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Highlights:

- Of many biosensor types, few have been applied in plant biology
- Sensors suited to adaptation and application in plants are identified
- Genetically-encoded biosensors are proving powerful tools for auxin and a few metabolites, but these remain qualitative tools
- Real-time, quantitative biosensors are the way forward

Summary

Biosensors come in an increasing array of forms and their development is defining the rate of advance for our understanding of many natural processes. Developmental biology is increasingly using mathematical models and yet few of these models are based on quantitative recordings. In particular, we know comparatively little about the endogenous concentrations or fluxes of signalling molecules such as the phytohormones, an area of great potential for new biosensors. There are extremely useful biosensors for some signals, but most remain qualitative. Other qualities sought in biosensors are temporal and spatial resolution and, usually, an ability to use them without significantly perturbing the system. Currently, the biosensors with the best properties are the genetically-encoded optical biosensors based on FRET, but each sensor needs extensive specific effort to develop. Sensor technologies using antibodies as the recognition domain are more generic, but these tend to be more invasive and there are few examples of their use in plant biology. By capturing some of the opportunities appearing with advances in platform technologies it is hoped that more biosensors will become available to plant scientists.

Introduction

The term biosensor has been attached to many technologies. They can be both qualitative or quantitative, genetically-encoded or solid-state physical devices, give real-time or time-fractionated data, *in vivo* or *ex vivo* and of many other designs. Developments in all these areas are advancing apace, led in different fields by the

need for cheap, reliable diagnostic tests, non-invasive recordings and by the ingenuity of researchers.

In essence, a biosensor couples a recognition event by a biological receptor to a transducer which converts this recognition event into a signal that can be captured and interrogated. Two common versions of these configurations are an enzyme coupled to an electrode with the output then electronic, and a ligand-sensitive receptor coupled to fluorescent proteins which are interrogated by confocal microscopy. Both these examples suggest measurements taken *in vivo*, and both can be real-time, but many other biosensor options are *ex-vivo* measurements and for these the versatility of antibodies as sensor units is prevalent, although molecular imprinting and many other recognition technologies may also be used. Current advances are in the areas of multiplexing sensors, high-throughput, DNA aptamer sensors, and increasing sensor robustness, resolution and sensitivity with the advent of nanotechnologies.

In order to limit our coverage, we will review progress in the development of biosensors for plant developmental signals and the abundant opportunities awaiting suitable sensors. In particular we will consider phytohormone signalling, although other systems will be covered in order to capture the breadth of biosensor applications. Examples of various biosensor systems are given in Table 1.

The ideal biosensor is selective, sensitive with a favourable signal to noise ratio, gives a quantitative (calibrated) dose-response curve over physiologically-relevant concentrations of analyte (usually over several orders of magnitude), gives a localised (spatially resolved) reading *in vivo*, has a fast-response yielding time-resolved data and yet is not invasive and does not disturb the endogenous responses of the system. Consequently, all biosensors are compromises! Yet the fact that this is true does not necessarily devalue the information they give. The impact of each compromise is determined by the experimental system and, generally speaking, the information they provide is an advance over what has gone before.

Biocatalytic electrochemical biosensors

Biosensor advances have been led by the glucose sensor, now sold globally for blood glucose monitoring by diabetics [1*]. Glucose oxidase has an FAD cofactor and, in early electrodes, the redox transfer of electrons from glucose was channelled via FAD into hydrogen peroxide in the presence of molecular oxygen. Peroxide was then detected at a platinum electrode. Biosensors based on similar oxidases or coupled enzymic reactions record current as peroxide is oxidised back to oxygen at the electrode at an operating potential of around 600 mV. They have been adopted widely, from sensors for pathogen detection, food quality testing (eg using lactate oxidase) [2] through to environmental monitoring, although there are few applications in plant biology. More recent developments incorporate mediators, electron transfer intermediates tethered to the electrode or available to diffuse freely in the electrode matrix, often a silicate sol. The use of mediators bypasses the production of hydrogen peroxide to allow more sensitive, longer-lasting and less damaging sensor systems [1*]).

