast, MALDI-MSI, based on soft ionization principles, can ionize plastic macromolecules without disrupting their molecular structure. Thus, MALDI-MSI holds potential for the accurate quantification of MPs [33]. However, several challenges remain in establishing methods for detecting MPs using MALDI-MSI.

1) How to remove interference from embedding agents: The difficulty in preparing biological samples for slicing without embedding agents. Embedding agents used to support the slicing of biological tissues may produce background interference. If the embedding material is necessary, its background noise should be identified by carefully comparing it with the characteristic peaks of the target molecules. Cryosectioning without embedding is the best choice, which requires finding the optimal slicing conditions (including adjusting the method and duration) for freezing the samples. Besides, some semi-synthetic plastics, such as Celluloid, which is made from cellulose, may have molecular structures and MS signals similar to those of plant biological

tissues. This may cause extra difficulties in conducting MS-based detection of such substances.

2) How to improve imaging speed. High resolution of the instrument and oversized sample areas can result in excessively long imaging times. Introducing machine learning and deep learning techniques can aid in developing more efficient and rapid MSI data processing programs.

3) How to improve the accuracy of MSI quantification. Inaccuracies in quantifying MPs may arise due to matrix interference and ion suppression in biological samples. Attempting internal standard correction for quantification or employing multiple calibration methods, such as thermal decomposition coupled with gas chromatography-MS or thermal alkaline/acid-assisted liquid chromatography-MS, can enhance the accuracy of quantification results from MSI.

CRediT authorship contribution statement

Y.L.: writing–original draft, review, editing & funding acquisition; X.Y.S., Y.W.: writing–review & editing; Y.F. Z.: resources, formal analysis; J.J.Z.: formal analysis, validation, writing–review & editing; P.W.: formal analysis; X.F.C.: supervision, resources, validation; B.S.X.: supervision, writing; L.W.: methodology, conceptualization, supervision, writing–review & editing, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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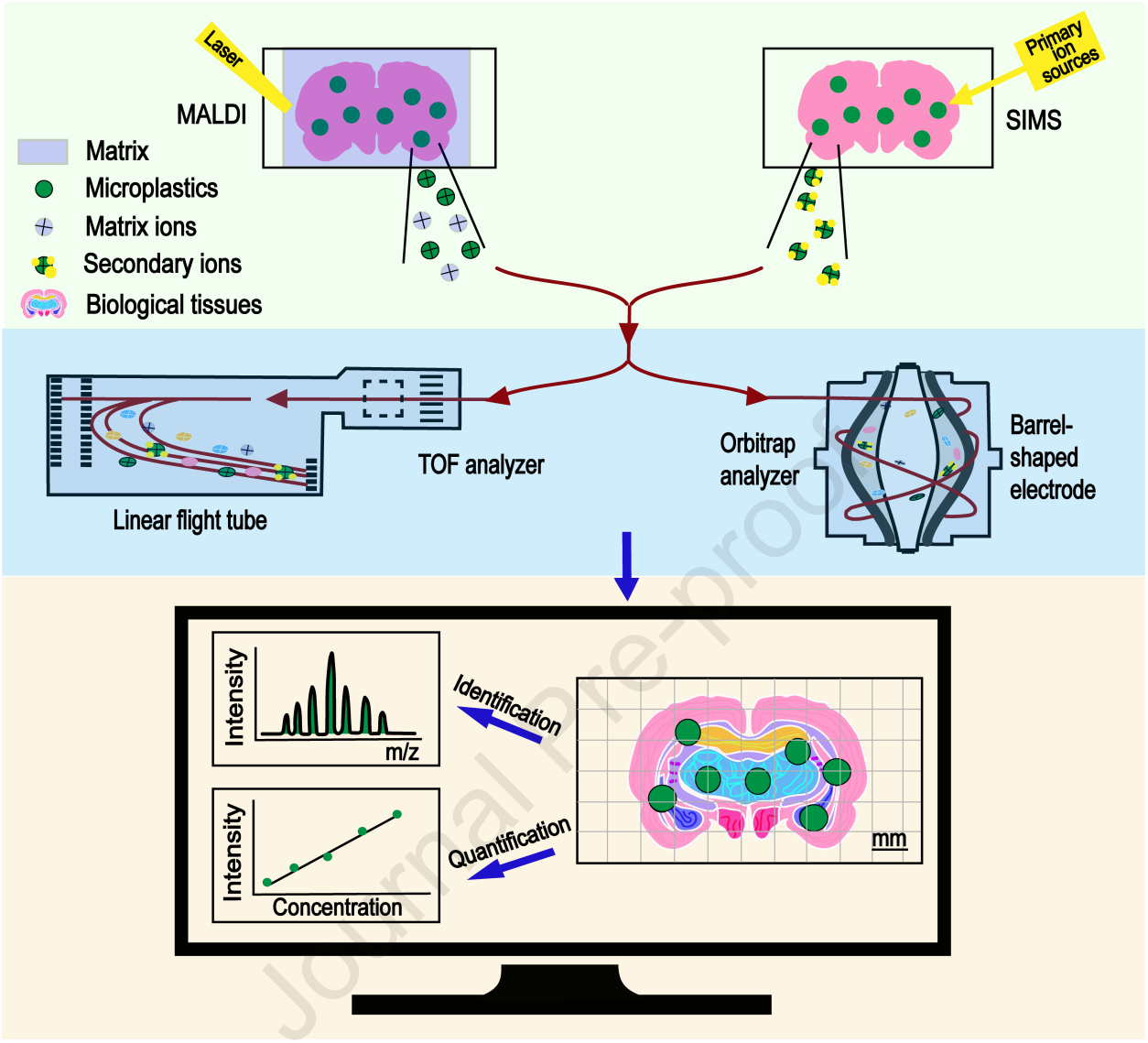

