Video Article

Measuring Fluxes of Mineral Nutrients and Toxicants in Plants with Radioactive Tracers

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Abstract

Unidirectional influx and efflux of nutrients and toxicants, and their resultant net fluxes, are central to the nutrition and toxicology of plants. Radioisotope tracing is a major technique used to measure such fluxes, both within plants, and between plants and their environments. Flux data obtained with radiotracer protocols can help elucidate the capacity, mechanism, regulation, and energetics of transport systems for specific mineral nutrients or toxicants, and can provide insight into compartmentation and turnover rates of subcellular mineral and metabolite pools. Here, we describe two major radioisotope protocols used in plant biology: direct influx (DI) and compartmental analysis by tracer efflux (CATE). We focus on flux measurement of potassium (K^+) as a nutrient, and ammonia/ammonium (NH_3/NH_4^+) as a toxicant, in intact seedlings of the model species barley (*Hordeum vulgare* L.). These protocols can be readily adapted to other experimental systems (e.g., different species, excised plant material, and other nutrients/toxicants). Advantages and limitations of these protocols are discussed.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51877/>

Introduction

The uptake and distribution of nutrients and toxicants strongly influence plant growth. Accordingly, the investigation of underlying transport processes constitutes a major area of research in plant biology and agricultural sciences^{1,2}, especially in the contexts of nutritional optimization and environmental stresses (e.g., salt stress, ammonium toxicity). Chief among methods for the measurement of fluxes in plants is the use of radioisotopic tracers, which was developed significantly in the 1950s (see e.g.,³) and continues to be widely used today. Other methods, such as measurement of nutrient depletion from the root medium and/or accumulation in tissues, use of ion-selective vibrating microelectrodes such as MIFE (microelectrode ion flux estimation) and SIET (scanning ion-selective electrode technique), and use of ion-selective fluorescent dyes, are also widely applied, but are limited in their ability to detect net fluxes (i.e., the difference between influx and efflux). The use of radioisotopes, on the other hand, allows the researcher the unique ability to isolate and quantify unidirectional fluxes, which can be used to resolve kinetic parameters (e.g., K_M and V_{max}), and provide insight into the capacity, energetics, mechanisms, and regulation, of transport systems. Unidirectional flux measurements made with radiotracers are particularly useful under conditions where the flux in the opposite direction is high, and the turnover of intracellular pools is rapid⁴. Moreover, radiotracer methods allow measurements to be conducted under fairly high substrate concentrations, unlike many other techniques (see 'Discussion', below), because the traced isotope is observed against a background of another isotope of the same element.

Here, we provide detailed steps for the radioisotopic measurement of unidirectional and net fluxes of mineral nutrients and toxicants in intact plants. Emphasis will be made on flux measurement of potassium (K^+), a plant macronutrient⁵, and ammonia/ammonium (NH_3/NH_4^+), another macronutrient which is, however, toxic when present at high concentrations (e.g., 1-10 mM)². We will use the radioisotopes $^{42}K^+$ ($t_{1/2}$ = 12.36 hr) and $^{13}NH_3/^{13}NH_4^+$ ($t_{1/2}$ = 9.98 min), respectively, in intact seedlings of the model system barley (*Hordeum vulgare* L.), in the description of two key protocols: direct influx (DI) and compartmental analysis by tracer efflux (CATE). We should note from the outset that this article simply describes the steps necessary to perform each protocol. Where appropriate, brief explanations of calculations and theory are provided, but detailed expositions of each technique's background and theory can be found in several key articles on the subject^{4,6-9}. Importantly, these protocols are broadly transferable to flux analysis of other nutrients/toxicants (e.g., $^{24}Na^+$, $^{22}Na^+$, $^{86}Rb^+$, $^{13}NO_3^-$) and to other plant species, albeit with a few caveats (see below). We also stress the importance that all researchers working with radioactive materials must work under a license arranged through their institution's ionizing radiation safety regulator.

Protocol

1. Plant Culture and Preparation

1. Grow barley seedlings hydroponically for 7 days in a climate-controlled growth chamber (for details, see ¹⁰).

NOTE: It is important to consider examining plants at a variety of developmental stages, as nutrient requirements will change with age.