Opportunities for biocatalytic sensor applications in plants

One of the beauties of enzyme-based electrochemical sensors is that the biosensor is designed on a molecular recognition event that has evolved naturally, often with high

selectivity and of an appropriate affinity for working under endogenous substrate concentrations. A survey shows that several of the plant hormones are catabolised by redox enzymes, raising the potential for selective, enzyme-based biosensors. Auxin, cytokinins and gibberellins are all deactivated by oxidases, for example. IAA oxidase remains somewhat elusive as a specific enzyme, with many peroxidases able to oxidise IAA but with low substrate specificity. Gibberellin is deactivated by GA 2-oxidases [3]. These 2-oxoglutarate-dependent dioxygenases (as is the widely used glutamate oxidase) [4] express in soluble, active form from *E. coli* and so might be suitable for GA sensors, especially with the benefit of recent advances in carbon microfiber electrodes and mediators [1*, 5*]. Cytokinin oxidase is better classed as a dehydrogenase [6], but it is both active and exceedingly stable after expression in heterologous systems. Early work using AtCKX2 has indicated that electrochemical sensors for cytokinin are feasible (Kowalska, Frebort and Napier, unpublished).

Several commercial biosensors use redox enzymes from plants. For example, superoxide dismutase is used for antioxidant assessment in health monitoring [7] and tyrosinase (monophenol monooxygenase) for monitoring pollution by phenolics [8]. Laccase (a copper-containing oxidase) is used in food monitoring to detect plant flavonoids [9]. No record can be found of the use of these sensors to study plant development.

It has become clear that extracellular ATP is an extracellular signal in plants as well as animals, and is involved in elongation growth [10*] and defence responses [11]. One elegant biosensor which could be applied to plant signalling is the ATP biosensor [12] based on a coupled enzyme system. The enzymes are immobilised in a porous layer of polymerised silane on a platinum electrode. The electron flow induced by regeneration of the final, redox enzyme in the pathway is monitored by voltammetry. The electrodes are small, 25-100 μm , fast and stable. Extracellular plant ATP has been studied using a non-electrochemical, genetically-encoded biosensor (see below). By fusing luciferase to a cellulose-binding peptide [10*] the sensor became locked onto the cell wall, allowing monitoring of apoplastic ATP in roots. The system does not need the infusion of luciferin, but the signal appeared selective and was sensitive to micromolar ATP concentrations. Although it could not be truly quantitative, this sensor gave high spatial resolution and was temporally dynamic. Other genetically-encoded biosensors are discussed below.

Non-enzymatic electrochemical sensors

Having illustrated the potential and noted the adaptability of enzyme-based (biocatalytic) sensors, it is also true that their application has limitations, not least the fact that access to a specific, stable enzyme is required for each analyte of interest. Consequently, other electrode-based sensor systems have been developed, non-enzymatic electrochemical sensors and affinity-based sensors [1*].

One electrochemical biosensor system is noteworthy, that based on the direct oxidation of the auxin IAA at the electrode surface at high electrode operating potentials [13]. Early carbon (graphite) pastes [13] have given way to carbon nanotube-coated platinum electrodes [14] which essentially increase contact area and electrical connectivity. By incorporating the self-referencing vibrating microelectrode technique, selectivity and sensitivity were improved [15**]. These auxin-sensitive microelectrodes are positioned close to the tissue surface and the tip vibrates in the

boundary solution layer to sample the auxin signal at two positions 10 μm apart. At the position closest to the tissue auxin measurements will represent the activity of auxin as it is lost from the cell wall. This signal is referenced against that from the electrode at its outer extreme of movement, a site representing close to background concentrations of auxin. By calculating the difference (self-referenced) signal, the sensor tends to subtract out noise, cancelling contributions from competing ions etc. giving a measurement of auxin activity at the surface. By calibration and using the dimensions of the electrode with Ficks laws on diffusion, local auxin flux can also be calculated.

