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Seeing is better than believing: visualization of membrane transport in plants

Markus Geisler

Recently, the plant transport field has shifted their research focus toward a more integrative investigation of transport networks thought to provide the basis for long-range transport routes. Substantial progress was provided by of a series of elegant techniques that allow for a visualization or prediction of substrate movements in plant tissues in contrast to established quantitative methods offering low spatial resolution. These methods are critically evaluated in respect to their spatio-temporal resolution, invasiveness, dynamics and overall quality. Current limitations of transport route predictions-based on transporter locations and transport modeling are addressed. Finally, the potential of new tools that have not yet been fully implemented into plant research is indicated.

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Introduction

Because you have seen me, you have believed;
blessed are those who have not seen and yet have believed.[1]
John 20: 29

The catalyzed movement of substrates across membranes, commonly referred to as transmembrane transport, has always been a hotspot in plant biology [1]. Throughout the 80s and 90s, the plant transport community concentrated mainly on biochemical characterization of single transporter isoforms with a focus on a kinetic level (the 'queen of transport experiments' [2]). In the last two decades, deep insights into transporter expression, trafficking, regulation and their role in plant physiology were provided. Recently, plant scientists have shifted their interest toward a more integrative investigation of long-range transport routes or transport networks [3–5] that is of water, nutrients or some hormones (for an overview, see Figure 1).

Transport was classically quantified on a whole organ or plant level using radiotracers, external microelectrodes or cell-type specific analyses of transport substrates (for reviews on these methods, see Ref. [6]). While these techniques allow for a high level of temporal resolution, they are limited in the spatial aspect. Therefore, direct and indirect techniques have been developed in order to image either local substrate concentrations or intra and intercellular transport *in planta*. Here, we present current methods that allow for a quantitative visualization of transmembrane transport and compare them with classical transport measurements and *in silico* techniques.

Inference of transport routes by transporter imaging

For several substrates, putative transport routes have been deduced from polar transporter localizations [3,5,7–10]. These cell-biological approaches have been partially backed up by analyses of reporter expression and transport modeling (see below).

The best-understood example is probably the polar transport of the auxin, indole-3-acetic acid (IAA), in the Arabidopsis root tip provided by a network of plasma membrane localized im- and exporters of the ABCB, AUX1/LAX and PIN-FORMED (PIN) families [3,11]. Different degrees of tissue-specific and polar locations of members of these families have been established by immunolocalization and fluorescent protein fusions (see Figure 3) that are in line with current models of a reversed fountain auxin flux pattern (see Figure 1; [8]). Similarly, the boric acid channel, NIP5;1, and the boron exporter, BOR1, have been localized in a polar fashion in the plasma membranes facing toward soil and stele, respectively, and are therefore suspected to function in the uptake and translocation of boron to support growth of various plant species (see Figure 1; [7]). Likewise, the ABCG-type SL transporter, PDR1, exhibits an asymmetrical localization in the plasma membrane of petunia root

[1] Please note that this bible citation is not at all meant as a religious statement but a stylistic element acknowledging the effort of the transport field to promote techniques allowing for a quantitative visualization of transport, which is the content of this review.