- One day prior to experimentation, bundle several seedlings together to make a single replicate (3 plants per bundle for DI, 6 plants per bundle for CATE). Bundle seedlings by wrapping a 2-cm piece of Tygon tubing around the basal portion of the shoots, and securing the tubing with tape to create a "collar".

NOTE: The number of plants per bundle may vary based on experimental conditions^{10,13,14}. Bundling is done to improve statistics and measuring accuracy, particularly when root mass and/or specific activity are low.

2. Preparation of Experimental Solutions/Materials

NOTE: The following is typically performed 1 day prior to experimentation.

- For DI, gather the following: Pre-labeling, labeling, and desorption solutions (for details, see¹¹), centrifugation tubes (for spin-drying of plant samples), and sample vials (for plant material and specific activity [S_o ; see below]). Aerate and mix all solutions.
- For CATE, gather the following: Well-mixed, aerated labeling and elution solutions (for details, see¹⁰), efflux funnels, centrifugation tubes (for spin-drying of plant samples), and sample vials (for eluates, plant samples, and determination of S_o and dilution factor [D_f ; see below]).

3. Prepare Radiotracer

CAUTION: The following safety steps should be taken prior to working with radioactivity.

- Ensure that the requirements of the radioactive materials license are understood and followed. Wear proper safety equipment (*i.e.*, goggles, gloves, lab coat, lead vest/collar) and dosimeters (*e.g.*, TLD ring and badge). Set up shielding (*i.e.*, Plexiglas and lead bricks) and perform radioactive work behind it. Ensure that a Geiger-Müller counter is present in order to routinely monitor for contamination.
- Preparation of $^{42}\text{K}^+$
 - Place a clean, dry beaker on the balance. Zero the balance.
 - Remove vial of tracer (20 mCi of $^{42}\text{K}_2\text{CO}_3$, in powder form) from packaging and pour tracer into the beaker. Take note of the mass.
 - Pipette 19.93 ml of dH_2O , followed by 0.07 ml of H_2SO_4 , into the beaker. This will drive the following chemical reaction:

$$^{42}\text{K}_2\text{CO}_3 (\text{s}) + \text{H}_2\text{SO}_4 (\text{l}) + \text{H}_2\text{O} (\text{l}) \rightarrow ^{42}\text{K}_2\text{SO}_4 (\text{l}) + \text{CO}_2 (\text{v}) + 2\text{H}_2\text{O} (\text{l})$$
 - Calculate the concentration of the radioactive stock solution, given the mass and molecular weight of K_2CO_3 , and the volume (20 ml).
 NOTE: If working with $^{13}\text{NH}_3/^{13}\text{NH}_4^+$ The tracer is produced in a cyclotron via the proton bombardment of the oxygen atom of water (typically resulting in 100-200 mCi activity; for production details, see¹²). Because the quantity of $^{14}\text{NH}_3/^{14}\text{NH}_4^+$ is extremely low in these solutions, the N concentration of the stock solution is negligible.

4. Direct Influx (DI) Measurement

- For $^{42}\text{K}^+$, pipette the amount of radioactive stock solution required to reach the desired final concentration of K^+ into the labeling solution.
 - For $^{13}\text{NH}_3/^{13}\text{NH}_4^+$, pipette a small amount (<0.5 ml) into the labeling solution. Allow the labeling solution to mix thoroughly (via aeration).
- Pipette a 1 ml sub-sample of labeling solution into a sample vial and repeat three times (4 samples in total).
 - Measure radioactivity in vials (in "counts per minute", cpm), using a gamma counter. Ensure that the counter is programmed such that cpm readings are corrected for isotopic decay (this is particularly important for such short-lived tracers).
 - Calculate S_o (expressed as cpm μmol^{-1}) by averaging the counts of the four samples (cpm ml^{-1}) and dividing by the concentration of substrate in solution ($\mu\text{mol} \text{ml}^{-1}$).
- Immerse roots in pre-labeling (non-radioactive) solution for 5 min, to pre-equilibrate plants under test conditions (see *e.g.*,^{10,13,14} for variations in pre-label time).
- Immerse roots in labeling (radioactive) solution for 5 min.
 NOTE: Labeling times can vary based on experiment^{3,4,7-10}.
- Transfer roots to desorption solution for 5 sec to remove the bulk of surface-adhering radioactivity. Transfer roots into a second beaker of desorption solution for 5 min to further clear roots of extracellular tracer.
- Dissect and separate shoots, basal shoots, and roots.
- Place roots in centrifuge tubes and spin samples for 30 sec in a low-speed, clinical-grade centrifuge (~5,000 x g) to remove surface and interstitial water.
- Weigh roots (fresh weight, FW).
- Count radioactivity in plant samples (shoot, basal shoot, and root; see step 4.2.1).
- Calculate the flux. Calculate influx into the plant using the formula

