Direct metabolomics for plant cells by live single-cell mass spectrometry

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Live single-cell mass spectrometry (live MS) provides a mass spectrum that shows thousands of metabolite peaks from a single live plant cell within minutes. By using an optical microscope, a cell is chosen for analysis and a metal-coated nanospray microcapillary tip is used to remove the cell's contents. After adding a microliter of ionization solvent to the opposite end of the tip, the trapped contents are directly fed into the mass spectrometer by applying a high voltage between the tip and the inlet port of the spectrometer to induce nanospray ionization. Proteins are not detected because of insufficient sensitivity. Metabolite peaks are identified by exact mass or tandem mass spectrometry (MS/MS) analysis, and isomers can be separated by combining live MS with ion-mobility separation. By using this approach, spectra can be acquired in 10 min. In combination with metabolic maps and/or molecular databases, the data can be annotated into metabolic pathways; the data analysis takes 30 min to 4 h, depending on the MS/MS data availability from databases. This method enables the analysis of a number of metabolites from a single cell with rapid sampling at sub-attomolar-level sensitivity.

INTRODUCTION

The function of a plant cell is realized by the interplay of thousands of molecules including small organic molecules, proteins and nucleic acids. Thus, identification and tracing of these molecules provide important insights into plant cell function. However, unlike DNA, proteins and metabolites cannot be amplified; therefore, most proteomics and metabolomics experiments involve homogenizing large numbers of cells. This approach compromises the spatial information of individual cells in plant tissue, which results in only spatial averages. Single-cell experiments have shown that these averages ignore crucial details about individual cell behavior[1–26].

MS analyses of single plant cells

Live imaging methods are effective for observing the dynamics of single molecules in living single cells[27–39]; however, they require that the target molecules be known before probing and that the number of probed molecules be limited. For the detection of a larger number of molecules, including unknown molecules, MS is a preferred method, but the mass spectrometer needs sufficient sensitivity to detect micromolar concentrations of metabolites in samples that are in sub-picoliter volumes. Single-cell MALDI-TOF MS is one popular example[40–46], as it can detect a wide range of molecules, from small molecules to macromolecules—i.e., from metabolites to proteins. However, laser irradiation of the whole single cell primarily ionizes the membrane lipids, abundant small molecules and housekeeping proteins, thus masking many other molecules from detection.

We proposed the 'video mass spectroscope' in 1999 (ref. 47), and we have applied an electrospray ionization method that is soft and cyclopedic to detect and identify molecules by MS/MS or MS^n analyses[47,48]. In 2007, we finally established a direct, rapid and comprehensive mass spectrometric detection method that generated hundreds to thousands of molecular peaks from a live single cell or even a single organelle. This was achieved by sucking out the cell content with a metal-coated microcapillary, i.e., nanospray tip, under video-microscopy observations and by directly feeding the content into a mass spectrometer via a nano-electrospray ionization plume[49–57]. We later called this method 'live MS'[52], as it provides direct and rapid *in situ* detection of live cells, including plant cells[49–57] (also see **Supplementary Notes 1–3**). The principle of live MS is shown in **Figure 1**, and its experimental procedures are shown in **Figure 2**. In particular, for plant analysis, many isomers need to be separated. This separation can be achieved by connecting an ion mobility separation device after the ion source.

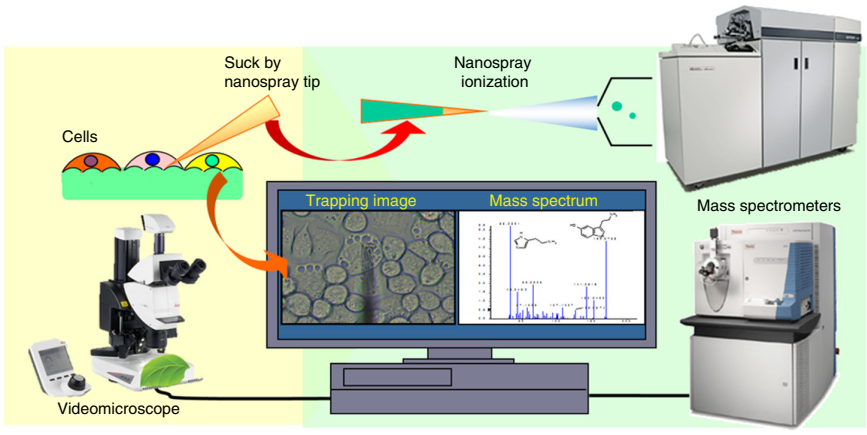


Figure 1 | Schematic principle of live plant single-cell MS. The live target single-cell content in a plant tissue is sucked by the nanospray tip under a stereomicroscope, and the ionization solvent is added from the other end of the tip. A voltage is then applied between the tip and the mass spectrometer to start the nanospray and to feed the sample content directly into the mass spectrometer[49–52].

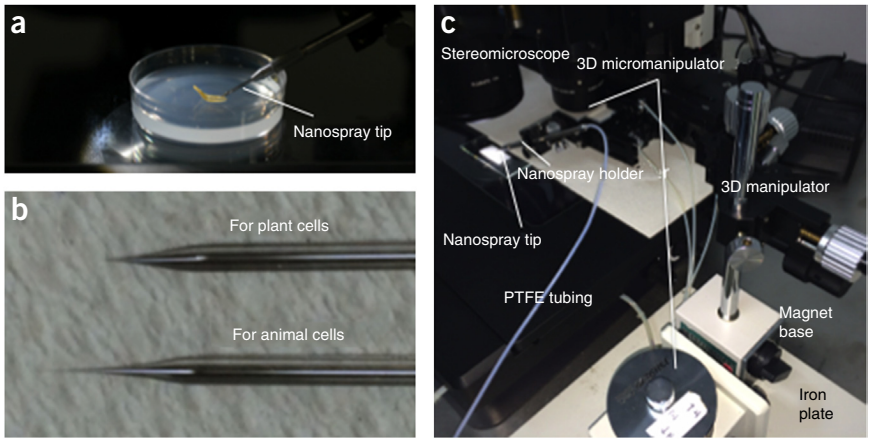


Figure 2 | Experimental setup. (**a**) Sampling of the plant tissue with gel support. (**b**) Shapes of the nanospray tip for plant cells (top) and animal cells (bottom). (**c**) Manipulating system for cell trapping by a nanospray tip.

Figure 3 shows an example of separating catechin isomers ((−)-epigallocatechin gallate (EGCg) and (−)-gallocatechin gallate (GCg)) from a single tea leaf cell by using differential mobility spectrometry (DMS)[55].

We compare the performance of mass spectrometers currently used in single-cell MS analyses for plant cells in **Table 1**. At this stage, live MS is not sufficiently sensitive to detect proteins, except those with dominant small peptides (MW <1,500 Da); the copy number of proteins in a cell seems to be much less than that of small molecules. Furthermore, the nano-electrospray ionization causes a dispersed multicharge of the proteins that spreads into several molecular peaks.