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Figure 1

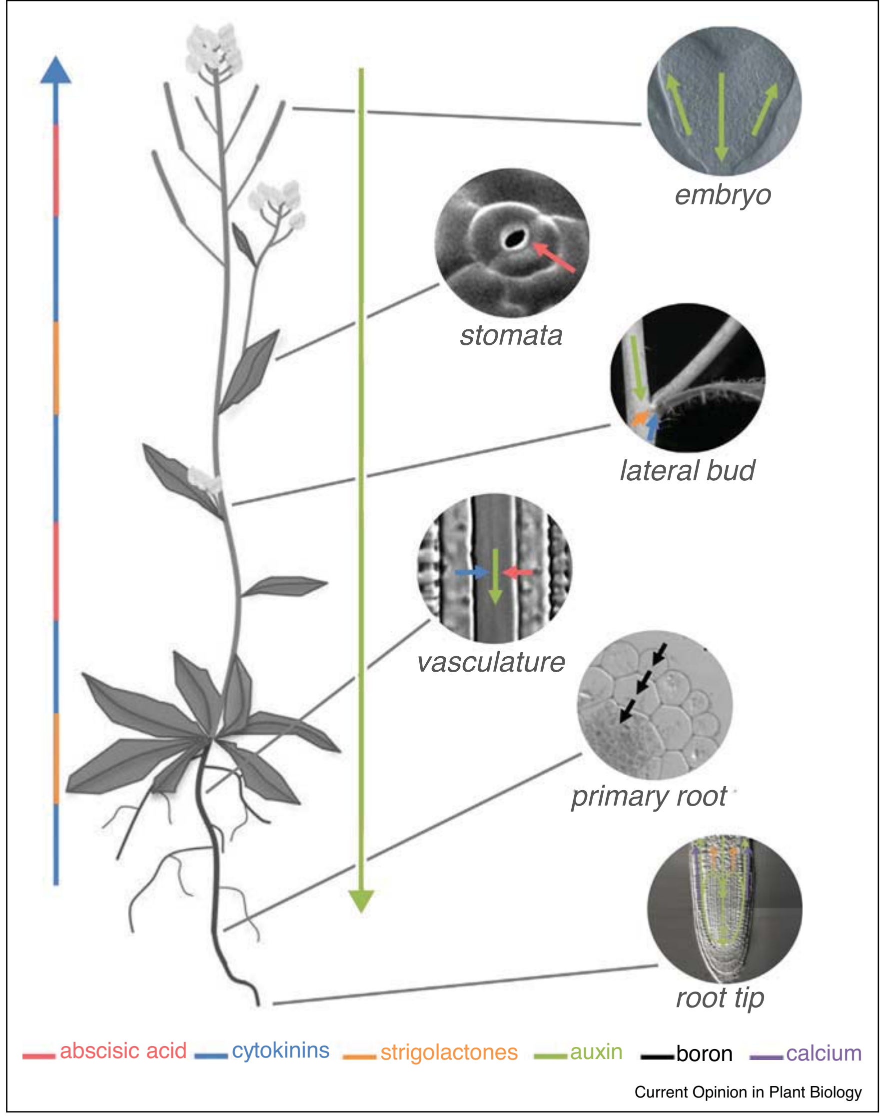

Illustration of trans-membrane, long-range transport routes in plants.
Exemplary long-range transport routes of indicated phytohormones, boron and calcium are indicated by arrows. Note that only long-distance signaling of Ca^{2+} where it acts as a second messenger but not as a nutrient is indicated. In contrast, boron paths are indicating nutrition routes. Overall figure outline is based on [5]; usage of Arabidopsis model was granted by Mary Lou Guerinot.

tips indicating a directional cell-to-cell transport process at least in this region of the root (see Figure 1; [5]). However, presence of a transporter on a cellular subdomain does not automatically reflect local activity levels. Moreover, this approach failed so far in the prediction of transport routes for non-polar transporters known to be involved in long-distance transport [12].

Quantification of transporter activity or regulation

A better proxy might be the recently developed *transport activity sensors*, such as AmTrac and MepTrac [13,14], NiTrac1 and PepTrac [15]. Although these transport activity sensors do not strictly monitor transport, and have not yet been tested *in planta*, they will most likely become valuable, especially in the context of analytes for which no tracers exist.

An alternative approach might be imaging transporter regulation, such as by protein phosphorylation [16]. AGC kinases were shown to phosphorylate and thus alter transport activities of auxin transporters of the PIN and ABCB families [16–20], but have not yet been imaged *in planta*. A future alternative might be offered by novel GFP-based kinase reporters, called SPARK (Separation of Phases-based Activity Reporter of Kinase). SPARK

offers imaging of fast kinase kinetics and is reversible [21••] but has not yet been adapted to plant systems.

Visualization of transporter substrates: *in vivo* biochemistry

While some transporter substrates (like some secondary metabolites) are by nature fluorescent and can be followed directly, most are not and thus need either be applied as a radiolabeled or fluorescent version (see below) or require an indicating tool [22]. *In vivo* biochemistry [23] has provided us with a series of cell-biological techniques that allow for an imaging of either local substrate concentrations or intra and intercellular transport *in planta* (see Figure 2). However, it is important to keep in mind that most of these techniques indicate local substrate concentrations that are then used as a proxy for transport.