$$\Phi = Q^*/S_o w t_L$$
 where Φ is the flux ($\mu\text{mol} \text{g}^{-1} \text{hr}^{-1}$), Q^* is the quantity of tracer accumulated in tissue (cpm, usually in root, shoot, and basal shoot, combined), S_o is the specific activity of the labeling solution (cpm μmol^{-1}), w is the root fresh weight (g), and t_L is the labeling time (hr).
 NOTE: More sophisticated calculation can be made to account for simultaneous tracer efflux from roots during labeling and desorption, based on parameters obtained from CATE (see below; for details, see⁴).

5. Compartmental Analysis by Tracer Efflux (CATE) Measurement

- Prepare labeling solution and measure S_o (see steps 4.1 - 4.2, above).
- Measure dilution factor (D_f).

NOTE: Often, the position of the sample relative to the detector in the gamma counter can influence the quantity of radiation measured. See discussion for details.

1. After measuring S_0 , add 19 ml of H_2O to each sample (such that final volume = eluate volume = 20 ml). Count radioactivity in each 20-ml sample (see step 4.2.1).
2. Calculate D_f by dividing the average cpm of the 1-ml samples by the average cpm of the 20-ml samples.
3. Immerse roots in labeling solution for 1 hr.
4. Remove plants from labeling solution and transfer plants to efflux funnel, ensuring all root material is within the funnel. Gently secure plants to side of efflux funnel by applying a small strip of tape over the plastic collar.
5. Gently pour the first eluate into the funnel. Start timer (counting up).
6. Open the spigot and collect the eluate in the sample vial after 15 sec (note: elution time will vary; see below). Close the spigot. Gently pour the next eluate into the funnel.
7. Repeat step 5.6 for the remainder of the elution series, which follows, from the first to the final eluate: 15 sec (four times), 20 sec (three times), 30 sec (twice), 40 sec (once), 50 sec (once), 1 min (25 times), for a total elution period of 29.5 min
NOTE: Desorption series can vary based on experimental conditions^{7-10,13,14}.
8. Once elution protocol is complete, harvest plants (steps 4.6 - 4.8, above).
9. Count radioactivity in eluates and plant samples in the gamma counter (multiplying the reading for each eluate by D_f , see 5.2).
10. Plot tracer release (cpm g (root FW)⁻¹ min⁻¹) as a function of elution time. For steady-state conditions, perform linear regressions and calculations of fluxes, half-lives of exchange, and pool sizes (for details, see⁶⁻⁹).

Representative Results

Figure 1 shows isotherms found using the DI technique (with ^{13}N), for the influx of NH_3 into roots of intact barley seedlings grown at high (10 mM) NH_4^+ , and either low (0.02 mM) or high (5 mM) K^+ . The isotherms display Michaelis-Menten kinetics when NH_3 fluxes are plotted as a function of external NH_3 concentration ($[NH_3]_{ext}$; adjusted by changes in solution pH¹³). NH_3 fluxes were significantly higher at low K^+ than at high K^+ . Analysis of Michaelis-Menten kinetic parameters showed that the K_M remained relatively stable between K^+ levels (150 vs. 90 μM at low and high K^+ , respectively), while V_{max} is strongly reduced at high K^+ (205 vs. 80 $\mu mol\ g^{-1}\ hr^{-1}$). Thus, the data indicate that K^+ level regulates nitrogen transport (V_{max} effect), but not by direct competition between K^+ and NH_3 for binding sites of transporters (K_M effect). Rather, K^+ may regulate NH_3 fluxes by other means, such as through modulation of aquaporin activity (for details, see¹³).