At present, we are using two types of spectrometers: the Orbitrap mass spectrometers (Orbitrap/MS) XL and Velos Pro for nontargeted molecular explorations and identifications, and the triple quadrupole mass spectrometer (tripleQ/MS; AB Sciex 5500) for target molecular detections at high sensitivity.

Advantages of live MS for single live plant cell analysis. There are three key advantages to live MS. First, it can directly detect molecules (mainly small molecules) from a target living single

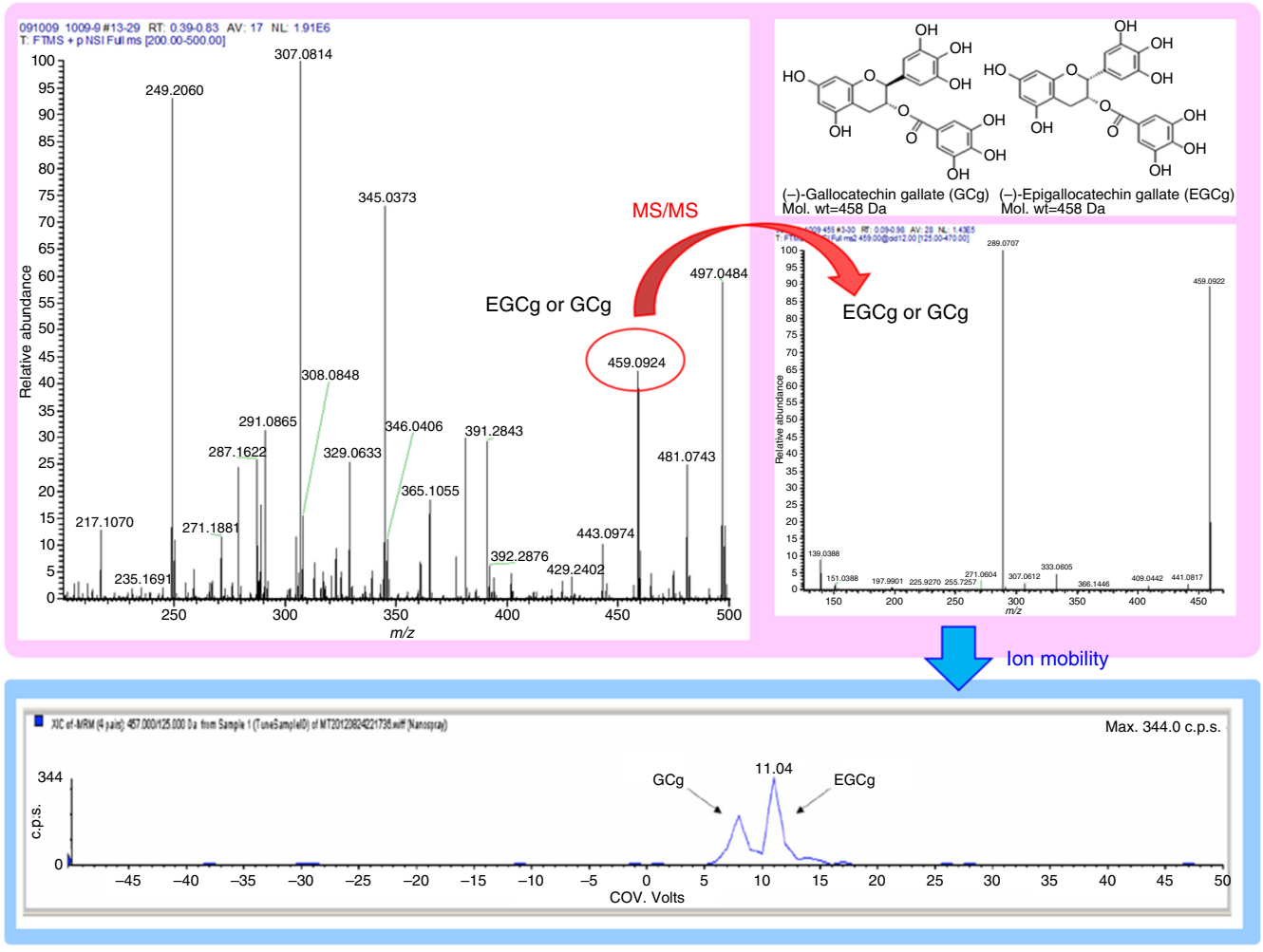


Figure 3 | Isomer separation of catechins from a single plant leaf cell by ion mobility-tripleQ/MS. Catechin isomers are not separated in the tea leaf single-cell analysis (upper left), nor in the MS/MS spectrum (upper right); however, with ion mobility separation, the two molecular peaks are separated (bottom)[55]. c.p.s., counts per second.

TABLE 1 | Comparison of performances of current plant single-cell MS analyses.

Method (ionization)	Target cells	Sampling size	Detected molecules	Isomer separation performed	Molecular identification performed	Live or pretreated	Refs.
Live MS (nano–electrospray ionization (ESI))	Plant leaf, stem (pith, cortical, mesophyll and epidermal), flower and animal cells	1–20 μm, even a single organelle	From abundant to minor small molecules (e.g., metabolites, amino acids, sugars, lipids, hormones and so on)	Performed with an ion mobility device	MS/MS–MS^n	Live cells	49–57, 20–23
MALDI	Single whole cells in plant tissue	<100 μm	Central metabolites	–	Possible by TOF/TOF	Treated with matrix	54,20, 22,41
Leaf spray	Plant leaf cells	Whole plant leaf	Small molecules on leaf surface cells	–	MS/MS–MS/MS^4	Live tissues	58
Laser ablation ESI	Plant epidermal cells	30–40 μm	Amino acids and saccharides	–	MS/MS	Large live cells	20,23, 25,59

cell (mainly vacuoles) under observation. Second, hundreds to thousands of molecular peaks from a single cell can be detected within 10 min. These peaks can be studied using MS/MS in the same run, as the signals are usually continuous for at least several minutes. Finally, characteristic molecules can be discovered and compared by multivariate statistical analyses or *t* tests between two sets of spectra at different sites or times in the sampling.

Experimental design

Plant preparation. To suck the single-cell component out using the nanospray tip, normally a leaf, stem or root is cut from a plant that is grown under specific conditions and immediately put on the stage of a stereomicroscope. To target cells from specific areas of the sample within a live plant, the sampling point should be set rigidly with supports (e.g., by a gel block or a plate) to ensure that the needle of the nanospray tip is inserted into the pointed cell under microscopic observation (**Fig. 2a**). Quick and direct sampling of fresh samples is desirable to minimize internal biochemical changes.

