Methods to Visualize Elements in Plants

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Topical Review

One sentence summary: We compare the wide range of techniques for visualizing element distribution in plants in order to assist researchers in selecting the method most useful for their particular research program.
**Methods to visualize elements in plants**

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A.v.d.E. wrote the section on SEM- and TEM-EDS, P.M.K., A.v.d.E., E.L., and P.W. wrote the section on XFM, A.v.d.E. and J.S.L. wrote the microPIXE, D.P.P. and S.H. wrote the section on LA-ICP-MS, K.L.M. wrote the section on NanoSIMS, E.L. wrote the section on autoradiography, P.M.K. and A.v.d.E. wrote the section on laser confocal microscopy. P.M.K. coordinated the overall drafting of the manuscript. All authors edited and approved the final version of the manuscript.

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Abstract
Understanding the distribution of elements in plants is important for researchers across a broad range of fields, including in plant molecular biology, agronomy, plant physiology, plant nutrition, and ionomics. However, it is often challenging to evaluate the applicability of the wide range of techniques available, with each having their own strengths and limitations. Here, we compare scanning/transmission electron microscopy-based energy-dispersive X-ray spectroscopy (SEM-EDS, TEM-EDS), X-ray fluorescence microscopy (XFM), particle-induced X-ray emission (microPIXE), laser ablation ICP-MS (LA-ICP-MS), nanoscale secondary ion mass spectroscopy (NanoSIMS), autoradiography, and confocal microscopy with fluorophores. For these various techniques, we compare their accessibility, their ability to analyze hydrated tissues (without sample preparation) and suitability for in vivo analyses, as well as comparing the most important analytical merits such as resolution, sensitivity, depth of analysis, and the range of elements that can be analyzed. We hope that this information will assist other researchers to select, access and evaluate the approach that is most useful for use in their particular research program.

Keywords: Element distribution; mapping; method selection; resolution; sensitivity
Introduction

Visualizing elements in plants is essential for a broad range of studies, including those aiming to improve plant nutrition and crop productivity, improving the nutritional content of edible portions of plants for human nutrition, and for reducing concentrations of harmful contaminants in food and in the broader environment. Accordingly, gaining a detailed understanding of the distribution and chemical form of target elements in plants is critical in plant molecular biology, agronomy, plant nutrition, plant physiology, and ionomics.

A variety of approaches can be used for visualizing the distribution of elements within plants. These techniques have their own advantages and disadvantages, and for many researchers, selecting the most appropriate technique and to evaluate the data from individual techniques can be challenging. For example, these various techniques differ in the range of elements that can be analyzed, their detection limits, ability to be quantitative, their resolving power, and whether specimens can be examined fresh or frozen hydrated (without sample preparation) or whether dehydration (such as freeze-drying) prior to analysis is required. In the present review, we aim to compare a suite of techniques that are suitable for mapping the distribution of elements within plants. Specifically, we compare scanning electron microscopy-based energy-dispersive X-ray spectroscopy (SEM-EDS) and transmission electron microscopy-based EDS (TEM-EDS), X-ray fluorescence microscopy (XFM), particle-induced X-ray emission (microPIXE), laser ablation ICP-MS (LA-ICP-MS), nanoscale secondary ion mass spectroscopy (NanoSIMS), autoradiography, and confocal microscopy using fluorophores as element-specific labels. For all experimental approaches, it is necessary to consider the methods used for sample preparation. However, this is not discussed in detail here, but rather, the reader is referred to other reviews.

This review builds upon and complements previous reviews, such as that of van der Ent et al. (2018b) who examined the use of X-ray-based approaches in hyperaccumulator plants, Kopittke et al. (2018) who examined synchrotron-based approaches in plants, Persson et al. (2016a) who examined LA-ICP-MS, Zhao et al. (2014) who discussed the types of research questions enabled by synchrotron-based approaches and mass spectrometry approaches, and Moore et al. (2012a) who examined NanoSIMS and complementary approaches for examining elemental distribution in plants. In the present review, we aim to provide a comprehensive comparison of the advantages and disadvantages of a broad range of the most frequently used techniques and methodologies, thereby enabling the reader to select the approach that is most applicable for use in their particular experiment.
Importance of visualizing elements in plants

The study of the distribution of elements in plants is critical for many research questions. These are briefly considered below before discussing the approaches that can be used to achieve this.

Functional characterization in molecular plant biology

Studies that link elemental imaging with genetic approaches are critical for characterizing genes that influence elemental homeostasis. Such studies provide opportunities for the analyses of gene × environment interactions in planta, for example by comparing transporter phenotypes. A range of the approaches described below have been used for functional characterization in molecular biology, with most studies focusing on micronutrients although some also focusing on macronutrients. For example, using a mutant of Arabidopsis thaliana unable to synthesize the metal chelator nicotianamine, it was found with LA-ICP-MS that the mutant accumulated Zn and Mn in the tissues surrounding the vascular cylinder, whilst Fe was confined to the cortical cell walls in the mutant despite being primarily in the epidermis of the wild-type (Persson et al., 2016a). In another recent study, XFM was used to investigate Arabidopsis, finding that METAL TOLERANCE PROTEIN 8 (MTP8) determined the distribution of Fe and Mn in seeds (Chu et al., 2017). In this latter study, the use of XFM to image element distribution in vivo avoided potential issues associated with green fluorescent protein imaging in quiescent seed tissue. In another example, XFM was used by Punshon et al. (2013) to show differences in the Ca localization and speciation in a calcium oxalate deficient5 (cod5) mutant of Medicago truncatula. These authors reported that knockout of COD5 prevented biogenic crystal formation by altering Ca distribution and the form of Ca oxalate. Finally, Kim et al. (2006) examined Fe in Arabidopsis seeds, finding that when the Fe uptake transporter VIT1 is disrupted, Fe did not accumulate in the provascular strands of the embryo.