Chemical indicators

Chemical indicators are fluorescent molecules that respond to the binding of substrates by changing their fluorescence properties ([24]; see Figure 2). They exist for different metal cations (such as zinc, potassium, sodium, magnesium, calcium), protons and chloride and their loading and membrane targeting has been optimized by generating hydrophobic versions that are cytoplasmically cleaved and activated. In plants, the most commonly used pH-indicator and calcium-indicator are BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein) [25] and Fura-2/Calcium Green variants, respectively [24]. The latter can be either non-ratiometric (such as Calcium Green) or ratiometric (Fura-2), allowing one to correct for unequal dye loading, photo-bleaching or focal plane shifts. However, a common problem with chemical indicators in plant cells is that dye loading is hindered by the cell wall or compartmentalization in organelles (mainly vacuoles) avoiding an equal distribution [22,26].

Therefore, genetically encoded (GE) indicators, *reporters* and *sensors* [27–30] have been developed that indirectly or directly indicate local substrate concentrations, respectively (Figure 2). They allow for selective targeting to subcellular compartments (by insertion of signal sequences) or to cell types (by choice of promoters) [31].

Reporters

Expression-based (or signaling) reporters consist of natural or synthetic promoters fused to a reporter gene. Many but not all plant hormones, including auxin, ABA, cytokinin (CK), ethylen, jasmonic acid (JA) and salicylic acid can be visualized in this manner (for reviews, see Refs. [23,27,30–32]). Since publication of the prototype artificial auxin-responsive element, *DR5* [33], several variants have been developed (for a review, see Ref. [22]). DR5 activation is slow (ca. 2 hour) and reflects an auxin

Figure 2

	Chemical Indicator	Reporter	Sensor	Fluorescent substrate	Click-chemistry	Imaging of radioactivity
Principle						
Readout type	Change of fluorescent properties	Promoter activation, reporter degradation	FRET, change of fluorescence intensity	Fluorescence	Fluorescence	(Digital) Radiography, Micro-autoradiography
Dynamic readout	+	+/-[1]	+	+	(+)	-
Subcellular resolution	+/-	-	(+)	+	+	+/-
Calibration	+/-	+/-[1]	+	-	-	-
Noninvasive	+	(+)[2]	(+)[2]	+	-	-

Current Opinion in Plant Biology

Comparison of methods used to derive transmembrane, long-range transport in plants.
Indicated approaches are evaluated with respect to their readout type and dynamic range, their invasiveness, their offered subcellular resolution and their correlation between the amount of detected target molecule and readout (calibration). Target molecules are in blue; fluorescent proteins are depicted as rounded rectangles. Note that principles are illustrative examples and might be not complete; brackets indicate limitations of methods. Figure outline is inspired by Ref. [27]. [1] Dynamic range and calibration varies between reporter types; [2] Reporter and sensors require genetic access and are thus not suitable for non-model plants (for details, see text).

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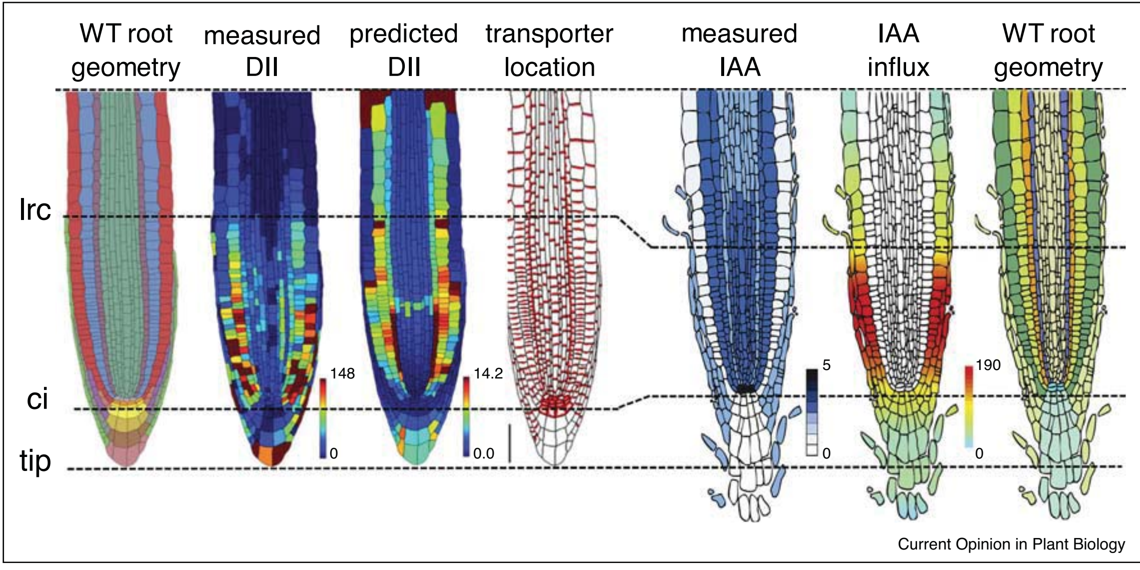
Figure 3