Direct molecular detection from a live plant single cell by live MS. Live MS of a plant sample is quite simple: (i) the sample is set under the stereomicroscope with a 3D micromanipulator; (ii) the cell contents are sucked out using a nanospray tip or microcapillary using a 3D manipulator; (iii) the ionization solvent is applied from the open rear end of the cell-trapped tip; (iv) a high voltage is introduced between the tip and the inlet of the mass spectrometer and the contents are fed directly into the mass spectrometer by nanospray ionization; and (v) hundreds to thousands of molecular peaks are acquired.

Data analysis of the mass spectra. The molecular peaks (*m/z*, relative intensity and noise) of the mass spectra are filed in text format on Excel. For molecular identification, the exact mass of a molecular peak and its MS/MS or MS/MS^n (ref. 51) spectra are compared with spectra in public databases, such as KEGG (Kyoto Encyclopedia of Genes and Genomes; http://www.genome.jp/kegg/), MassBank (http://www.massbank.jp) or MetFrag (http://msbi.ipb-halle.de/MetFrag), to annotate the molecule. Statistical analyses, such as principal component analysis (PCA) and *t* tests, are applied to the group data to extract characteristic molecular peaks for recognizing differences between cell groups. Molecular difference in cell localization, cell stages, environmental conditions and so on can be determined.

MATERIALS

REAGENTS

- Ultrapure water (Organo, Puric-ω)
- Acetonitrile, LC-MS grade (Thermo Fisher Scientific, cat. no. A955-212, CAS (75-05-8)) **! CAUTION** Acetonitrile is harmful and flammable, and it should be handled in a fume hood.
- Methanol absolute, LC-MS grade (Kanto Chemical, cat. no. 25185-76, CAS (67-56-1)) **! CAUTION** Methanol is toxic and flammable, and it should be handled in a fume hood.
- Formic acid (FA), puriss p.a. eluent additive for LC-MS, (Sigma-Aldrich, cat. no. 56302-50ML, CAS (64-18-6)) **! CAUTION** FA is corrosive and volatile, and it should be handled in a fume hood.
- Ammonium formate, puriss p.a. eluent additive for LC-MS (Sigma-Aldrich, cat. no. 55674-50G-F, CAS (540-69-2))
- Ammonium hydroxide, eluent additive for LC-MS, ≥25% in H_2O (Sigma-Aldrich, cat. no. 44273-100ML-F, CAS (1336-21-6)) **! CAUTION** Ammonia is caustic, hazardous and volatile, and it should be handled in a fume hood.
- Poly-tyrosine solution (Thermo Fisher Scientific, cat. no. CS0272L, CAS (31724-37-5)) for calibration of the mass spectrometers
- Nitrogen gas, nitrogen gas separator (System Instruments, N2 Supplier Model 24F (for Thermo mass spectrometer) or Anest Iwata, Smart Air SLP-221ECD (for AB Sciex mass spectrometer)) for the collision cell and curtain gas (AB Sciex) of the mass spectrometers

TABLE 2 | Performance in current mass spectrometers.

Mass spectrometer type	Sensitivity	Resolution	Through-put
TripleQ/MS	++++ (targeted mol.)	+	++ (+)
Q-TOF/MS	+++	++	+++
Orbitrap/MS	+++	+++	++ (+)
FT-ICR/MS	++	++++	+

+: OK, ++: good, +++: better, ++++: best, assuming current technology level.

- Helium gas, 99.999% pure for ion trapping and collision-induced dissociation (CID)
- Nanospray ionization solvents (see Reagent Setup)

EQUIPMENT

- Stereomicroscope (Leica M205 FA with a fluorescence light source EL6000 or M205 C) with a DFC310 FX CCD camera for image display and Leica Application Suite version 3.2 for recording
- Micromanipulator (Narishige MHW-103 on MMN-1) set on the magnet stand
- Nanospray tips (1–1.2 mm outer diameter, 35 mm length with filament) with 1-µm top bore diameters and their top end/point curved a short distance to make them stiff enough to insert into hard plant tissue (Cellomics tip, 1 µm for plant cells, HUMANIX; **Fig. 2b**). Homemade pulled capillaries with filaments can be also made by a puller (Narishige PN-31). These capillaries need cleaned electrodes to be inserted into the sample solvent every time a high voltage is applied for nanospray generation[49]
- Pipettes (Eppendorf, Research plus, adjustable 0.5–10 µl) and microfilament tips (Eppendorf, GELoader tips)
- LTQ-Orbitrap XL and Velos Pro mass spectrometers equipped with an offline nanospray ion source (Thermo Fisher Scientific)
- QTRAP 5500 triple quadrupole mass spectrometer equipped with the SelexION ion mobility attachment (AB Sciex) and a modified offline nanospray ion source (see Equipment Setup)
- Software: Xcalibur Qual Browser version 2.1 (Thermo Scientific) and Analyst version 1.5.1 (AB Sciex) for MS data management, MZmine version 2.0 or MarkerView version 1.2.1 (AB Sciex) for alignment of the mass spectra. For *t* tests and principal component analysis (PCA), we used MarkerView (AB Sciex) **! CAUTION** Some commercially available software for *t* tests and PCA analyses for LC-MS data (e.g., SIEVE (Thermo Fisher Scientific) cannot be used for offline nanospray data, because the data do not have retention time. Mass spectra data can be converted to text file in any equipment software. This allows you to perform data analyses with free public software for PCA and *t* test (e.g., R (GNU GPL)) or *t* test only by Excel, and annotation using public databases as shown in this paper
- Database: MassBank and MetFrag for MS/MS spectra, and KEGG for metabolite mapping.

REAGENT SETUP

Plant growth conditions *Raphanus sativus* was grown from seeds for 5 d in a dark chamber at 24 ± 1 °C and then changed to designated light irradiations. For the light-wavelength dependence test, we irradiated various colors of LED light sources (5 W) for 48 h. For phototropism analysis, blue LED was irradiated from the vertical direction.

Box 1 | Orbitrap/MS setup ● TIMING 30 min–1 h

Procedure for setting up the Orbitrap/MS XL or Velos Pro

1. Check that the gas sources and the heat pump chiller are working correctly, and then precondition the MS instrument for at least 1/2 h at these settings.
2. Set the offline nanospray ion source to MS, and turn on the monitor of the ion source inlet viewing video camera.
3. Set MS parameters as follows: resolution at 100,000, applied ion source voltage ~1 kV (for positive ion mode) and –0.8 kV (for negative ion mode), mass detection range as you like (for metabolites, e.g., *m/z* 50–500 or 100–700), and a capillary temperature of 200 °C.
4. Set the nanospray tip on the ion source, which includes only the calibration solvent or trapped cell content at the top of the tip and ionization solvent, which includes internal standard(s), introduced from the rear end of the tip. Move the top of the tip 2–6 mm toward the center of the MS inlet by observing the ion source monitor. For calibration, put 3 µl of poly-tyrosine solution into a nanospray tip. Check the mass spectrum at *m/z* 182, 508, 997 for positive ion mode and *m/z* 180, 506, 995 for negative ion mode, and click the calibration button.

! CAUTION Touching the ion source during measurement could cause electrocution.