Improving plant nutrition and productivity

Understanding element distribution in plants is also important for improving plant mineral nutrition and productivity. As an example, consider the application of foliar fertilizers to improve plant growth in soils containing low levels of plant-available nutrients. However, the mechanisms by which foliar-applied nutrients move across the leaf surface and are translocated and assimilated remain unclear. To examine this research question, Zn fertilizer was applied to the surface of a leaf of wheat (Triticum aestivum) and changes in leaf Zn
concentrations were measured \textit{in vivo} for up to 24 h (Doolette et al., 2018). Although some translocation of the foliar-applied Zn was observed, it was found that the Zn had only limited mobility regardless of the form of Zn applied. Similar results were also reported by Tian et al. (2015). In a similar manner, autoradiography has been used to examine the translocation of foliar-applied Zn over time \textit{in vivo} in whole plants of wheat (Read et al., 2019). These authors found that the use of $^{65}$Zn-labelled compounds allowed for time-resolved analyses of Zn distribution in live plants, reporting that $^{65}$Zn was translocated throughout the plant (including to the grain, where it is important for human nutrition) following its foliar application.

\textit{Improving human nutrition through foodstuffs}

In order to improve human nutrition through higher quality foods, an understanding of nutrient concentration and distribution within foodstuffs is essential. This is because the nutritive value of foods depends not only upon the total elemental concentration, but also its distribution and molecular speciation. Accordingly, biofortification strategies need to consider both distribution and speciation of nutrients within the foodstuff tissue. As an example of a study aiming to improve human nutrition, grains of buckwheat (\textit{Fagopyrum esculentum}) were examined using microPIXE (Pongrac et al., 2011). These authors found that the inner layers of the pericarp were enriched in K, Mn, Ca, and Fe while the outer layer was enriched in Na, Mg, P, S, and Al, and thereby by altering the milling approach it was possible to alter the nutritional content of the grain. Furthermore, for both wheat and rice grain, it is known that whilst micronutrients tend to accumulate in the bran layers (i.e. the aleurone, tegument, and the pericarp), those elements which are generally more mobile within the phloem (such as K, Mg, P, Fe, Zn, and Cu) tend to accumulate to higher concentrations in the aleurone layer (de Brier et al., 2015). In this regard, Wang et al. (2011) used LA-ICP-MS to examine the stable Zn isotope $^{70}$Zn in wheat grain, finding that there are two barriers to Zn transport in wheat grain: between the stem tissue rachis and the grain, and between the maternal and filial tissues in the grain.

Not only is distribution important in influencing the nutritional value of foods, but it is also necessary to understand how the co-localization of different elements within foods impacts on nutrient availability to humans. For example, co-localization of micronutrients with P (often present as phytate) likely reduces micronutrient availability in the human gut, with such co-localization observed in sweetcorn and maize (\textit{Zea mays}) (Cheah et al., 2019) and wheat (Moore et al., 2012b).
Understanding toxic elements in plants and tolerance mechanisms

Understanding the behavior of toxic elements in plants, their impacts on the plant growth, their translocation through the plant and accumulation in human foodstuffs, and the mechanisms that plants use to tolerate these toxicants is of critical importance. First, to illustrate the importance of understanding elemental distribution in crop plants, consider the problem of Al toxicity. Soluble concentrations of Al are elevated in the acid soils that comprise ca. 3.95 billion ha of the global ice-free land (Eswaran et al., 1997). Although Al is highly toxic to plant root growth, much remains unknown about how it exerts its toxic effects. In this regard, NanoSIMS has been used to examine Al distribution in root tissues of soybean (Glycine max), finding that Al accumulates almost entirely in the walls of cells in the rhizodermis and outer cortex (Kopittke et al., 2015). These authors reported that this Al in the cell wall of young, elongating roots was toxic and caused a rapid reduction in root elongation. Interestingly, in tea (Camellia sinensis), a known accumulator of Al, much of the Al accumulated in the cell walls of the leaves, with this seemingly associated with a tolerance mechanism (Tolrà et al., 2011). As another example, the accumulation of As in foods is of interest due to the consumption of this carcinogen by humans. The distribution of As in roots of Arabidopsis was examined using XFM, confirming the localization of a new arsenate reductase (HAC) which limits As accumulation in the tissues (Chao et al., 2014).

Another area of major research interest has been in the use of imaging techniques to understand how hyperaccumulating plants are able to tolerate high concentrations of metal(loid)s in their tissues. Nickel hyperaccumulator plants (which make up the majority of hyperaccumulator plants known globally) have been the most intensively studied (Reeves et al., 2018). In most species studied to date, Ni is concentrated in the epidermal cell vacuoles of the leaves (Küpper et al., 2001; Bhatia et al., 2004; Kachenko et al., 2008; van der Ent et al., 2017). Hyperaccumulation spans several length-scales, from whole plants down to organs, tissues, individual cells, cellular organelles and transporter molecules, and information at all of these scales is important for understanding the mechanisms involved with hyperaccumulation (van der Ent et al., 2017).

**X-ray fluorescence-based approaches for visualization**

With X-ray fluorescence-based approaches, elements are detected based upon their characteristic fluorescent X-rays. These fluorescent X-ray are generated by passing the specimen through a focused beam of high-energy X-rays (XFM), electrons (SEM- and TEM-based EDS) or protons (PIXE). This beam excites a range of different elements (depending on
the energy of the incident X-rays, electrons, or protons) which are detected and quantified by a detector to determine elemental concentrations in the specimen. The movement of the specimen through the incident beam in x-y creates a raster map in which each point represents a pixel with concentration data (or relative element intensity) for a range of elements. High-energy X-rays (≥ 15 keV) have a great penetrative power, and will pass through plant specimens (both sectioned tissues, and potentially even through entire, intact plant tissues), whereas electrons and protons will only penetrate 5–50 µm into a specimen. In principle, the incident X-ray beam does not destroy the sample, hence the method is typically considered ‘non-destructive’. However, as X-rays are ionizing radiation, and hence depending on the energy and dwell on the sample, damage might occur due to the formation of free radicals which are highly reactive and damaging to the tissue being analyzed. Furthermore, the incident X-ray beams do not generate heat in the specimen in contrast to electron and proton beams, which consist of particles and have a far greater potential to damage the specimen during scanning. Obtaining sufficient element sensitivity while keeping dwell low enough not to cause beam-induced damaged can be challenging in PIXE (Laird et al., 2019).