Auxin electrodes were sensitive between around 0.1 and 50 micromolar IAA [14], shown to give readings from plant extracts in accord with extraction and analytical techniques, to be stable over hours of recording, and the vibrating electrode version has been used to quantitate fluxes from root apices [15]. These are clearly useful tools, and the flux measurements are in accord with models of auxin movement in this tissue. Unfortunately, the application of these electrodes has been limited, due mainly to operational restrictions. The more advanced self-referencing electrode, for example, may only be suited to records at the surface of 'permeable' tissues like the root apex because of its need for free vibrational movement in the boundary layer, but it is non-invasive.

Affinity-based biosensors

Recognition need not be harnessed to catalysis. Receptor proteins can provide some of the same properties as enzymes, and well-selected antibodies provide highly selective and high affinity recognition for almost any soluble analyte. Receptors are not always suitable, often due to lability or requirement for a membrane. Antibodies on the other hand are robust and versatile and have been adapted into diverse immunosensor systems. Affinity recognition need not be based on biomolecules as the recognition element and some robust sensor systems make use of molecular imprinted materials and volume-sensitive hologram-based biosensor surfaces [16]. The most recent addition to the family of affinity-based sensor technologies is the development of nucleic acid fragments as recognition species, especially the use of selected or designed aptamers (eg short 20-40 nucleotide, single stranded DNA) which bind the analyte [1*,17*]. In all cases there is no enzymatic activity to measure for signal transduction, but a range of quantitative analytical methods are widely available including electrochemical, such as by impedometrics [18*], versions of activity mapping by atomic force microscopy [19**], and optical such as by hologram [20], fluorescent tags or various biophysical platforms, such as microbalance and surface plasmon resonance (Table 2).

In almost all cases the assay formats for affinity-based sensors have been *in vitro* and so to get any record of the response timescale a series of time-fractionated samples must be prepared. Consequently, affinity-based assays generally do not give a dynamic, interactive record and may miss transient changes. There is no doubt that for applications in eg diagnostics or environmental monitoring this is adequate and appropriate. Cost-effective, high-throughput systems are available, some with parallel, multiplexed assays [1, 21, 22].

The plant biology world does have some very good antibodies to eg plant hormones and these are starting to be developed into biosensors. An electrochemical

impedance-based electrode based on an antibody has been described for ABA [23]. This is reported to have a sensitivity range of 1 nM to 1 μ M, although there was an unfavourable pH dependence and a slow time resolution because it relied on binding equilibrium being reached. A sensor for auxin based on a competitive immunoassay recorded by QCM (quartz crystal microbalance) has also been described [24] and a voltametric electrode for gibberellins [25], each with similar good dynamic ranges, but poor time resolution. They have yet to yield novel biological information.

Outside the world of diagnostics, time resolution is often a limitation in immunological biosensors because of the need to allow binding to approach equilibrium, usually a matter of tens of minutes. However, redesigning the familiar competition-based assay to analyse data from the dissociation kinetic (as opposed to the normal binding/association curve) does allow quantitative real-time recordings (Badescu and Napier, unpublished). Of course, an assay of this type is *ex vivo*, but the assay platform combined with continuous sampling using microfluidics and microdialysis gives an antibody-based, quantitative real-time biosensor. The versatility, cost and availability of antibodies for immunological sensors carry user advantages. Plant developmental biology could undoubtedly benefit from harnessing the power of these immunosensor systems.

Genetically-encoded biosensors – promoter::reporters

One reason why solid-state sensors have not been widely exploited in plants has been the widespread success of genetically-encoded sensors. Genetic reporters sensitive to the activity of particular hormones have been available for a number of years, and amongst the most widely used has been the auxin-sensitive reporters based on the synthetic promoter DR5 [26]. The first generation used DR5 fused to β -glucuronidase (GUS) and this reporter gene has helped describe some of the many sites of auxin accumulation, in particular after gravitropic stimulation. More recently, optical sensor systems based on green fluorescent protein (GFP) and other FPs have become the fusion partners of choice.