▲ CRITICAL STEP Repeat the measurement until the mean residual mass deviation is <3 ppm. Next, stop the measurement and discard the nanospray tip.

▲ CRITICAL STEP Close the cover of the ion source so as not to touch the high voltage. You can put some tape or measure the grid on the ion source monitor screen to repeatedly position the nanospray.

5. Start the measurement (high voltage is simultaneously applied to the tip at this point) and check the TIC to determine whether the nanospray has been generated. Adjust the position of the top point of the nanospray tip to obtain the highest and most stable TIC. Prepare the MS measurement method file for detecting target molecules at high sensitivity. If you have standard reagents for your targeting molecules, you can tune the conditions (by changing, e.g., S-lens RF level or collision energy) and set the method parameter file to obtain higher sensitivity.

▲ CRITICAL STEP If you put the top of the tip too close to the inlet point of the mass spectrometer, a sudden spark will be generated, which will crack the top part of the nanospray tip. This sparking procedure is often intentionally used to widen the top bore when the top of the tip is plugged by a cell's contents. It is, however, difficult to get the desired shape and bore size at the top point.

? TROUBLESHOOTING

6. For MS^n analysis, start the collision by CID in the ion trap and check the spectrum at each step to determine which fragments are detected. High-energy collision dissociation (HCD), which produces a higher number of product ions, can be applied at various energy levels but only at the final step of MS^n. If you have standard reagents, you can obtain MS^n spectra to compare with the sample and tune the collision conditions of the MS.

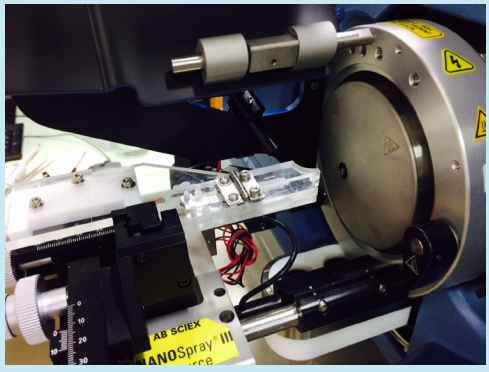
▲ CRITICAL STEP Higher-order MS^n analysis needs higher precursor molecular peak intensities.

Box 2 | TripleQ/MS setup ● TIMING 30 min–1 h

SRM by tripleQ/MS is suitable for highly sensitive detection of tiny amounts of single cellular metabolites. In SRM mode, the target parent ion is initially selected by the first mass filter Q1, and the daughter ions after the collision cell are selected by Q3 and then detected. To get a higher signal, the instrument parameters can be optimized by analyzing standard molecules before each series of samples. In our laboratory, we use the AB Sciex 5500 for SRM (MRM mode) analysis.

Nanospray ion source

As offline nanospray ion sources are not available for the AB Sciex 5500, we modified the online AB Sciex nanospray source as shown here. In place of the online nanospray emitter, we made a groove on a plastic holder of the nanospray tip in which we put a thin metal sheet for an electrical connection from a high voltage point of the MS body to the nanospray tip.



Procedure for optimizing SRM for the analysis of specific compounds

1. Prepare standard solutions of the target compounds.
2. Set the nanospray tip, which contains the standard sample or the trapped cell sample, on the offline nanospray ion source of a tripleQ/MS (in this case, we used QTRAP 5500 (AB Sciex)). Details about setting the position of the top of the nanospray tip are described in **Box 1**.

? TROUBLESHOOTING

3. Use the ion source settings below for the positive and negative ion modes of the QTRAP 5500. Usual starting parameters are as follows: curtain gas (CUR) 5.0, ion spray voltage (IS; positive/negative) +1,800/–1,500, ion source gas 1 (GS1) 0.0 and ion source gas 2 (GS2) 0.0.
4. Start the measurement and check the target ion peak from the mass spectrum.
5. Start compound optimization to find the combination of the parent ion (Q1) and daughter ions (Q3), the tuning for declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP). Create an SRM (MRM) instrument method based on the Q1/Q3 transition parameters.
6. Start the sample measurement with these best-tuned parameters.

Sampling As the coefficient of variation (CV) of this method with internal standards is 5–25% (determined in an experiment using 3–5 samples), which inversely depends on the peak intensity, the differences in peak intensities even between two sampled cells should be meaningful. However, we recommend using more than three samples. When taking the average of metabolites in samples with similar cell morphology or stage using fluorescent probes, we usually make 3–5 samplings within 15 min, which keeps all samples in the same condition, without drying. Video-microscopic observation is very important for judging the cell stage and/or condition. It is important to note that single-cell analysis is not for taking an average (e.g., of metabolite intensities using many cells), but for unveiling why and which cells are different.

MS calibration solution Commercially available poly-tyrosine solution was diluted to 50% with methanol for mass calibration before the experiment. Lock mass calibration was also performed using the molecular peaks of contaminating plasticizers (e.g., phthalates) from the plastic tubing. In single-cell analysis, these peaks seemed to be stable in every sample, and they were intense enough to be recognized by the equipment for the mode of lock-mass calibration (an operation to estimate and correct any *m/z* fluctuations for every obtained scan). Because of this feature, the use of the same brands and sequenced lots of plasticware and nanospray tips throughout each sequence of experiments is recommended. Intensity calibration can also be done by adding an internal standard to the ionization solvent.

Nanospray ionization solvent with internal standard Prepare an ionization solvent with the following composition: 50–90% (vol/vol H_2O) methanol solution or acetonitrile solution with 0.1–1% (vol/vol) formic acid (for positive ion mode), 0.1–1% (vol/vol) ammonia hydroxide solution (for negative mode) or 1–10 mmol/liter ammonium formate (for positive and negative switching mode). The solution should also contain the desired internal standards. In our experiments, we used 1 ng/ml 15 N labeled amino acids (cell-free amino acid mix (20 aa; U-15N, 96–98%), Cambridge Isotope Laboratories, cat. no. NLM-6695-PK) or stable isotopes of the target metabolites. The proportion of the organic solvent can be adjusted depending on the polarity of the target molecules. It is worth bearing in mind that ionization efficiency becomes lower at lower concentrations of organic solvent.

▲ CRITICAL After trial experiments, you will know which of the metabolite's peaks are detectable and of interest among all the detected molecular peaks. Next, in the final experiments, it is best to use stable isotope–labeled versions of these molecules as internal standards in order to better correct for ionization efficiencies. For untargeted analyses, you should ideally select more than one molecule or a molecule that shows multiple peaks, but that do not overlap with the sample peaks, in order to cover the detection *m/z* range.

! CAUTION Solvent mixing should be done in a fume hood.