Comparison of imaged, modeled and measured auxin transport in the root tip.
Measured DII-VENUS signals and transporter locations (here: PINs) were extracted from confocal images and are compared with modeled DII-VENUS distribution and predicted auxin fluxes (all taken from Ref. [89•]). As reference, imaged auxin (transporter) distributions are compared with heat map presentation of IAA influx profiles measured using an IAA-specific microelectrode [97] or of high-resolution IAA contents measured by MS after cell-type sorting [97,98]. Note that the latter two quantitative transport techniques are not described in this review. Cell types labeled on the schematics of wild-type root geometry (left and right) are given; for identity refer to Refs. [89•] and [97]. Note that the alignment of schematics is based on position of root tips, columella initials (ci) and end of lateral root caps (lrc).

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response maximum, and is thus mainly suitable for the root tip [22,34••,35].

Reporters-based on the principle of SCF-mediated proteasomal degradation of transcriptional repressors in the signaling pathway of several hormones were developed increasing the temporal (but also spatial) resolution of expression-based reporters. The blueprint consisted of the DII degron sequence of Aux/IAA repressor proteins of auxin signaling, which was fused to a nuclear targeted VENUS [36]. This module provides a decrease of DII-VENUS reporter fluorescence in tens of minutes upon auxin perception and is therefore suitable to image unequal auxin distribution in bending roots [36]. Combining the degron motive fusion with an auxin-insensitive reporter gene resulted in ratiometric reporters. For example, R2D2, now offers semi-quantitative auxin imaging at very reasonable spatio-temporal resolution [37•,38,39••]. Degradation-based reporters have also been developed for GA (GFP-RGA; [40]), JA (Jas9-VENUS; [41]) and SL (StrigoQuant; [42]).

Despite tremendous improvements in expression and degradation-based reporters with respect to speed and sensitivity, their indirect mode of action allows only for — at best — semi-quantitative *in vivo* imaging of local substrate concentrations. Further, reporters display final outputs that integrate over transport, biosynthesis and metabolism. An even more limiting aspect is that their sensitivity is primarily determined by the expression of the employed signaling machinery and thus cell and tissue-specific [34••].

Sensors

A series of genetically encoded activity sensors that are able to report directly and within seconds changes in calcium, proton, energy metabolite, heavy metal or macronutrient concentrations have been established [27,31]. Like reporters, sensor are used to assess resident substrate concentrations that are then often equalized as transport activities [27,43].

The photoprotein aequorin, from jellyfish has been widely used in plants for more than two decades in order to study calcium dynamics by means of emitted bioluminescence [44]. However, due to its low cellular sensitivity, aequorin was slowly replaced by FRET-based sensors (Figure 2; [45]), such as Cameleon [24]. Versions of Cameleon with improved calcium binding affinity and dynamic range [23,27,28] (YC3-60, [46] and YC-nano), were recently used for demonstrating long-range calcium signaling in the root and between shoot and root, respectively [47–49]. The intensiometric calcium sensor, R-GECO1, exhibits an increased (>10×) sensitivity compared with YC3.6 in response to elicitors of the innate immune response to pathogens, flagelin22 and chitin [50••], and R- was used to image auxin-induced calcium

gradients in root-hair tips and long-distance calcium waves between root cells [51••].

For local pH measurements, single fluorescent protein sensors, such as pHluorins [52], using the pH sensitivity of EGFP are mainly employed [53•]. The pH sensor, pHusion, allows for a ratiometric quantification of cytoplasmic and apoplastic pH [28]. Recently, a pHusion variant allowed measuring pH in and outside of the TGN/early endosome network demonstrated the role of the V-ATPase for protein secretion [54••].