SEM- and TEM-based EDS

Using SEM- and TEM-based EDS (Figure 1 and Figure 2), samples are scanned using an incident electron beam in order to produce the characteristic fluorescent X-rays. These approaches are the most commonly used methods for examining elemental distribution in plant tissues.

Given that most SEM- and TEM-based EDS systems operate under a high vacuum, the plant tissue specimen must be totally dehydrated (and coated with carbon to make it conductive for electrons) prior to analysis (Figure 2). However, where a cryo-SEM is available, it is possible to examine frozen plant tissue specimens in the hydrated state. Appropriate specimen preparation for cryo-SEM and cryotransfer remains extremely challenging technically, and detection limits are poorer than for dehydrated specimens. In addition, it is also increasingly possible to analyze living plants using environmental SEM (ESEMs), although there are issues with sample size restrictions and beam damage (Danilatos, 1981; McGregor and Donald, 2010). In the majority of studies, specimen dehydration (typically by freeze-drying or lyophilization) is required, and this has the potential to cause artefacts due to elemental redistribution (see van der Ent et al. (2018b) for a full discussion of considerations).
When imaging a specimen, a SEM can typically achieve a resolution of ca. 1-50 nm. However, when examining elemental composition using SEM-based EDS, the resolution is considerably poorer due to the interaction of the electrons with the sample, typically being in the order of 2-5 µm and worsening with increasing accelerating voltage. For TEM-based EDS, the resolution is better than for SEM-based EDS because the use of TEM requires the plant tissues to be cut as ultrathin sections (ca. 60-100 nm in thickness), thereby greatly reducing problems associated with the depth of penetration. Thus, for TEM-based EDS, it is possible to achieve a resolution of ca. 100 nm.

A comparatively large range of elements can be detected use SEM- and TEM-based EDS, typically B to U when examining across the K-, L-, and M-edges. However, the detection limit of the technique is rather poor, generally 0.1-1 wt% for most elements, which severely limits its field of application for plant-based studies. As indicated earlier, the analysis is surface-sensitive, given that the electron beam penetrates only a few microns into the sample (SEM) or because ultrathin sections are used (TEM). Accurate quantification for SEM is extremely difficult, and often not attempted, as the method is highly sensitive to sample-specific characteristic (bulk composition, density, and so forth) and hence calibration standards during the analysis are essential (Tylko et al., 2010). However, no commercial ‘biological standards’ for SEM have yet been developed.

It is clear that SEM- and TEM-based EDS are useful approaches where concentrations of the element of interest are high and where the risk of elemental redistribution upon sample dehydration is low. However, their overall usefulness for visualizing elements in plants is comparatively low except for hyperaccumulators or plants where elemental concentrations are high (Figure 1 and Figure 2). Some recent examples of studies using SEM- and TEM-EDS include for analyses of contaminants on surfaces and in longitudinal sections of leaves of *Tilia cordata* (Mantovani et al., 2018), the distribution of Cd in root cross sections of *Taraxacum ohwianum* (Cheng et al., 2019), the distribution of Ni and Co in leaves of *Glochidion cf. sericeum* (van der Ent et al., 2018a), and the distribution of CuO nanoparticles in the xylem of maize (Wang et al., 2012).

*Synchrotron-based X-ray fluorescence microscopy*

X-ray fluorescence microscopy can be either synchrotron-based or laboratory-based (Figure 1, Figure 3, and Figure 4), with these systems having several important differences. Both synchrotron-based and laboratory-based systems use X-rays for the incident beam in order to
produce fluorescent X-rays for elemental mapping. Here, we first focus on synchrotron-based
XFM.

There are currently approximately 50 synchrotrons in the world, although not all have XFM
beamlines. The most frequently used beamlines for plant analyses include (but are not limited
to) the XFM beamline at the Australian Synchrotron (Australia) (for example, see Kopittke et
al. (2018)), 13IDE at the Advanced Photon Source (USA) (for example, see Doolette et al.
(2018)), the XFM beamline and the hard X-ray nanoprobe beamline at Brookhaven National
Laboratory (USA) (for example, see Li et al. (2019b)), and ID21 at the European Synchrotron
Radiation Facility (France) (for example, see Pradas del Real et al. (2017)).

Generally, XFM is conducted at ambient temperature and pressure with no theoretical
restrictions on sample size. As a result, plants can be examined hydrated, and even in vivo
analyses are possible if the entire plant can be mounted in front of the X-ray beam (Blamey et
al., 2018b; Doolette et al., 2018). Nevertheless, it is imperative that care is taken to ensure that
the incident X-ray beam does not result in artefacts in the specimen during analysis, which
can cause localized structural damage and redistribution of elements. This is particularly
important when examining hydrated samples, with these being especially sensitive to
radiation damage. Unfortunately, few studies state whether they have explicitly determined
whether sample damage occurs and whether there is a concomitant redistribution of elements
(Jones et al., 2020).