The term auxin biosensor has been used to describe the DR5-GFP reporter [27]. It was used to define the temporal change in auxin activity maximum in the root columella and lateral root cap cells after gravitropic stimulation. Many other groups have also capitalised on the value and versatility of DR5-GFP and versions of it. Coupled with confocal microscopy and co-localisation with reporters for auxin transport proteins or IAA biosynthesis enzymes, for example, these biosensors have provided us with beautiful and detailed accounts of very localised and temporal auxin signals [28**].

The importance of promoter-reporter fusions in developmental biology is not under question, and there is no doubt that DR5 and similar biosensor drivers have contributed greatly to our understanding of plant signalling, but we should ask whether this type of biosensor is as good as we can get.

There are many attractions to the use of genetically-encoded, promoter-driven biosensors. They are free of the need to puncture or otherwise wound the host cells, they can be driven by sensitive, signal-dependent promoters and many can be monitored in living cells in real time. However, they are not perfect. DR5 is considered a fairly faithful reporter for IAA activity [26] but it is also induced by

brassinolide and some tissue selectivity is known [29,30]. Reporter systems for other hormones have been less instructive and even less analyte-specific [31].

There is also the question about time-responsiveness. Clearly, from induction to functional reporter there is a time delay. In gravitropism, auxin activity changes in the root could be detected by DR5::GFP only after 1.5 hours [27]. The need for more rapid sensors might be questioned for some analytes, yet there is no doubt that auxin, for example, redistributes rapidly and polar flow is generally recorded at around 10 mm/h, which means it may cross hundreds of cells each hour. Further, it is unwise to assume that because we have not previously been able to measure rapid changes in analytes that they do not exist or do not matter. GFP is generally a stable protein and so transience in the amplitude of a signal and the frequency of transients may also be missed, yet might be vital for message decoding as they are for intracellular calcium concentrations [32].

Reservations about some of the characteristics of the fluorescent proteins has led to novel, improved versions. Mutagenesis of YFP yielded Venus, a variant with reduced sensitivity to pH and chloride ions, and a much faster maturation time [33]. Triplicated FPs targeted to specific intracellular compartments [34*] have been used to good effect to increase signal amplitude and precision, but the complexities of driving the sensor signal via promoter activation, transcription, translation, maturation and degradation make the promoter-driven sensors qualitative reporters, not quantitative.

Genetically-encoded biosensors – other options

The optical biosensor field has been led and enlightened, in particular, by calcium sensitive reporters such as aequorin and its successors the cameleons [35]. The cameleons utilise the large conformational rearrangement induced by calcium binding to calmodulin to change the proximity of paired FPs fused at either end, frequently CFP and YFP or their variants, and the sensor is interrogated using FRET microscopy. Second generation versions were improved for selectivity by using engineered versions of calmodulin and a calmodulin-binding peptide in the bridge between FPs so that on binding calcium the sensor folds up on itself to promote FRET [35]. Other modifications generated a set of cameleons with differing calcium dose-dependencies to allow measurement over a wide set of concentration ranges suited to differing cellular compartments. FRET can be ratiometric allowing the recordings to be calibrated and quantitative. Furthermore, because expression of the biosensor is constitutive, there is no time delay before a signal can be recorded. Temporal changes can be recorded on the timescale of less than a millisecond [36**], although data acquisition timescales are frequently of the order of seconds due to constraints in the microscope. Likewise, dissociation and reversal of the signal is rapid making these biosensors very dynamic tools.

Examples of the use of cameleons in plants include the spiking signatures of cytoplasmic calcium induced by nodulation in *Medicago* [37] and intranuclear calcium spikes in root hairs [38**]. It should be noted that other calcium-sensitive chemical dyes such as Oregon Green are also still extremely useful even though they require single cell injections [39]. These allow analyses in plants without the need for transformation.