EQUIPMENT SETUP

Stereomicroscope setup Plant samples can be set on the base mount of a stereomicroscope and observed with maximum zooming and magnification

Box 3 | Ion mobility-tripleQ/MS setup ● TIMING 30 min–1 h

DMS is a technique that separates ions in a DMS-cell installed under atmospheric pressure. When separation voltages (SVs) are applied, an ion in the DMS mobility cell moves to the electrode because of the difference between mobility in the high and low electric fields (mobility difference). To compensate for the orbit of the moving ion, a compensation voltage (COV) is applied. The combination of this SV and COV is specific to each compound analyzed, and it is used to separate ions by spatial molecular shapes. It can be applied for the separation of isomers, and this is one of the main reasons for performing it in plant metabolomics[55].

In our laboratory, A QTRAP 5500 system equipped with SelexION is used for measurements to separate isomers such as (–)-epigallocatechin gallate (EGCg) and (–)-gallocatechin gallate (GCg) from single tea leaf cells[55].

Method development procedure for isomer separation by using an ion-mobility attachment

1. Optimize the SRM (MRM for AB Sciex) transition for target molecules on Analyst software. Re-adjust this condition for a nanospray ion source with a nanospray tip. The conditions are shown as follows.

Source and gas parameters:
CUR: 10, collision gas (CAD): low, IS: −1,800 to −2,200 (−2,000.0), TEM (gas is not heated for nanospray): 0, GS1: 10, GS2: 0 and interface heater (IHE): on.

SRM parameters:

Q1 Mass	Q3 Mass	Dwell time (ms)	DP	EP	CE	CXP
456.873	125.0	150	−90	−10	−50	−17
456.873	168.9	150	−90	−10	−24	−19

Declustering potential, DP; entrance potential, EP; collsion energy, CE; collision cell exit potential, CXP.

2. Immediately after single-cell trapping, dip the top of the nanospray tip in 50% 2-propanol and then add 3 µl of 50% acetonitrile (containing 0.1% (vol/vol) formic acid) from the back end of the tip.

▲ CRITICAL STEP For this kind of open nanospray ion source, the modifier cannot be introduced by the default machine setting. This method overcomes the modifier deficit.

3. Set the DMS parameters:

Modifer	None
MoC	Low
Separation voltage	3,200–3,800 (3,500)
Compensation voltage	0
DMS offset	5.0
DMS resolution enhancement (DR)	0

! CAUTION Although the vaporized gas of acetonitrile and 2-propanol is in small amount, keep the exhaust port of the MS machine open.

at 160× and 640×, with internal systems for transmitting light or fluorescence excitation. To acquire clear images of the samples, another LED light source for irradiating the surface of the samples is frequently efficient. ! CAUTION Light sources that emit heat waves are not recommended, as they risk drying the cell samples quickly.

Micromanipulator setup Mount a Narishige MMO-203 3D-micromanipulator onto a MMN-1 3D manipulator with an injection holder (or nanospray holder; H1-7 for a 1.2-mm glass capillary) whose top end holds the nanospray tip or hand-pulled capillary (**Fig. 2c**). Place the systems on a magnet base that itself is locked in on an iron plate (300 × 200 × 5 mm^3). Connect the injection holder with 1 m of polytetrafluoroethylene (PTFE) tubing (inner and outer diameters of 0.9 mm and 2 mm, respectively). Attach a terminal tube connector for a disposable syringe (25–50 ml) to the tubing for suctioning and pumping. Any other micromanipulator model can be used.

Orbitrap/MS setup The sensitivity and resolution of mass spectrometers are key to the detection and identification of many compounds from a tiny amount of single-cell contents. Spectrometers for the electrospray ionization of tripleQ/MS, Q-TOF/MS, Orbitrap/MS and Fourier transform–ion cyclotron resonance (FT-ICR)/MS can be used for single-cell MS. **Table 2** shows our evaluations of current performances in mass spectrometers. To balance the three properties shown in **Table 2**, we are now mainly using Orbitrap/MS for cyclopedic molecular detection and tripleQ/MS for targeted sensitive molecular detection. The setup of the Orbitrap/MS is described in **Box 1**.

TripleQ/MS setup See **Box 2**.

Ion mobility–tripleQ/MS setup See **Box 3**.

Other MS setup Other types or models of mass spectrometers can be used for this method. As sensitivity is very important, equipment should be kept clean with no accumulation of ions on the ion optics and detectors. Any type of tripleQ/MS is useful for sensitive target molecular analyses. Q-TOF–type mass spectrometers are very useful for nontarget molecular exploration and identification. In any case, you may have to develop an offline nanospray ion source. The image in **Box 2** shows a handmade setup and gives a good example even only setting of electric grip works to setting the nanospray tip and applying high voltage. You should design the ion source to avoid electrocution.

PROCEDURE

Plant sample preparation ● TIMING 2–5 min

1| Take out the sample (leaf, stem or root) from the target plant, and record the time and condition of the plant (take a photo before sampling, if necessary) (**Fig. 4**). Cut the sample into slices of ~1 mm with a razor. For a leaf sample, peel the epidermal layers away using double-sided tape to directly access the plant cells, or cut the leaf at a slanted angle to expose many layered cells. Rinse the sliced cut face with distilled water. Even without cutting, the use of a suction procedure is possible; however, epidermal contaminations should be taken into account.

2| Set the cut sample on a glass slide with double-stick tape or in an agar gel, and set the plate in the center view of the stereomicroscope base and adjust the magnification (for a stem slice, magnification is 100-fold to 640-fold; for leaf cells, we use 640-fold magnification, as cells in a leaf are smaller than those in a stem). Select the target cell and move it into the center of view by manipulating the *xy* base stage of the microscope.
▲ **CRITICAL STEP** Observe the refraction of light from the round cell shape to check that the target cell has not been damaged by the razor cut.

Single-cell sampling ● TIMING 1–5 min

3| Attach the nanospray tip to the nanospray holder of a micromanipulator, and set the top point of the tip above the center of the objective lens and check the view (above the sampling point) to move the top point to the real center of view at the upper focusing point (**Fig. 3**).

4| Descend the top point of the tip until it is near the sampling cell surface. The focused image of the top of the tip will finally merge with that of the sample surface, which means that the tip's top point and cell surface are in the same focal point.
▲ **CRITICAL STEP** Centering the top of the tip and the target cell in the microscopic view is important for the success of this procedure.
? TROUBLESHOOTING

5| Touch the target single cell with the top of the tip, and push it through the cell wall. Suck the cell content (hundreds of femtoliters) into the top point of the tip from the cell by squeezing the syringe. Remove the tip from the cell and then remove the trapped nanospray tip from the nanospray holder. Record the time of sucking by photo or by video to check the timing and the sucked location of the cell precisely.
? TROUBLESHOOTING

6| Use tips with the same specifications to ensure that the sample volumes are within 10% for the comparison of intensity data between samples. This precaution is taken because the trapped volume at the top of the tip depends on the surface tension of this area, which itself depends on the shape of the top point. The total cost and throughput of this procedure is ~$5–6 per sample, which is mainly the cost of the nanospray tip, and 3–10 min (or less) per sample.