The Frommer group was driving forward the development of FRET sensors for of a wide range of sugars [23,27,31]. Beside a quantification of intracellular concentrations, these sensors also permitted the kinetic characterization of individual sugar transporters [1] and the identification of novel transporters [55]. Sensors for transport of ATP; [56,57], ROS, heavy metals (eCALWY) (for references, see Refs. [27,31]) and different macronutrients have been developed (for a review, see Ref. [58]). Very recently, ATP sensing in living plant cells using the FRET-based sensor, ATeam1.03-nD/nA [56], revealed concentration gradients in plant tissues and stress-related dynamics of energy physiology [57].

Hormone imaging by sensors has been achieved first for ABA: two independent groups have developed sensors consisting of a FRET pair that is linked by an ABA-responsive sensory module, called ABACUS [59•] and ABAleon [60•], respectively. Both sensors have proven to be useful in demonstrating kinetics of ABA transport and dynamics upon application of external ABA, [30,32,59•]. The nuclear localized GA sensor, GIBBERELLIN PERCEPTION SENSOR 1 (GPS1), allowed sensing of nanomolar concentrations in Arabidopsis [61].

The intensiometric calcium sensor, R-GECO1, was shown to exhibit an increased (>10×) sensitivity compared with classical YC3.6 in response to elicitors of the innate immune response to pathogens, flagelin22 and chitin [50••]. R-GECO1 was used to image auxin-induced calcium gradients in root-hair tips but also long-distance calcium waves between root cells [51••].

Fluorescently labelled substrates

Fluorescently labeled substrates have only recently been used in plants (Figure 2). The fluorescent BR analog, Alexa Fluor 647-castasterone (AFCS) [62], GA-fluorescein [63] and different fluorescent auxins [34••,35,64] have been developed. Moreover, a quantum dot-based fluorescent probe, CdTe-JA, was employed to label JA binding sites in tissue sections of mung bean and Arabidopsis seedlings [65]. Recently, different fluorescent SL analogs were successfully designed: while EGO10A-BP and CISA-1 [66] are constitutively fluorescent variant making it first choice for transport studies, Yoshimulactone Green or GC242 were constructed to exhibit only fluorescent after cleavage by the SL receptor [67].

Bioactive auxin derivates tagged with fluorescein (IAA-FITC) or rhodamine (IAA-RITC) are distributed symplasticly and apoplasticly, respectively [35]. Hayashi *et al.* designed fluorescent auxin analogs to be actively transported but to be inactive for auxin signaling [34••], while very recently new fluorescently labeled auxins exhibit an anti-auxin activity [64]. The former is important because auxin signaling is known to influence auxin transporter expression and location [34••]. NBD-IAA (7-nitro-2,1,3-benzoxadiazole-IAA) revealed a similar auxin distribution as found with DII-based reporters [34••] but lacked signals in the quiescent center known to be mainly produced by auxin biosynthesis and not transport. A limitation of NBD-auxins is that they are for unknown reasons only working in root and not in shoots [34••]. Recently, NBD-NAA, whose nuclear import is restricted, was used as a tool to dissect cytoplasmic and ER delivery of nuclear auxin concentrations [39••]. This indicates their usefulness in analyzing intracellular transport, which is usually hard to uncover using classical tools.

Click-chemistry of transport substrates

In animal models, the problem of bulky fluorescent tags interfering with binding to transporter (or receptor) proteins has been partially solved by 'click chemistry' [68]. This technique triggers the selective, covalent connection between a molecule of interest and a detection tag *in situ* (Figure 2). A variety of labeling tags can be used then for microscopy or analytics.

To our knowledge only one study reported on the distribution of a mobile substrate using this technology in plants: the azido derivative of indole-3-propionic acid (IPA, an active auxin), IPA-N_3, was used as an auxin tracer and provided some evidence of the presence of auxin binding sites in the apoplast of elongating cells [69••]. IPA-N_3 was detected mainly in the outer cell layers of the root tip and does thus not fully reflect the auxin accumulation sites. This seems to be less a penetration but a fixation problem [69••]. However, these technical issues will be overcome and as such 'click chemistry' might soon be the method of choice despite the fact that it requires a fixation step preventing *in vivo* imaging.