For XFM, given the penetrating nature of the X-rays (both the incident X-rays as well as the
fluorescent X-rays), elemental distribution can often be examined throughout the entire
thickness of plant tissues. However, the depth of analysis varies greatly depending upon the
element of interest, being determined by the energy of the corresponding fluorescent X-rays
(with these having a lower energy than the incident X-rays). This is best illustrated with the
following examples. For Ca, with a K-edge emission line of 3.7 keV, 50 % of the
fluorescence will be absorbed in a plant sample ca. 70 µm thick and 90 % in a sample ca. 200
µm thick. In contrast, for Se, with a K-edge emission line of 11.2 keV, 50 % of the
fluorescence will be absorbed in a sample ca. 2000 µm thick and 90% in a sample ca. 6500
µm thick. In other words, assuming a root with a thickness of 1000 µm, only the Ca in the
surface 100-200 µm can be detected (with the Ca in the vascular tissue being ‘invisible’)
while Se will be detected across the entire depth of the root cylinder. Thus, great care needs to
be taken when comparing the distribution of various elements, especially in thicker samples.
Most synchrotron-based XFM facilities tend to have a resolution in the order of 20 nm to 1 \( \mu \text{m} \) (Li et al., 2019b). The time required to conduct analyses also varies greatly. For synchrotron-based systems with fast detector systems, the dwell is now routinely \( \leq 1 \) ms, meaning that a 1-megapixel image can be collected in ca. \( \leq 17 \) min. The elements which can be examined depend upon a wide range of factors. Often, elements can be accessed from P (2.1 keV) to Ag (25 keV), while higher Z elements can potentially be examined using the L-edges. The detection limit varies widely depending upon the facility as well as the element being analysed. For elements such as Mn, Fe, and Zn, the detection limit is excellent, being in the order of ca. 1 mg/kg or even lower. However, the detection limit decreases for the lower Z elements, including P, S, and K, often being ca. 10-1000 mg/kg, which is a function of the smaller X-ray cross-sections (resulting in lower fluorescence yields) and operation of most XFM beamline in air which absorbs low-energy X-rays. For synchrotron-based XFM, analyses are potentially fully quantitative, for example using the GeoPIXE software package which produces quantitative self-absorption corrected maps which are line overlap-resolved and in which the background is subtracted (Ryan, 2000).

Given that XFM allows analyses of plants \textit{in vivo} with no (or minimal) sample preparation and with good sensitivity (Figure 3), this technique is being used to examine an increasing number of diverse problems. This includes studies where it is imperative to avoid sample processing (such as freeze drying) or where repeated measurements of living plants are required \textit{in vivo}. It is especially useful for studies focusing on trace metals and metalloids, such as Mn, Zn, Fe, Cu, As, and Se, and to a lesser extent the macronutrients P, S, K, and Ca (Figure 3). Some recent examples of studies using XFM include analyses of the METAL TOLERANCE PROTEIN8 (MTP8) (Chu et al., 2017; Eroglu et al., 2017; Basiri-Esfahani et al., 2019), kinetic analyses of living leaves of cowpea (\textit{Vigna unguiculata}) exposed to toxic levels of Mn (Blamey et al., 2018b), analyses of Zn movement following foliar fertilization in wheat (Doolette et al., 2018), and analyses of nanoparticles in plants (Martinez-Criado et al., 2016). A potential new, and yet unexplored use, is for high-throughput screening of plant mutant libraries. This is especially exciting given the non-destructive nature of XFM analyses as well as the ability to examine changes in the spatial distribution of elements instead of examining only bulk concentrations (Figure 3). Finally, another potential advantage of XFM analyses is the potential to combine imaging with speciation through the use of X-ray absorption near edge structure (XANES) spectroscopy (Kopittke et al., 2018), although this is
beyond the scope of the present review [see Wang et al. (2015) for an example for examining the distribution and speciation of Se in rice and wheat tissues].

Laboratory-based X-ray fluorescence microscopy

The strengths of XFM are evident from the research output from studies undertaken at synchrotrons. However, the restrictive nature of access to synchrotron XFM facilities is recognised by many users as a limiting factor in using XFM in their research, and where laboratory-based facilities are available, this can overcome that limitation. However, we are not aware of many laboratory-based XFM systems, with this being a current restriction for their use. The authors are aware of systems used for the investigation of plants at microXRF facility at The University of Queensland (UQ, Australia) (Figure 4), Washington State University (USA) (Fittschen et al., 2017), and the Maia Mapper at the Advanced Resource Characterisation Facility of CSIRO in Australia (Ryan et al., 2018). The latter is perhaps the most advanced laboratory XFM system as it can image up to \(\sim 80\) M pixels over a \(500 \times 150\) mm\(^2\) sample area using the Maia detector array. However, it has been developed for drill core sections and polished rock slabs, not biological applications. The UQ microXRF facility has been specifically developed for biological applications, and has dual microfocus sources (focussing to 5 and 25 \(\mu\)m), two large silicon drift detectors (SDD) of 150 mm\(^2\) and can scan areas up to \(300 \times 300\) mm in air, vacuum or helium atmosphere. It also has a cryo-stage (50 \(\times\) 50 mm active area held at -50°C) to analysis of samples in frozen-hydrated state.

These new laboratory-based XFM facilities do not fully replace synchrotron-based XFM for a number of specialised applications, but will bridge the gap between what is currently possible in the laboratory environment and the capability of synchrotron facilities. Furthermore, it allows researchers to combine the strengths of both facilities, for example by whole organism mapping at their laboratory followed by investigation of target cells at the synchrotrons and hence strengthen the outcome of both platforms.

Laboratory-based XFM systems essentially offer unlimited access (within the institutional constraints of availability and financial considerations) as required by experimental needs. In addition, many laboratory-based systems provide vacuum and helium purge capabilities that might not be available at synchrotron-based beamlines, thereby offering improved capability for measurement of light elements such as Al, Si, S, and P.
However, these laboratory-based systems offer a worse spatial resolution, often in the range of 5-50 µm (compared to 20 nm to 1 µm for synchrotron-based systems). In addition, laboratory-based systems typically use a concave focussed polychromatic X-ray source with Bremsstrahlung background, with this having important differences to a monochromatic, highly parallel X-ray source in synchrotron-based XFM. For example, there is no energy tunability in laboratory-based systems, and hence X-ray absorption spectroscopy not possible. Finally, the substantially lower X-ray flux for laboratory-based systems (typically 1000- to 10,000-times less bright) results in longer dwell times (50 to 100 ms per pixel) compared to synchrotron-based systems (0.5 to 5 ms per pixel).