MS measurement of a single cell ● TIMING 2–10 min

7| Use the gel loader tip to insert 3 μl of ionization solvent into the back end of the nanospray tip (**Fig. 4**). To take out the air gap between the trapped sample and the ionization solvent in the tip, the nanospray tip is snapped by grabbing the end part of the tip, or by putting it into a microcentrifuge (<2,000*g*) in the direction of its top point for a few seconds. The ionization solvent includes the internal standard.

8| Set the nanospray tip on the ion source and start the measurement with the desired method file. Check the total ion chromatogram (TIC) to confirm whether the nanospray is properly generated or not. Adjust the position of the top point of the nanospray tip to get the most stable and highest TIC. Use a starting application of high but not sparking voltage to start a smooth nanospray.

The typical duration of the detectable period of signals is 3–10 min, which should be sufficient to obtain full scan data or *m/z* range divided and accumulated full scan spectra and also MS/MS spectra.
! CAUTION Touching the ion source during the measurement could cause electrocution.
? TROUBLESHOOTING

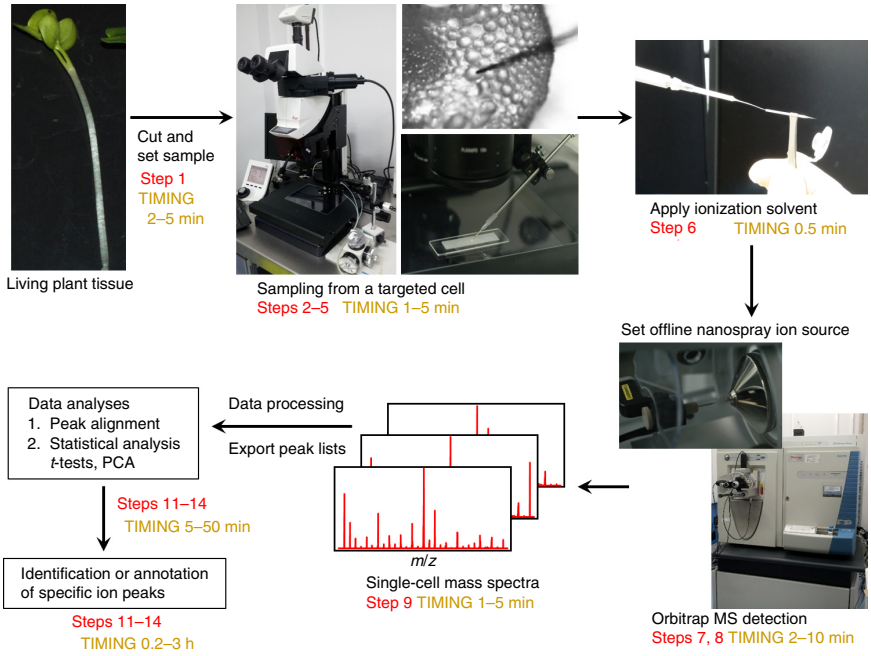


Figure 4 | Schematic procedure of live single plant cell MS.

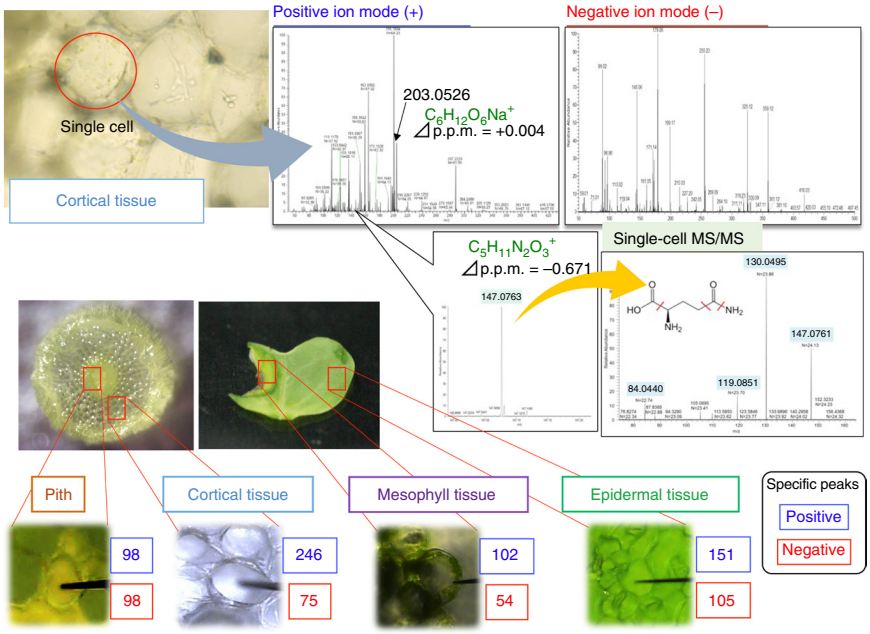


Figure 5 | Results of live single plant cell MS. Within the detected mass spectrum of a single cell in the cortical part of a radish sprout, hexose and glutamine were identified by MS/MS using the LTQ-Orbitrap Velos Pro. By applying a *t* test between tissue samples, a number of tissue-specific molecular peaks were extracted. Blue numbers (positive ion mode) and red numbers (negative ion mode) show the number of site-specific molecular peaks obtained from the *t* test analysis[56].

9| (Orbitrap/MS) Accumulate the spectra over the spray time, which usually lasts several minutes to >10 min. During the nanospray ionization of the contents of the single cell, MS/MS, MS^n and selected ion monitoring (SIM) can be performed in addition to the normal full-scan mode, as shown in **Figure 5**.
? TROUBLESHOOTING

10| (TripleQ/MS) Set selected reaction monitoring (SRM) (MRM for AB Sciex Analyst software) parameters with optimized Q1/Q3 pairs and their parameter transitions, and then start the SRM measurement. The ion mobility separation of isomers can be performed with the obtained SRM data. Export the peak lists of the targeted compounds, which contain *m/z* values of the Q1 and Q3 ions, and peak intensity, and use the data for calibration and quantification.
? TROUBLESHOOTING

Data analysis: mass spectra alignment ● TIMING 10–30 min

11| For Orbitrap/MS data, display *m/z*, intensity and relative noise in columns of the peak table at the appropriate mass range in Thermo Xcalibur Qual Browser (**Fig. 5**). Right-click the peak table and choose the clipboard (Exact Mass), and then paste in the Excel spread sheet for all data. We usually delete the relative column and any raw data for which intensity/noise is ≤3 in order to cut off noise for Orbitrap/MS data, but not for tripleQ/MS data, which contain less noise. Save the remaining data in Excel as a tab-delimited text format file.

12| Use MarkerView or MZmine2 (http://mzmine.sourceforge.net) software to align the *m/z* values among the data sets being compared, and then acquire an aligned peak list as a table in Excel.