DI is also useful for the capturing of relatively fast changes in influx due to nutritional shifts, or to the application of pharmacological agents. For example, **Figure 2** highlights the rapid plasticity of the K^+ -uptake system in roots of intact barley seedlings grown at moderate (0.1 mM)- K^+ and high (10 mM)- NH_4^+ conditions. Here, we observed a ~350% increase in K^+ influx within 5 min of NH_4^+ withdrawal from the external solution. This "ammonium withdrawal effect" ("AWE") was found to be sensitive to the K^+ -channel blockers tetraethylammonium (TEA^+), barium (Ba^{2+}), and cesium (Cs^+). Using DI and electrophysiological measurements in several *Arabidopsis* genotypes, we were able to conclusively ascribe the vast majority of the AWE to changes in activities of the *Arabidopsis* K^+ channel, AtAKT1, and high-affinity K^+ transporter, AtHAK5¹⁴.

Figure 3 plots the steady-state efflux of $^{42}K^+$, over time, from roots of pre-labeled barley seedlings grown at low (0.1 mM) K^+ and moderate (1 mM) NO_3^- . These traces show how the CATE method can reveal rapid and significant changes in efflux upon application of various pharmacological/nutritional agents. Substantial, immediate inhibition of K^+ efflux was observed upon either an application of 10 mM Cs^+ , a K^+ -channel blocker, or a sharp increase in K^+ provision (from 0.1 to 10 mM). These results are consistent with molecular studies describing the unique gating properties of outward-rectifying K^+ channels¹⁵. By contrast, application of 10 mM NH_4^+ rapidly and strongly stimulated K^+ efflux. This effect can be explained by the activation of outward-rectifying K^+ channels via depolarization of the electrical potential gradient across the plasma membrane of root cells¹⁶, which is known to occur upon introduction of NH_4^+ ¹⁷. Thus, using this method, we have been able to demonstrate, *in planta*, that K^+ channels mediate K^+ efflux in roots of barley¹⁰.

Lastly, **Table 1** shows CATE parameters extracted from measurements of steady-state $^{42}K^+$ efflux ($[K^+]_{ext} = 0.1\ mM$) in barley seedlings grown either with 1 mM NO_3^- or 10 mM NH_4^+ , the latter representing a toxic scenario. The high NH_4^+ condition brings about a suppression of all K^+ fluxes, and a significant decline in cytosolic K^+ concentration ($[K^+]_{cyl}$), which is normally homeostatically maintained at ~100 mM under healthy growth conditions¹⁸ (as observed, e.g., in **Table 1**, under NO_3^- supply).

Figure 1. ¹³NH₃ influx isotherms reveal how K⁺ supply regulates nitrogen transport. NH₃ influx as a function of varying external concentrations of NH₃ ([NH₃]_{ext}) in intact roots of barley seedlings grown at high (10 mM) NH₃/NH₄⁺ and either low (0.02 mM, red) or high (5 mM, blue) K⁺. Michaelis-Menten analyses of isotherms reveal that high-K⁺ provision has relatively little effect on the substrate affinity (*i.e.*, K_M) of NH₃-uptake transporters, but significantly reduces the transport capacity (*i.e.*, V_{max}; see 'Representative Results'). Note, changes in [NH₃]_{ext} were established by shifting the external solution pH with NaOH, and thus the NH₃:NH₄⁺ ratios, as per the Henderson-Hasselbalch equation. Error bars indicate SEM of 4-7 replicates. (Reproduced from Coskun *et al.* Rapid ammonia gas transport accounts for futile transmembrane cycling under NH₃/NH₄⁺ toxicity in plant roots. *Plant Physiol.* **163**, 1859-1867 (2013).)