The depth of accumulated knowledge acquired from successive generations of sensors for intracellular calcium signalling is well reviewed [32] and developments continue [40**]. However, other metal ions are also amenable to genetically-encoded (and other) biosensors, although these have yet to be deployed in plants. Zinc enjoys a set of possible sensors based on both natural zinc-binding protein domains, such as metallothioneins, and on synthetic motifs [41*, 42*] and magnesium sensors can be envisaged based on engineered Troponin C [43]. Staining embryos of Norway spruce with zinc-sensitive chemical probes indicated that intracellular zinc mediates programmed cell death in plants [44]. More versatile analysis with biosensors may reveal additional roles in developmental responses.

Following on the heels of the cameleons has been the elegant engineering of bacterial periplasmic sugar-binding proteins to yield a set of optical metabolite biosensors. As above, the signal is transduced by the fusion of a conformationally-active centre, the metabolite binding domain, with FPs to give FRET reporters [45]. The biggest collection is the FLIPs (FLUorescent Indicator Proteins) from the Frommer laboratory and these are sensitive to physiologically-relevant concentrations of a set of pentoses, hexoses and disaccharides as well as some amino acids and phosphate [46**]. Such is the power of the FRET sensors that they have greatly improved the science of fluxomics, the record of changes in steady state concentrations over time, with some FRET sensors now allowing more than one analyte to be monitored at one time [45].

One other ratiometric biosensor that has been deployed in plant biology is the redox-sensitive roGFP. In this case excitation efficiency is sensitive to redox state, allowing calibration and a quantitative output. The probe has been used to report on the local redox state after wounding [47*], in various cellular compartments and from inside mitochondria during respiratory challenges [48*].

As discussed above for enzymatic sensor systems, FRET and other fluorescence-based reporters are restricted in use because they have relied on the availability of analyte-specific binding proteins or binding domains. The ability to combine the generic availability of antibodies with FRET-sensitive reporter cassettes would be highly valuable, although unfortunately still a dream. Alternative, widely applicable strategies are starting to emerge however. Many proteins are rapidly degraded once ubiquitinated and this may offer a way to get dynamic data for a wide variety of plant signals for which proteolysis is the natural regulatory process. For example, combining the degron domain of an Aux/IAA protein fused to Venus YFP under a constitutive promoter may yield a much more dynamic reporter for auxin than DR5 (T. Vernoux, ENS Lyon, France; unpublished). Where the size of an FP is troublesome, tetracysteine tags can be used with fluorescent biarsenicals [49]. Although this does require absorption of the fluor from a bathing medium, valuable data have been acquired from both luciferase and aequorin which each need exogenous cofactors.

Opportunities and targets

Modelling development

Plant developmental signalling has been led forward jointly by analytical biochemistry, in particular the advances of mass spectrometry [50**], and qualitative optical sensors. Until the advent of cell sorting to produce quantitative maps of eg IAA, all quantitative techniques lost spatial resolution of the analyte. Even with cell-

specific analysis the opportunities for fluxomics are lost. As noted, the use of qualitative optical sensors has allowed activity mapping with high spatial and increasingly better time resolution. These methods, along with the subcellular distribution maps of PIN and AUX proteins have provided information for several mathematical models of eg auxin signalling and channelling [51, 52**]. However, all such models work around the problem of having no quantitative data on auxin concentration and assume its location. Typically, data for other plant developmental signals trails that for auxin. Biosensors clearly have the potential to illuminate and refine both models and the physiology they mimic.

Biosensors in plant disease detection

Stepping away from hormonal control systems, there are many other areas of plant science for which biosensors could be rewarding. Systemic signalling could be amenable to any of the sensor systems described above [53*] and recent peroxide sensors could help study stress responses [5*], but there are also possibilities in disease detection and diagnosis. Label free biosensors that can detect and quantify specific plant pathogens on-field might enable farmers to target application of pesticides precisely, reducing their use [21*].