PCA and *t* test ● TIMING 5–20 min

13| Input the text format data to MarkerView software in order to classify the group and to extract the cell stage or site's specific molecular peaks (**Figs. 5** and **6**). Two groups of data sets can be compared using a *t* test. If there are more than two data sets to compare, PCA can be performed, or a *t* test between two sets of data of interest can be performed. The overall spectrum profile can be compared by PCA by using the scores plot, which shows clusters of points (each point is for one mass spectrum) to visualize grouping of the stages or sites of cells. The group-specific peaks can be shown as scattered points in a loadings plot. Central points (molecular peaks) are not specific, but scattered points from the center are specific or unique molecular peaks to a specific group. These group-specific molecular peaks can be more clearly extracted by applying a *t* test between two sets of group data at *P* values of <0.05.

Molecular identification ● TIMING 10–60 min for known molecules, and days or even months for unknown molecules

14| Use databases to identify the detected molecular peaks with the exact mass. In the KEGG database, peak identity is judged if the value is within the tolerance mass range of ±3 p.p.m. (**Fig. 5**). Candidate molecular formulae can be identified with greater certainty when the spectra have related peaks that are calculated as the protonated, Na^+- or K^+-adducted forms in positive mode, and H^- in negative mode. The combination check of the precursor and its possible adduct(s) is very helpful for molecular identification by the exact mass. Annotated compounds are written with the KEGG compound ID in Excel[57].

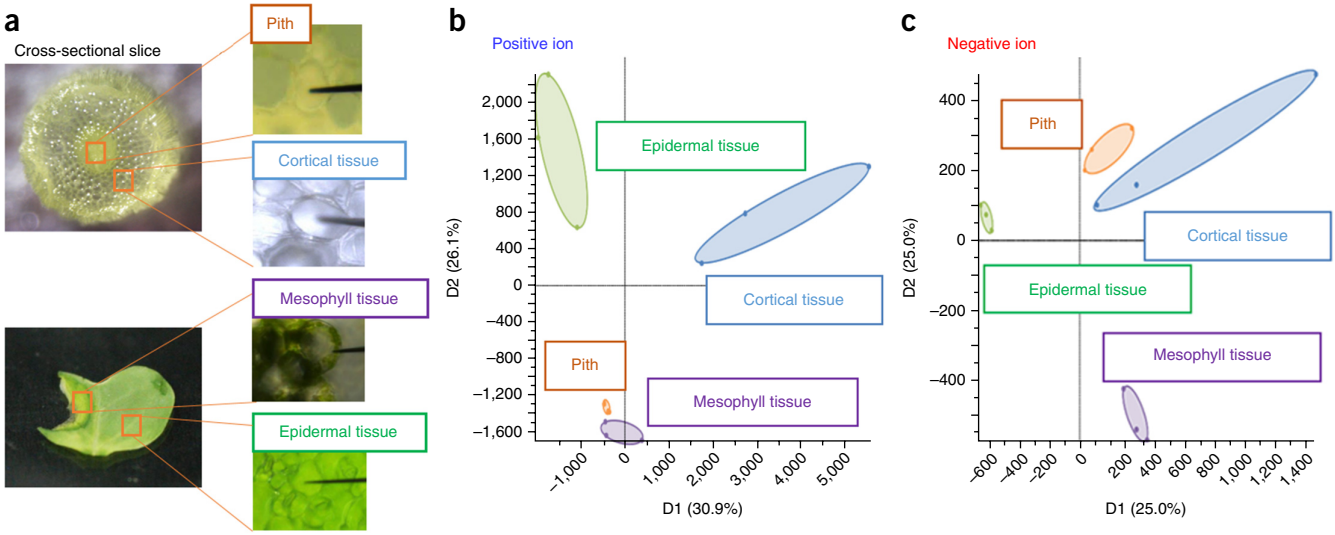


Figure 6 | Principal component analyses of live single plant cell mass spectra. (**a**–**c**) The spectra of each tissue shown in **a**, showing the clusters in score plots for positive (**b**) and negative (**c**) ion mode detections; these clusters indicate that each tissue has different or specific components in their spectra[56].

15| Conduct MS^n analysis for more direct molecular identifications. When we cannot perform MS^n analysis with standard samples, we compare the MS/MS or MS^3 spectra of target molecular peaks to the MassBank and MetFrag public databases to confirm the molecular identification. The previously mentioned adduct check of the precursor peak is also important for annotation of the MS^n spectra.

16| Adopt ion mobility separation if the molecular peaks cannot be identified by coexisting isomer(s), as shown in **Box 3** and **Figure 3**.

Metabolic pathway analysis ● TIMING 0.5–3 h

17| Use KEGG metabolite maps using KEGG (application programming interface) API for the pathway analysis. Specify the metabolite pathway number and KEGG compound ID, and then visualize the objects of the metabolite matched with the KEGG compound ID on the specified metabolite pathway using KEGG API. In addition, this can mark each object of metabolites as a given color, so we can visually compare data sets on a colored metabolite pathway as active or inactive metabolic reactions by specified rules, such as an increase or decrease in the amount of metabolites.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
4	It is difficult to set the tip to the point of interest on the tissue	The top of the tip is not in the center of view	In the upper position of sample, set the top of the tip in the center of the microscopic view under lower magnification and then change the magnification to the final one. Finally, gradually move the tip down by keeping it in the center of view
5	The top of the tip does not insert into the cell, but it fractures on contact with the cell	The shape of the top point is not strong enough for plant cells	Change the top shape of the tip such that it is shorter and more curved
	Hard to see the cell	Light irradiation is not good	Adjust the angle of surface irradiation light (LED is preferable) to get a clear image of cells
8	No or small TIC signals	Top of the tip is blocked or narrowed	Clean the top point of the tip using pure water or move the top of the tip closer to MS inlet or increase the applied voltage by taking care not to make a spark

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
9	No target molecular peaks	Concentration is too low	Change the mode from full scan to SIM or use tripleQ/MS if the target is known
10	No concentration dependency	Peak overlapping	Change the SRM transition to another daughter ion or change the ionization solvent (if solvent peaks appear overlapped)
Box 1 (step 5), **Box 2** (step 2)	No or small TIC signals	Positioning of the top of the tip is not good	Center the tip position again and tune the position. (Minimize the curtain gas of tripleQ/MS, which requires delicate positioning)

● TIMING

Steps 1 and 2, plant sample preparation: 2–5 min
Steps 3–6, single-cell sampling: 1–5 min
Steps 7–10, MS measurement of a single cell: 2–10 min
Steps 11 and 12, data analysis: mass spectra alignment: 10–30 min
Step 13, PCA and *t* test: 5–20 min
Steps 14–16, molecular identification: 10–60 min for known molecules, and days or even months for unknown molecules
Step 17, metabolic pathway analysis: 0.5–3 h
Box 1, orbitrap/MS setup: 30 min–1 h
Box 2, triple/MS setup: 30 min–1 h
Box 3, ion mobility-tripleQ/MS setup: 30 min–1 h

ANTICIPATED RESULTS

Single plant cell trapping and molecular peak detection

When single plant cell trapping by nanospray tip is successful, the cell content will automatically flow into the top of the tip, as seen in **Supplementary Video 1**. Plant cells usually look swollen in shape because of their internal osmotic pressure. After the cell is penetrated by the tip, the cell shape starts to deflate with no or flat light reflection. It is possible, therefore, to check that only a single cell has been sampled by watching out for changes in the shapes of the adjacent cells.