Figure 2. NH₄⁺ withdrawal significantly stimulates channel-mediated K⁺ influx. K⁺ influx at steady state, and upon withdrawal of NH₄⁺, in roots of intact barley seedlings grown at low (0.1 mM) K⁺ and high (10 mM) NH₄⁺. The effect of K⁺-channel blockers (10 mM TEA⁺, 5 mM Ba²⁺, and 10 mM Cs⁺) on the stimulated K⁺ influx is pronounced. Asterisks denote different levels of significance between -NH₄⁺ and treatment pairs (*0.01 < P < 0.05, ***P < 0.001; one-way ANOVA with Dunnett's multiple comparison post-hoc test). Asterisks in parentheses denote level of significance between control and -NH₄⁺ pair (Student's *t*-test). Error bars indicate SEM of >4 replicates. (Reproduced from Coskun *et al.* Capacity and plasticity of potassium channels and high-affinity transporters in roots of barley and Arabidopsis. *Plant Physiol.* **162**, 496-511 (2013).)

Figure 3. K^+ efflux is channel-mediated under low- K^+ conditions. Steady-state $^{42}K^+$ efflux in roots of intact barley seedlings grown at low (0.1 mM) K^+ and moderate (1 mM) NO_3^- , and the immediate effects (at $t = 15.5$ min; see arrow) of 10 mM CsCl, 5 mM K_2SO_4 , and 5 mM $(NH_4)_2SO_4$ on efflux. Each plot represents the mean of 3-13 replicates (SEM <15% of the mean). (Reproduced from Coskun *et al.* Regulation and mechanism of potassium release from barley roots: an *in planta* $^{42}K^+$ analysis. *New Phytol.* **188**, 1028-1038 (2010).)

$[K^+]_{ext}$ (mM)	N source (mM)	Influx ($\mu\text{mol g}^{-1} \text{hr}^{-1}$)	Efflux ($\mu\text{mol g}^{-1} \text{hr}^{-1}$)	Net Flux ($\mu\text{mol g}^{-1} \text{hr}^{-1}$)	E:I Ratio	Pool Size (mM)	Half-life (min)
0.1	1 NO_3^-	7.22 ± 0.23	1.86 ± 0.18	5.36 ± 0.18	0.25 ± 0.02	98.84 ± 14.08	28.18 ± 3.40
	10 NH_4^+	1.89 ± 0.13	0.57 ± 0.05	1.32 ± 0.10	0.30 ± 0.01	28.39 ± 3.40	32.50 ± 4.69

Table 1. Steady-state K^+ fluxes and compartmentation under various N provisions. Steady-state flux and compartmental analysis of barley seedlings grown at 0.1 mM K^+ , and either moderate NO_3^- (1 mM, as Ca^{2+} salt) or high NH_4^+ (10 mM, as SO_4^{2-} salt). Errors indicate $\pm$ SEM of >8 replicates. (Reproduced from Coskun *et al.* Regulation and mechanism of potassium release from barley roots: an *in planta* $^{42}K^+$ analysis. *New Phytol.* **188**, 1028-1038 (2010) and Coskun *et al.* Capacity and plasticity of potassium channels and high-affinity transporters in roots of barley and Arabidopsis. *Plant Physiol.* **162**, 496-511 (2013).)

Discussion

As demonstrated in the examples above, the radiotracer method is a powerful means of measuring unidirectional fluxes of nutrients and toxicants *in planta*. **Figure 1** shows that NH_3 influx can reach in excess of $225 \mu\text{mol g}^{-1} \text{hr}^{-1}$, which is perhaps the highest *bona fide* transmembrane flux ever reported in a plant system¹³, but the magnitude of this flux would not be visible if only net fluxes were measured. This is because a large efflux of NH_3 occurs at the same time as influx, in a futile cycling scenario that can result in a pronounced underestimate of influx that increases with labeling time¹³. By supplementing the tracer technique with electrophysiological analysis, we were able to demonstrate that under the conditions of **Figure 1**, both influx and efflux of ^{13}N is primarily of the neutral gas NH_3 , and not of its conjugate acid NH_4^+ (for details, see¹³). This is the first *in planta* demonstration of rapid NH_3 gas fluxes in roots, and as such, provides important preliminary evidence towards unraveling the transport mechanism that lies at the heart of NH_3/NH_4^+ toxicity in higher plants^{2,13}. Molecular work in heterologous expression systems has demonstrated that NH_3 can flow via aquaporins in plants¹⁹, and the data from **Figure 1**, along with recent pharmacological evidence, has begun to corroborate such findings at the level of the intact organism¹³.