Currently antibodies are the preferred detection systems since they offer all the advantages noted above and many pathogen-specific antibodies are available. However, most transducing technologies remain lab-based instruments requiring specialist technicians, such as surface plasmon resonance (SPR), quartz crystal microbalance (QCM) and cantilever-based sensors. Field units need development [21*]. There is better news for volatile sensors and both electronic nose and field asymmetric ion mobility spectrometry (FAIMS) have been shown to be useful early disease and infestation sensors [55*, 56].

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References:

1. * Ronkainen NJ, Halsall HB, Heineman WR: Electrochemical biosensors. *Chem Soc Rev* 2010, 39:1747-1763.
A balanced and well presented review and explanation of electrochemical sensor technologies
2. Haccoun J, Piro B, Tran LD, Dang LA, Pham MC: Reagentless amperometric detection of L-lactate on an enzyme-modified conducting copolymer poly(5-hydroxy-1,4-naphthoquinone-co-5-hydroxy-3-thioacetic acid-1,4-naphthoquinone). *Biosens Bioelectron* 2004, 19:1325-1329.
3. Thomas SG, Phillips AL, Hedden P: Molecular cloning and functional expression of gibberellins 2-oxidases, multifunctional enzymes involved in gibberellins deactivation. *Proc Natl Acad Sci USA* 1999, 96:4698-4703.
4. Kulagina NV, Shankar L, Michael AC: Monitoring glutamate and ascorbate in the extracellular space of brain tissue with electrochemical microsensors. *Anal Chem* 1999, 71:5093-5100.
5. * Sanford AL, Morton SW, Whitehouse KL, Oara HM, Lugo-Morales LZ, Roberts JG, Sombers LA: Voltammetric detection of hydrogen peroxide at carbon fiber microelectrodes. *Anal Chem* 2010, 82:5205-5210.
Improved sensitivity and especially selectivity for hydrogen peroxide, plus good spatial and temporal resolution makes this electrode system well suited to biological monitoring. However, application will need invasive sampling.
6. Frebortova J, Fraaije MW, Galuszka P, Sebel M, Pec P, Hrbac J, Novak O, Bilyeu KD, English JT, Frebort I: Catalytic reaction of cytokinin dehydrogenase: preference for quinines as electron acceptors. *Biochem J* 2004, 380:121-130.
7. Prieto-Simon B, Cortina M, Campas M, Calas-Blanchard C: Electrochemical biosensors as a tool for antioxidant capacity assessment. *Sensors Actuators* 2008, 129:459-466.
8. Tembe S, Inamdar S, Haram S, Karve M, D'Souza SF: Electrochemical biosensor for catechol using agarose-guar gum entrapped tyrosinase. *J Biotechnol* 2007, 128:80-85.
9. Jarosz-Wilkolazka A, Ruzgas T, Gorton L: Use of laccase-modified electrode for amperometric detection of plant flavonoids. *Enz Microbial Technol* 2004, 35:238-241.
10. * Kim S-Y, Sivaguru M, Stacey G: Extracellular ATP in plants. Visualisation, localization, and analysis of physiological significance in growth and signalling. *Plant Phys* 2006, 142:984-9992.
A clever application of a natural ATP sensor, the luciferase enzyme. By fusing it with a cellulose-binding peptide it accumulates in the cell walls. Controls were run with other purine ribosides to test the specificity of the response. Temporal resolution was good and in the minutes, but ATP quantification is not possible.
11. Chivasa S, Tome DFA, Murphy AM, Hamilton JM, Lindsey K, Carr JP: Extracellular ATP. A modulator of cell death and pathogen defence in plants. *Plant Sign & Behav* 2009, 4:1078-1080.
12. Llaudet E, Hatz S, Droniou M, Dale N: Microelectrode biosensor for real-time measurement of ATP in biological tissue. *Anal Chem* 2005, 77:3267-3273.