**microPIXE**

With microPIXE (Figure 1 and Figure 5A), an ion beam of protons is used as the incident beam, generating fluorescent X-rays in the sample. For microPIXE, the resolution is generally in the range of 1-3 µm, or occasionally slightly better. PIXE excites the K-lines of virtually all elements, and hence it is generally possible to measure elements in the range of 1.5–60 keV (corresponding to Al to W), with this being considerably wider than that achieved using XFM. Moreover, very light elements in the range of 0.05-1.3 keV (corresponding to Li to Mg) can be analysed with particle-induced gamma-ray emission (PIGE). As such, PIXE opens up imaging of metals such as Ag and Cd and the metalloids Sb, Te and I which are very difficult to measure with synchrotron XFM. The detection limit using microPIXE is excellent, typically in the range of lower mg/kg, with analyses also generally being fully quantitative with the use of techniques (such as Rutherford backscattering spectrometry, RBS) to determine sample matrix composition. The new PIXE Maia facility at the University of Melbourne in Australia combines the benefits of PIXE with those of the revolutionary Maia detector array able to process 4–9 M events/s (measured on a plant specimen) (Laird et al., 2019) or up to ~900 times greater than that typically used in the previous single detector system (Laird et al., 2013).

Based on this discussion, it is clear that microPIXE is useful for examining elements in a wide range of systems. Given that it can analyze a wider range of elements than many other approaches (such as XFM), it is especially valuable for examining light elements (such as Na, Mg, or Al) as well as heavier elements (such as Cd or the rare earth elements, as shown in Figure 5A) which often cannot be analyzed with other approaches, all with a good detection limit (Figure 1 and Figure 5A). Some recent studies using microPIXE include for the analysis...
Mass spectrometry-based approaches for visualization

For mass spectrometry-based approaches, small portions of the sample are progressively removed during scanning for analysis. Because analysis is by mass spectrometry, not only are these generally highly sensitive techniques, but they also allow isotopic analyses. However, given that small portions of the sample are removed for analysis during scanning, these mass spectrometry based approaches are considered destructive.

LA-ICP-MS

LA-ICP-MS uses a focused laser to ablate the surface of the sample (Figure 1 and Figure 5B,C). These ablated particles are then transported to an ICP-MS in a stream of He-gas, for both elemental and isotopic analysis.

LA-ICP-MS offers excellent detection limits (< 1 mg/kg) and a very wide range of elements (Li-U). Furthermore, multi-element analyses are routine, and stable isotope analyses are also possible as illustrated in Figure 5C. In addition, LA-ICP-MS offers a modest resolution (ca. 1 µm), being similar to microPIXE and SEM-based EDS. A wide range of elements can be examined using LA-ICP-MS, from Li to U. Sensitivity is greater than for X-ray based approaches, with the detection limit being sub-mg/kg for many physiologically-relevant elements (Persson et al., 2016a). Analyses are potentially fully quantitative, although this is not without substantial difficulties. Specifically, the laser beam interaction with the sample will vary according to the sample properties, resulting in changes in the amount of analyte (sample) removed per pulse. For example, portions of the plant tissues which are heavily lignified will ablate less material than softer parts of the plant tissue, making full quantification challenging. Thus, analyses of plant tissues are generally considered to be semi-quantitative.

Although LA-ICP-MS analyses are generally conducted at ambient temperature and pressure in an inert argon atmosphere, extreme care must be taken to avoid sample desiccation due to the exposure of samples to the dry stream of argon gas when examining hydrated tissues, with this leading to experimental artefacts. As a result, most analyses utilizing LA-ICP-MS examine dehydrated samples and hence in vivo analyses are challenging. Nevertheless, it is indeed possible to analyze fresh (hydrated) tissues and living plants in some situations (Salt et
al., 2008; Klug et al., 2011), and protocols for sample preparations which produce intact, dry samples with unaltered ion distribution within tissue are available (Persson et al., 2016a).

LA-ICP-MS is therefore particularly useful in studies where high sensitivity is required with access to an extremely broad range of elements with good sensitivity (Figure 1 and Figure 5B,C). It is a surface-sensitive technique, which will offer advantages compared to XFM or microPIXE in some situations, but will be a disadvantage in others. The use of isotopic analyses is also a potentially useful advantage for examining the flux and distribution of exogenously applied isotopes. Recent studies to use LA-ICP-MS for the study of plants include for the imaging of Fe, Zn, and Mn in roots of Arabidopsis (Persson et al., 2016a), Mn in roots and grain of barley (Hordeum vulgare) (Long et al., 2018; Chen et al., 2019), Ca, Na, and K in stems and leaves of tobacco (Nicotiana tabacum) (Thyssen et al., 2017), Zn, S and P analyses of biofortified wheat grains (Persson et al., 2016b), Cd, Pb, Cu and Zn in roots of pea (Pisum sativum) (Hanč et al., 2016), for mapping the distribution of pollutants in leaves of sweet basil (Ocimum basilicum) (Ko et al., 2018), and for examining nutrient distribution in nodes of rice mutants (Yamaji and Ma, 2019).

**NanoSIMS**

In NanoSIMS (Figure 1 and Figure 6), ions are used as the incident (primary) beam, these ions collide with the sample surface and cause atoms, ions, and molecules from the sample surface to be ejected into the vacuum (sputtering). The ionized particles (secondary ions) are then collected and transported to a mass spectrometer for analysis. For NanoSIMS, the sputtering depth is ca. 5-20 nm (Hoppe et al., 2013) making it a very surface-sensitive technique.

Currently, there are considerably fewer NanoSIMS facilities in the world than there are synchrotrons. As a result, accessing a NanoSIMS for an experiment can potentially be difficult for many researchers. Nevertheless, the facilities most commonly used for investigations of plant tissues include those at the University of Manchester (England) and the University of Western Australia (Australia).