Figures 2 and 3 also provide excellent examples of the utility of measuring unidirectional fluxes with radiotracers. Using DI with $^{42}K^+$, we were able to demonstrate that ion channels are not responsible for steady-state K^+ uptake in roots of barley seedlings grown at low K^+ and high NH_4^+ , in contrast to the model system *Arabidopsis*¹⁴. Only when NH_4^+ was withdrawn did we see evidence for the engagement of K^+ channels (**Figure 2**). Although the net flux of K^+ is also stimulated by NH_4^+ withdrawal (as shown by increased tissue K^+ content¹⁴), only by measuring unidirectional influx were we able reveal the magnitude and rapid onset of this phenomenon. Moreover, by conducting DI measurements with mutants and pharmacological agents, we were able to identify which transport proteins were involved. Similarly, by applying nutritional and pharmacological agents while monitoring tracer efflux (**Figure 3**), we were able to characterize and identify mechanisms of K^+ efflux from barley root cells¹⁰. Thus, techniques such as DI and CATE can be instrumental to the understanding of transport characteristics for a critical macronutrient.

As noted in the protocol, often the position of the sample relative to the detector in the gamma counter can influence the quantity of radiation measured. Thus, if a 1-ml sample is "topped up" with 19 ml of H₂O, the counts measured (cpm) in the 20-ml sample can be significantly lower than in the 1-ml sample, despite having the same amount of radiotracer. Therefore, a D_f can be applied to correct for this apparent 'dilution' of radioactivity. This issue is often not explicitly stated by manufacturers of detection instrumentation and must be worked out by the individual researcher. Similarly, the effectiveness of shielding within detectors against ambient radiation (*i.e.*, from nearby samples within the counter) can be exaggerated by manufacturers, and such issues should be worked out for individual measuring systems.

A major advantage of the tracer technique is its non-invasiveness, which provides a means to measure fluxes, intracellular pool sizes, and exchange rates, under steady-state conditions. For example, with CATE, we could non-invasively quantify cytosolic concentrations of K⁺ (Table 1). This can be preferable to alternative methods such as impalement of cells with ion-selective microelectrodes¹⁸, which imparts physical and possibly chemical disturbances to the cell. In addition, the tracer technique is unique in that it provides a comprehensive view of fluxes and compartmentalization for whole organs and intact plants. This is important if one is interested in understanding whole-plant nutrient dynamics, toxicity, and ultimately, performance in the field. Lastly, radiotracer methods allows for very sensitive measurements to be conducted under fairly high substrate concentrations. Traditional depletion experiments and microelectrode techniques can experience issues of background interference and thus, may require that the external concentration of the substrate of interest is lowered well below that provided during growth. This could be problematic if one is interested in studying "steady-state" conditions of high substrate concentrations (such as with NH₃/NH₄⁺ toxicity or "high-K⁺" conditions; see above).

It should be noted that, like all techniques, measuring fluxes with radiotracers is not without its limitations. For example, the availability of radiotracers can be problematic, particularly for very short-lived isotopes like ¹³N that require close proximity to a production facility such as a cyclotron. Another major limitation is that at times, it can be difficult to discriminate between fluxes that are occurring across membranes and those occurring extracellularly. Such distinctions call for rigorous phase testing^{7,10,20}. In the case of K⁺ efflux, only after careful examination were we able to confirm that steady-state ⁴²K⁺ release from roots was occurring not across cell membranes at high [K⁺]_{ext} (>1 mM)¹⁰, but from extracellular spaces (*c.f.*, Figure 3). Such issues can be resolved by examining the effect of a wide range of pharmacological agents, or through thermodynamic analyses, which have shown, for example, that very high Na⁺ fluxes reported under saline conditions would be energetically unfeasible were they to proceed across cell membranes^{21,22}.

Disclosures

The authors have nothing to disclose.

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