13. Hernandez P, Galan F, Hernandez NL: Direct determination of indole-3-acetic acid in plant tissues by electrochemical techniques using a carbon paste modified with OV-17 electrode. *Electroanal* 1994, 6:577-583.
14. Wu K, Sun Y, Hu S: Development of an amperometric indole-3-acetic acid sensor based on carbon nanotubes film coated glassy carbon electrode. *Sensors Actuators* 2004, 96:658-662.
15. ** Mancuso S, Marras AM, Magnus V, Baluska F: Noninvasive and continuous recordings of auxin fluxes in intact root apex with a carbon nanotube-modified and self referencing electrode. *Anal Biochem* 2005, 341:344-351.
 Many of the problems associated with electrode-based systems are overcome by increasing sensitivity with nanotubes and by using self-referencing to improve specificity. Applied in the tractable system of the root apex where electrode access was non-invasive and the apoplast is amenable to the boundary layer, the data give the first dynamic record of auxin concentrations and flux in living tissue.
16. Kabilan S, Marshall AJ, Sartain FK, Lee M-C, Hussain A, Yang X, Blyth J, Karangu N, James K, Zeng J, Smith D, Domschke A, Lowe CR: Holographic glucose sensors. *Biosens Bioelectron* 2005, 20:1602-1610.
17. * Li D, Shlyahovsky B, Elbaz J, Willmer I: Amplified analysis of low-molecular-weight substances or proteins by the self-assembly of DNAzyme-aptamer conjugates. *J Am Chem Soc* 2007, 129:5804-5805.
 Early days for a technology that may come to dominate high-throughput biosensor applications for small molecules.
18. * Barton AC, Collyer SD, Davis F, Garifallou G-Z, Tsekenis G, Tully E, O'Kennedy R, Gibson T, Millner PA, Higson SPJ: Labelless AC impedimetric antibody-based sensors with pg ml⁻¹ sensitivities for point of care biomedical applications. *Biosensors Bioelectron* 2009, 24: 1090-1095.
 Advances in immunosensors will open up the biosensor world to all those who do not have selective enzymes or conformationally active receptors. This work shows that test and reference electrodes can be interrogated to give sensitive and specific signals for a series of antibodies to monitor both small and large analytes. Fabrication is a specialised job, but reproducible biosensors for most antigens is now possible. However, sampling protocols for living tissue still need developing.
19. ** Muller DJ, Helenius J, Alsteens D, Dufrene YF: Force probing surfaces of living cells to molecular resolution. *Nature Chem Biol* 2009, 5:383-390.
 Recent developments in atomic force microscopy allow not just surface topology to be recorded at high spatial resolution, but for activities and fluxes of specific analytes to be mapped onto the same surfaces. This review gives a fine overview. Although this can be done on living tissue, the problems of depth and access will mean that its application to plant cells will be challenging, but root hairs and other surface features, or isolated organelles, might be amenable.
20. Marshall AJ, Kabilan S, Blyth J, Lowe CR: Analyte-sensitive holograms for (bio)chemical analysis. *J Phys Condens Matter* 2006, 18:S619-S626.
21. * Skottrup PD, Nicolaisen M, Justesen AF: Towards on-site pathogen detection using antibody-based sensors. *Biosens Bioelectronics* 2008, 24:339-348.
 A useful review on the state of the art for diagnostic biosensors.