Imaging of radiolabeled substrates

Radio-autographic analysis of the distribution of transport has a long tradition in plants (e.g. [70]). Recently, polar auxin transport of Arabidopsis stem segments was visualized by detecting ^{14}C-IAA with a double-sided silicon strip detector [71]. In recent years, the micro-autoradiography method (MAR) has been established to visualize the distribution of ^{109}Cd and ^{33}P within sections of rice

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tissues [72]. Currently, only fixed material can be used, preventing any dynamic information about substrate distributions. As ^{14}C (and ^{3}H) emit beta particles at very low energy, most transport substrates could be labelled with suitable isotopes for autoradiography, which would expand the application properties of MAR (Figure 2; [72]).

Immunolocalization of transport substrates

ABA [73,74], IAA [22] and CK [75] have been visualized in different plant species by whole-mount immunolocalization using polyclonal and monoclonal antisera (Figure 2; [76]). Despite requiring of fixation, some of these antisera have become successful tools for the *in situ* localization of local maxima [75,77] or the translocation of hormones in plants [78–82]. Anti-IAA antibody signals were reported to recapitulate signals found with the *DR5* reporter in the mature and lateral root [77,79]. However, a part of these publications have been heavily debated with respect to their interpretation and technical quality [83].

Modeling of transmembrane transport over tissues

Early auxin transport models, called flux-based models, were inspired by Tsvi Sachs in the late 1960s and hypothesized that the flux of auxin through cell membranes reinforces itself [84,85]. The integration of experimentally determined patterns of transporter localization into simulations confirmed this in Arabidopsis [86–88,89•]. Gradient-based models were introduced [90,91], provided support for the assumed roles of auxin maxima in shoot development (for an excellent review, see Ref. [92]).

The primary motivation of most of these studies was to predict the movement of a transported substrate (here: auxin; Figure 3) within a developing tissue. In this regard, the development of the OnGuard platform for modeling transport processes in stomata has proven successful in uncovering previously unexpected features of guard cell physiology [93]. However, today's interest lies more in testing if a hypothesized role for a substrate can be supported by *in silico* data [94]. A recent study employing data-driven modeling of intracellular auxin fluxes between the ER or cytoplasm and the nucleus, respectively, indicated that the ER has a dominant role in controlling nuclear auxin uptake [39••]. This and other approaches argue that computer models can provide (or even go beyond) a 'proof of principle'. However, any conclusions-based on human-made, and as such subjective, algorithms will always require an experimental validation.

Conclusions and perspectives

Recently, the plant transport field has made a major conceptual shift away from a *single transporter* analysis toward a more *integrative transporter* approach, aimed at exploring transporter networks in order to understand their interplay and their incorporation into plant physiology. This progress has been made possible by the development of a series of techniques that allow visualization of local concentrations and movements of substrates in plant tissues. While early quantitative methods (like radiolabeled substrates, micro-electrodes and cell-type specific analyses of transport substrates) provide temporal resolution, visualization of transport by means of chemical indicators, fluorescent analogs and especially reporters and sensors provides good spatial information with reasonable resolution for many substrates. In that respect, doubting Thomas was right: seeing is better than believing.

Most tools have their individual advantages and limitations (see Figure 2). Some allow only for (semi)-quantitative analyses with high spatial but only low temporal resolution (such as reporters and sensors), while some indicators, sensors or fluorescent analogs were found to interfere with plant physiology by high substrate binding affinities or by competing with endogenous signaling [24,32,57]. However, these kinds of problems may to a certain degree be overcome by using non-plant substrate binding domains [95].

As usual, new technology and insight provides us with new challenges. For example, the transfer of sensors from single cell approaches to the plant level has been difficult due to the wide affinity/sensitivity range (nM-mM) that is required for some substrates. A solution might be the co-expression of spectrally non-overlapping, intensiometric sensors with different affinity ranges. Further, in contrast to intercellular transport, transmembrane transport between subcellular compartments has proven difficult to measure directly. In that respect, a directed targeting of sensors to individual subdomains of interest as shown recently [54••] and design of selective permeable fluorescent dyes [39••] will be helpful.

Another issue is the inadequate separation of transport from signaling/biosynthesis by some imaging tools, like reporters or sensors. In that respect it appears as if we have not yet fully used the potential of fluorescent transport substrates or click chemistry. In the future, structure-activity design will allow us to predict fluorophore positions and linker length [63,64] in order to direct uptake, stability and distribution of fluorescent substrates [96].

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