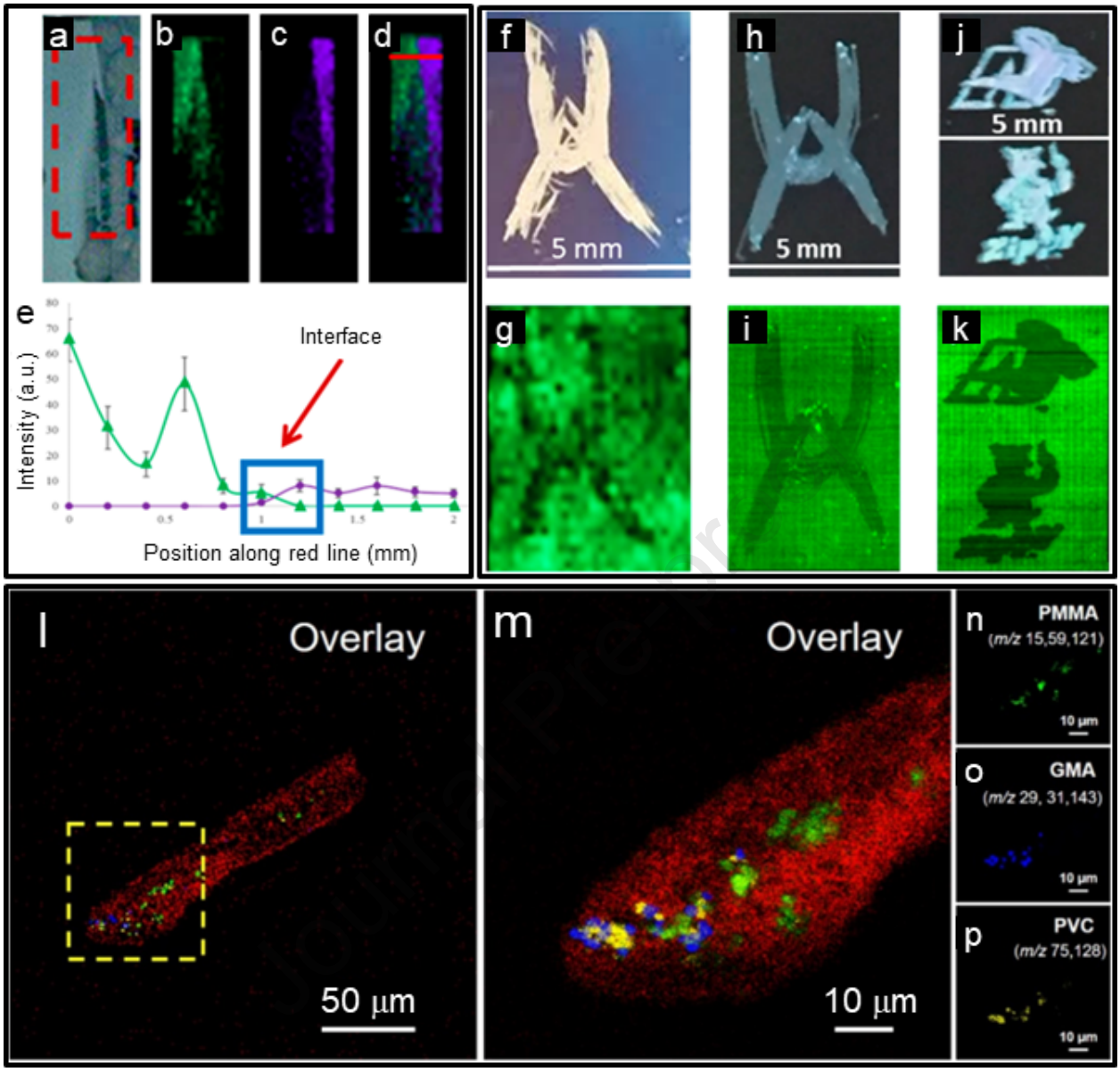
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p
PVC
(m/z 75,128)
10 μm

Highlights

- Mass spectrometry imaging holds promise for in situ imaging of microplastics in biological samples.
- Mass spectrometry imaging could provide in situ spatial distribution information of biological samples
- Mass spectrometry imaging could quantify the quality and quantity of MPs in biological samples.