NanoSIMS operates in an ultra-high vacuum, meaning that samples must first be dehydrated before analysis. Furthermore, NanoSIMS requires a flat surface, and hence it is typically only possible to examine sectioned tissues (Figure 6). As for other techniques where sample dehydration in required prior to analysis, extreme care must be taken to ensure that the
method used for sample processing does not cause experimental artefacts through redistribution of the elements of interest.

NanoSIMS offers an excellent lateral resolution, with analyses routinely conducted at resolutions as low as 100 nm (Figure 6). Using this technique, it is possible to analyse a very wide range of elements of relevance to plant studies, from H to U. The sensitivity is also very good, with the detection limit being low mg/kg range. The sensitivity for any given element depends upon the primary beam selected, with either an O\(^{-}\) beam or Cs\(^{+}\) beam available. The negatively charged primary beam (i.e. O\(^{-}\)) tends to favour the production of positively charged secondary ions, while the positively charged primary beam (i.e. Cs\(^{+}\)) tends to favour the production of negatively charged secondary ions. As a result, for elements such as Na, Mg, Al, K, Ca, Mn, Fe, and Zn, the O\(^{-}\) beam is generally preferred (Nuñez et al., 2017). In contrast, for elements such as Si, P, S, Cl, As, and Se, the Cs\(^{+}\) beam is generally preferred.

The main advantages of NanoSIMS are the excellent detection limit and spatial resolution, as well as the wide range of elements that can be analyzed. As a result, this approach is particularly suited to examining the sub-cellular distribution of elements within cross-sections of plant tissues (Figure 1 and Figure 6). Isotopic analyses are also possible using this approach [for an example in plants, see Moore et al. (2016)]. Some recent studies utilizing NanoSIMS include the study of Fe with amyloplasts in pea seeds (Moore et al., 2018), detoxification of Mn by Si in leaves of soybean and sunflower (Helianthus annuus) (Blamey et al., 2018a), determining the mechanisms by which foliar-applied Zn fertilizer moves across the leaf surface (Li et al., 2019a), as well as examining the distribution of Al in roots of soybean (Kopittke et al., 2015) with the mass of Al being too low to examine using XFM (Figure 6).

**Autoradiography**

Autoradiography (Figure 1 and Figure 7A) is the oldest of the techniques discussed here, having been used for plants since the 1920s (Hevesy, 1923). In autoradiography, radioactive isotopes are supplied to a plant, which are taken up and redistributed throughout the plant tissues. To then examine their distribution in the plant, an image is obtained of the decay emissions from the various plant tissues (Figure 7A). These decay emissions can be detected using an X-ray film, or more recently, using digital autoradiography.
Compared to some other approaches, such as XFM or NanoSIMS, access to autoradiography facilities is likely not too difficult. This approach also has a range of other advantages, including being able to examine hydrated plant tissues, including for *in vivo* analyses. Furthermore, autoradiography can be used to examine large samples, or even entire plants. Another major advantage is the ability to separate background isotopes of an element (i.e. those natively present in the plant tissues) from the radio-isotope of the same element added exogenously. The resolution achievable with autoradiography varies between ca. 25-1000 µm (Figure 1) and depends upon a range of factors (Zhang et al., 2008).

The greatest challenge for using autoradiography are the highly restrictive and complicated health and safety regulations in many jurisdictions for working with radio-isotopes. In addition to this limitation, it is only possible to examine a single element at a time (Solon et al., 2010). Furthermore, it is only possible to use this technique for elements where a suitable radioisotope exists. For studies of plants, these elements include $^{26}$Al, $^{32}$P, $^{33}$P, $^{35}$S, $^{45}$Ca, $^{51}$Cr, $^{54}$Mn, $^{55}$Fe, $^{59}$Ni, $^{67}$Cu, $^{65}$Zn, $^{73}$As, and $^{109}$Cd (Kanno et al., 2012).

The main uses of autoradiography are for *in vivo* studies or studies in which sample processing needs to be avoided. It also offers excellent detection limits and allows separation of background elements from those added exogenously as radioisotopes – this being critical when only a portion of the total element is of interest (Figure 1 and Figure 7A). Recent studies to use autoradiography to examine elemental distribution in plants include the study of $^{65}$Zn applied as foliar fertilizers in wheat (Read et al., 2019) and tracking $^{64}$Cu-labelled nanoparticles in *Lactuca sativa* (Davis et al., 2017).

**Laser confocal microscopy with fluorophores**

The final approach considered here is the use of laser confocal microscopy with element-selective fluorophores (Figure 1 and Figure 7B).

Given that many researchers are likely able to access laser confocal microscopy without substantial difficulty, this approach is one of the easier ones in terms of facility access, as most research institution will have laser confocal microscopes, especially in medical faculties where they are routinely used. This approach is also non-destructive, and can also be used on hydrated samples, including for *in vivo* analyses of living plants. The maximum resolution is similar to some other approaches, being ca. 1 µm.
All the approaches considered above have analyzed elemental composition directly. However, laser confocal microscopy relies on the binding of ion-selective fluorophores to the element of interest for their subsequent detection using excitation by specific wavelengths emitted by lasers (Figure 7B). This in itself represents a potential limitation of this technique, as fluorophores will generally only bind to free ions not already bound strongly to other plant ligands in the plant. Thus, the proportion of the total pool identified using the fluorophore can be uncertain. In addition, issues with penetration into the plant tissue are largely unknown and are hard to quantify. The range of elements that can be investigated using laser confocal microscopy is entirely dependent on the commercial availability of fluorophores with specific affinities for elements of interest. These include, but are not limited to: Zn (Zinpyr-1, FluoZin, TSQ), Ni/Co (Newport Green), Cu (Phen Green) and Pb/Cd (Leadmium Green).