Some examples of mass spectra obtained in experiments are shown in **Figure 7**. If the top of the tip is plugged by sucking the cell membrane or other cellular constituents, the plugged tip shows small peaks of only discharged surrounding air (**Fig. 7a**). When cell components are not successfully sucked into the tip, only solvent peaks can be detected (**Fig. 7b**,**c**). A good spectrum for a single plant cell will show many molecular peaks between the solvent peaks (compared with the same intensity scale of the vertical axis). In plant single-cell analysis, solvent peaks do not dominate, because the detected peaks of cell content are present at relatively high intensity.

Cell-specific molecular extraction and molecular identifications

As shown in **Figure 5**, positive and negative modes of mass spectra of many single-cell sites in a plant tissue (the pith and cortical parts of cell in a stem of a radish sprout, and mesophyll and epidermal parts in its leaf) can be detected. Align text filed data of these mass spectra to compare data sets by using MZmine2 or MarkerView. The aligned data are subjected to a *t* test by MarkerView software or Excel to extract the site-specific molecules. Between a pith and cortical cell in a stem tissue, 98 and 246 positive molecular ions are found to be pith- and cortical site–specific molecular peaks ($P < 0.05$), and 98 and 75 are negative molecular peaks, respectively. When these site-specific molecules are searched by exact mass in the KEGG metabolic map database, a first approximation of site-specific metabolite changes can be obtained. Such extracted molecular peaks should be further characterized. An example of an extracted molecular peak at *m/z* 147.0763 is shown in **Figure 5** (central), where it was identified as glutamine after being subjected to MS/MS analysis and consulting with the MassBank database (**Fig. 5** (right)).

Cell classification by PCA

To classify the data according to the locations where the cell contents are trapped, the cell-alignment metadata are included with the MS data and analyzed by PCA. **Figure 6** shows three measured spectra of each location of the plant tissue and the clusters in the scores plots. This result shows that the live single-cell mass spectra are similar to each other

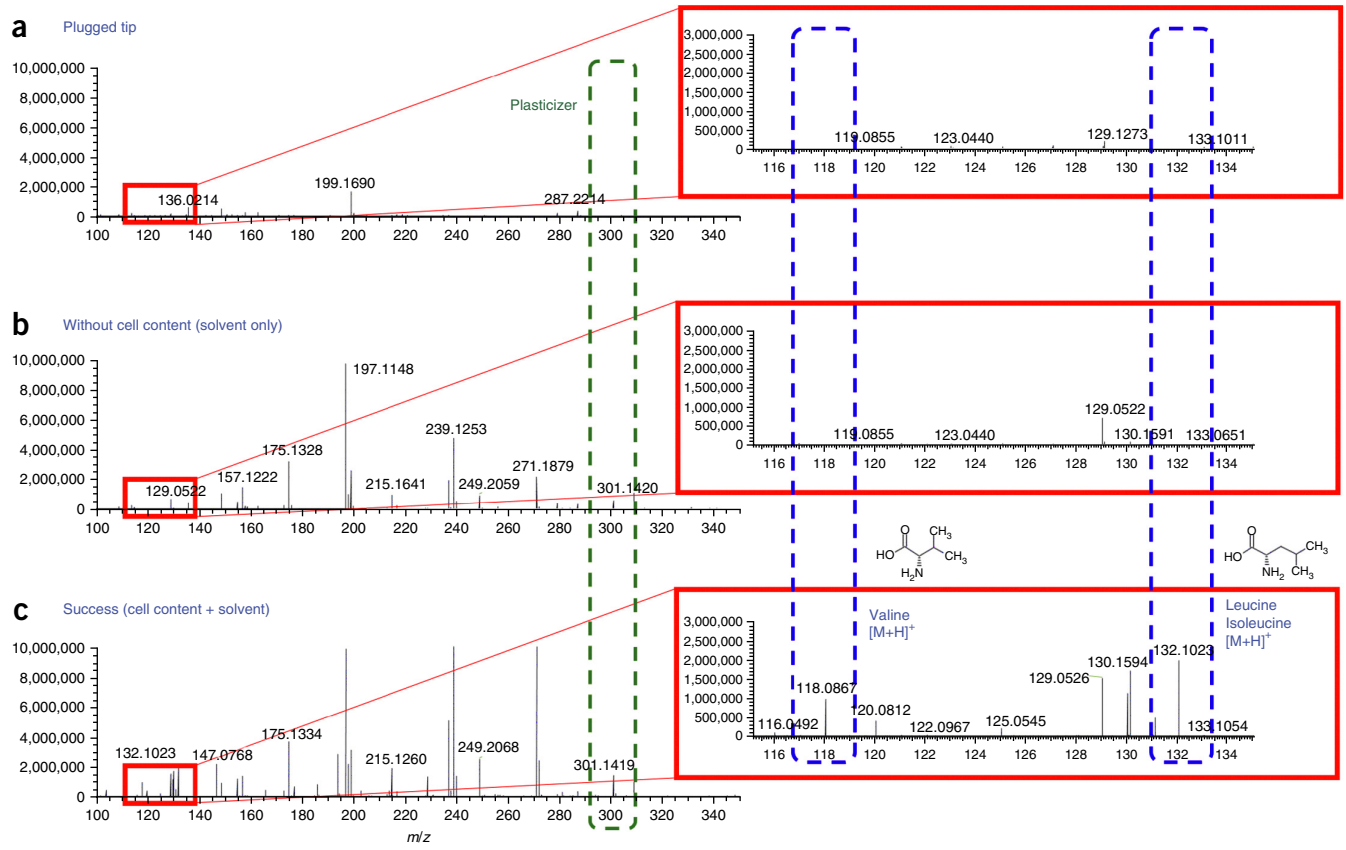


Figure 7 | Typical spectra in live single plant cell MS. (**a**) Plugged tip. (**b**) Sample is not sucked (solvent only). (**c**) Successful experiment shows molecular peaks of single-cell content.

when cells are measured from the same tissue area, and they are different from each other between different locations in a plant. We also use this analysis to measure the sample correctly from the target site of plant tissues.

Conclusion

We have established a rapid, direct molecular detection method for the analysis of large numbers of metabolites in a targeted site of a single live plant cell. Live MS can be applied to diverse plant cell biology and agricultural research topics, as it provides an easy means to investigate the molecular content of a cell with exceptional resolution. The resulting information should help to provide new details on the state of a cell, and it is expected to reveal undiscovered molecules and dynamic molecular mechanisms.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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