The use of laser confocal microscopy with element-selective fluorophores is particularly suited to cases where in vivo analyses are required for an element where a suitable fluorophore exists, with an excellent detection limit (Figure 1 and Figure 7B). However, questions still remain regarding the binding of the fluorophores and their penetration into the plant tissue. Recent studies using confocal microscopy with fluorophores include imaging the distribution Ni$^{2+}$ with the dye Newport Green in Alyssum murale (Agrawal et al., 2013) and A. lesbiacum (Ingle et al., 2008), the use of Zinpyr-1 for imaging the distribution of Zn$^{2+}$ Noccaea caerulescens (Kozhevnikova et al., 2017; Dinh et al., 2018) and in arabidopsis (Sinclair et al., 2007), and Leadmium Green for imaging Zn$^{2+}$ and Cd$^{2+}$ in Sedum alfredii and Picris divaricata (Lu et al., 2008; Hu et al., 2012).

**Concluding remarks**

Understanding the distribution of elements within plant tissues is critical for a range of research programs within plant science, including for functional characterization in molecular biology, improving plant nutrition and productivity, improving human nutrition, and understanding toxic elements in plants and tolerance mechanisms. For analyzing plants, a range of techniques are suitable but it can often be confusing as to which approach is best given their range of advantages and limitations. It is clear from this review that there is no single technique which is best. Rather, each of the techniques have their own strengths and weaknesses. By comparing the accessibility, ability to analyze hydrated tissues (without sample preparation) and conduct in vivo analyses, as well as comparing the resolution, sensitivity, depth of analysis, and the range of elements that can be analyzed for seven different approaches, we hope that this information will assist other researchers to select and
access the approach that is most useful for use in their particular research program. In addition, it will help with the use of correlative approaches in which the same sample is examined with multiple techniques to exploit the advantages listed here. Of central importance in the future will be the analyses of living plants (including \textit{in vivo} analyses) with minimal sample preparation at excellent resolution and with good detection limits across the wide range of physiologically-relevant elements – this requiring a strong correlative approach. The use of such correlative approaches will enable important research questions to be answered within the field of plant science.

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Advances box

- A wide range of approaches can be used to visualize the distribution of elements within plants, including SEM-EDS, XFM, microPIXE, LA-ICP-MS, NanoSIMS, autoradiography, and confocal microscopy with fluorophores.
- There have been rapid advances in these various techniques in recent years, with marked differences in accessibility, resolution, sensitivity, depth of analysis, and sample preparation requirements.
- Differences in the strengths and weaknesses of these approaches often makes it difficult for researchers to select the most appropriate method for their particular research program.
- These approaches are already allowing a diverse range of experiments to be conducted in situ, including in living plants, to examine functional characterization in molecular plant biology, to improve the nutrition and plants (and humans), and for understanding the behaviour of toxic elements.

Outstanding Questions box

- Will recent advances in techniques allowing the visualizing of elements in plants (including in living plants) provide new opportunities in research fields not yet realized?
- Given that comparatively few researchers have used elemental imaging approaches to examine functional characterization in molecular plant biology (for example, gene × environment interactions), can ongoing improvements in elemental imaging approaches be used to encourage such studies?
- Can correlative approaches, in which the same sample is examined with multiple techniques, be used to extend our knowledge by allowing exploitation of the advantages of those multiple techniques?
Figure 1. Comparison of seven broad techniques used for examining element distribution in plants. All values are indicative of typical systems.

Figure 2. Freeze-dried cross-section of a root of Conyza cordata examined using scanning electron microscopy-based energy-dispersive X-ray spectroscopy (SEM-EDS) showing elemental distribution maps of O, S, K, Ca, and Cl. The images were obtained with an incident beam of 15 kV. The specimen was prepared by Jolanta Mesjasz-Przybyłowicz.

Figure 3. Use of synchrotron-based X-ray fluorescence microscopy (XFM, Australian Synchrotron) for high-throughput screening of plant mutant libraries for Arabidopsis. The image in (A) is an optical micrograph. The images show the distribution of (B) Fe, (C) Mn, (D) Zn, and (E) Se in ca. 6000 seeds, with each image having a resolution of ca. 20 megapixels when displayed at full resolution. The image in (F) shows a small portion of a detailed scan for Fe showing some seeds differing in their Fe concentration and distribution. The ‘overview scans’ (B-E) had 10 µm pixel size with a dwell of 1 ms per pixel, while the detailed scans [a small portion shown in (F)] had 1 µm pixel size with a dwell of 7 ms per pixel. In total, an estimated 40,000 seeds were examined, with only ca. 6000 seeds shown here. Note that the analyses are non-destructive.

Figure 4. Analysis of a fresh hydrated shoot the selenium (Se) hyperaccumulator Neptunia amplexicaulis, using laboratory-based X-ray fluorescence microscopy (XFM) at The University of Queensland (Australia). Images show elemental maps of K, Ca and Se distribution, and map of sum of all X-rays (useful for observing structure of the sample).

Figure 5. (A) Particle-induced X-ray emission (PIXE) elemental map of an intact Noccaea caerulescens seed (pixel size 2 µm, dwell 5 ms per pixel). The Southern France accessions (St Laurent de Minier/Ganges) has the ability to hyperaccumulate Cd with up to 900 mg/kg Cd in the seeds. (B) Stele of a mature barley root examined using laser ablation inductively coupled plasma mass spectrometry [193 nm excimer laser, Analyte G2 (Teledyne Photon Machines, Bozeman, MT, USA) equipped with a Cobalt cell (Teledyne Photon Machines, Bozeman, MT, USA), with a pixel size of 2 µm] showing 24Mg distribution. (C) LA-ICP-MS images from the inner tissues of a mature barley root with a pixel size of 5 µm, showing 24Mg, 67Zn, 66Zn, and a light micrograph (the red square indicating the area analyzed using LA-ICP-MS). The root was first starved for Zn, then exposed to 67Zn stable isotope (94.3% enriched) for 2 h. The natural 66Zn/67Zn isotopic ratio is 6.8 (66Zn: 27.9%, 67Zn: 4.1 %, 66Zn/67Zn = 6.8). The
67Zn image shows how the Zn (added as 67Zn) is taken up and transported radially towards the stele.

**Figure 6.** Nanoscale secondary ion mass spectroscopy (NanoSIMS) analyses of a portion of a transverse cross section of soybean root exposed to 30 µM Al for 0.5 h with a RF plasma O source. The images for Al (left) and Na (right) were obtained with a pixel size of 0.3 µm and a dwell of 60 ms per pixel. For more information on plant growth and analyses, see Kopittke et al. (2015).

**Figure 7.** (A) Autoradiography on treated leaves of wheat to which 65Zn labelled foliar fertilizers had been applied as 65ZnCl₂, 65ZnEDTA, 65ZnO-NPs (nanoparticles), and 65ZnO-MPs (microparticles) (750 mg/L). The digital photograph (left) shows the leaves to which the Zn was applied. A total of 10 droplets were applied to each leaf before being washed from the leaf surface. For more information, refer to Read et al. (2019). (B) Zinpyr-1 (fluorescent indicator for Zn²⁺, green color) stained and auto-fluorescence of a leaf section of *Noccaea caerulescens* obtained using confocal fluorescence microscopy.
**Figure 1.** Comparison of seven broad techniques used for examining element distribution in plants. All values are indicative. The color of the shading indicates a ranking: green is a potential advantage of the technique, red is a potential disadvantage, orange is neither an advantage nor disadvantage, and white is not ranked.

| Accessibility       | SEM- and TEM-based EDS | XFM                  | microPIXE | LA-ICP-MS | NanoSIMS | Autoradiography | Confocal |
|---------------------|------------------------|----------------------|------------|-----------|-----------|-----------------|----------|
|                     | Easily accessible      | Generally difficult  | Generally difficult | Often accessible | Generally difficult | Often accessible | Easily accessible |
| Sample preparation  | Generally dehydrated   | Generally nil (living) | Generally dehydrated | Generally dehydrated | Generally dehydrated | Generally nil (living) | Generally nil (living) |
| In vivo analyses?   | Typically no           | Yes                  | Typically no | Typically no | No        | Yes             | Yes      |
| Non-destructive?    | Yes                    | Yes                  | No         | No        | No        | Yes             | Yes      |
| Maximum resolution (µm) | 2 (SEM) 0.1 (TEM)     | 0.015 (synch.) 5 (lab.) | 1          | 1         | 0.05      | 25-1000        | 1        |
| Scan speed (ms/pixel) | 1-100                  | 1 (synch.) 100 (lab.) | 1-5        | 5-250     | 200       | Full field      | Full field |
| Detection limit (mg/kg) | 1000                  | 100-0.1              | 1-10       | < 1       | 1         | N/A²            | < 1      |
| Elements            | B-U                    | P-Ag (synch.) Al-Mo (lab.) | Li-U      | Li-U      | H-U       | Suitable radioisotopes³ | Suitable fluorophores⁴ |
| Multi-element analyses? | Yes                    | Yes                  | Yes         | Yes       | Yes       | No              | Yes      |
| Stable isotope analyses? | No                     | No                   | Yes         | Yes       | Yes       | No              | Yes      |
| Analysis depth (µm) | 0.1-2 (SEM) Section (TEM) | 10-10,000          | 50-150     | 1-50⁵     | < 0.1     | 10-1000        | Light penetration |

¹ Scanning/transmission electron microscopy-based energy-dispersive X-ray spectroscopy (SEM-EDS, TEM-EDS), X-ray fluorescence microscopy (XFM), particle-induced X-ray emission (microPIXE), laser ablation ICP-MS (LA-ICP-MS), nanoscale secondary ion mass spectroscopy (NanoSIMS)

² Depends upon the activity and the integration time.

³ Only possible where a suitable radioisotope exists, which includes²⁶Al, ³²P, ³³P, ³⁵S, ⁴⁵Ca, ⁵¹Cr, ⁵⁴Mn, ⁵⁵Fe, ⁵⁹Ni, ⁶⁷Cu, ⁶⁵Zn, ⁷³As, and ¹⁰⁹Cd.

⁴ Depends upon the availability of fluorophores with specific affinities for elements of interest.
5 Depends upon energy.
**Figure 2.** Freeze-dried cross-section of a root of *Conyza cordata* examined using scanning electron microscopy-based energy-dispersive X-ray spectroscopy (SEM-EDS) showing elemental distribution maps of O, S, K, Ca, and Cl. The images were obtained with an incident beam of 15 kV. The specimen was prepared by Jolanta Mesjasz-Przybyłowicz.
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The natural $^{66}\text{Zn}/^{67}\text{Zn}$ isotopic ratio is 6.8 ($^{66}\text{Zn}: 27.9\%$, $^{67}\text{Zn}: 4.1\%$, $^{66}\text{Zn}/^{67}\text{Zn} = 6.8$). The $^{67}\text{Zn}$ image shows how the Zn (added as $^{67}\text{Zn}$) is taken up and transported radially towards the stele.
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A wide range of approaches can be used to visualize the distribution of elements within plants, including SEM-EDS, XFM, microPIXE, LA-ICP-MS, NanoSIMS, autoradiography, and confocal microscopy with fluorophores.

There have been rapid advances in these various techniques in recent years, with marked differences in accessibility, resolution, sensitivity, depth of analysis, and sample preparation requirements.

Differences in the strengths and weaknesses of these approaches often makes it difficult for researchers to select the most appropriate method for their particular research program.

These approaches are already allowing a diverse range of experiments to be conducted in situ, including in living plants, to examine functional characterization in molecular plant biology, to improve the nutrition and plants (and humans), and for understanding the behaviour of toxic elements.

Outstanding Questions box

Will recent advances in techniques allowing the visualizing of elements in plants (including in living plants) provide new opportunities in research fields not yet realized?

Given that comparatively few researchers have used elemental imaging approaches to examine functional characterization in molecular plant biology (for example, gene × environment interactions), can ongoing improvements in elemental imaging approaches be used to encourage such studies?

Can correlative approaches, in which the same sample is examined with multiple techniques, be used to extend our knowledge by allowing exploitation of the advantages of those multiple techniques?