22. Leonard P, Safsten P, Hearty S, McDonnell B, Finlay W, O’Kennedy R: High throughput ranking of recombinant avian scFv antibody fragments from crude lysates using the Biacore A100. *J Imm Method* 2007, 323:172-179.
23. Li Y-W, Xia K, Wang R-Z, Jiang J-H, Xiao L-T: An impedance immunosensor for the detection of the phytohormone abscisic acid. *Anal Bioanal Chem* 2008, 391:2869-2874.
24. Li J, Wu Z-W, Xiao L-T, Zeng G-M, Huang G-H, Shen G-L, Yu R-Q: A novel piezoelectric biosensor for the detection of phytohormone β -indole acetic acid. *Anal Sci* 2002, 18:403-407.
25. Li J, Xiao L-T, Zeng G-M, Huang G-H, Shen G-L, Yu R-Q: Immunosensor for rapid detection of gibberellic acid in the rice grain. *J Agric Food Chem* 2005, 53:1348-1353.
26. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ: Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* 1997, 9:1963-1971.
27. Ottenschlager I, Wolff P, Wolverson C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K: Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proc Natl Acad Sci USA* 2003, 100:2987-2991.
28. ** Pagnusset GC, Alandete-Saez M, Bowman JL, Sundaresan V: Auxin-dependent patterning and gamete specification in the *Arabidopsis* gametophyte. *Science* 2009, 324:1684-1689.
The definition of auxin activity maxima in just a few cells of the nucellus, and hence local auxin gradients, during early female gametophyte gives exquisite detail to developmental biology and the power of DR5 as an auxin biosensor. This is the latest of papers from many groups which have given similar insights at the shoot apical meristem and during lateral root emergence. However detailed the spatial resolution gets, these remain qualitative sensors.
29. Nakamura A, Higuchi K, Goda H, Fujiwara MT, Sawa S, Koshihara T, Shimada Y, Yoshida S: Brassinolide induces IAA5, IAA9, and DR5, a synthetic auxin response element in *Arabidopsis*, implying a cross talk point of brassinosteroid and auxin signalling. *Plant Physiol* 2003, 133:1843-1853.
30. Chandler J: Local auxin production: a small contribution to a big field. *BioEssays* 2009, 31:60-70.
31. Hagenbeek D, Quatrano RS, Rock CD: Trivalent ions activate abscisic acid-inducible promoters through an *ABII*-dependent pathway in rice protoplasts. *Plant Physiol* 2000, 123:1553-1560.
32. McAinsh MR, Pitman JK: Shaping the calcium signature. *New Phytol* 2008, 181:275-294.
33. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A: A variant of yellow fluorescent protein with fast and efficient maturation for cell biological applications. *Nature Biotech* 2002, 20:87-90.
34. * Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM: Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. *Current Biol* 2005, 15:1899-1911.
Describes the evolution of one of the most selective and sensitive of the

- DR5-based auxin sensors. Repeated FPs, use of DR5_{rev}, and a nuclear localisation motif all help create a controlled and sensitive qualitative biosensor. In concert with other auxin-relevant reporters, a picture of dynamic control of development by auxin is shown.
35. Palmer AE, Jin C, Reed JC, Tsein RY: Bcl-2-mediated alterations in endoplasmic reticulum Ca²⁺ analysed with an improved genetically encoded fluorescent sensor. *Proc Natl Acad Sci USA* 2004, 101:17404-17409.
 36. ** Okumoto S: Imaging approach for monitoring cellular metabolites and ions using genetically-encoded biosensors. *Curr Opin Biotech* 2010, 21:45-54.
A well worked review of the technology of genetically-encoded fluorescence-based metabolite biosensors and the methodology behind their use for fluxomics.
 37. Miwa H, Sun J, Oldroyd GED, Downie JA: Analysis of calcium spiking using a cameleon calcium sensor reveals that nodulation gene expression is regulated by the calcium spike number and the developmental status of the cell. *Plant J* 2006, 48:883-894.
 38. ** Sieberer BJ, Chabaud M, Timmers AC, Monin A, Fournier J, Barker DG: A nuclear-targeted cameleon demonstrates intranuclear Ca²⁺ spiking in *Medicago trunculata* root hairs in response to Rhizobial nodulation factors. *Plant Physiol* 2009, 151:1197-1206.
One big advantage of genetically-encoded biosensors is the capacity to direct them into specific compartments. The cameleons are archetypal calcium sensors, but this work shows how much information can be derived from biosensors when they are fully evolved. Not only are subcellular flows of analyte measured, but they are calibrated and analysed at high temporal resolution. The cells were analysed every 5 s to give a record of calcium spiking over tens of minutes.
 39. Capoen W, Herder JD, Sun J, Verplancke C, De Keyser A, De Rycke R, Goormachtig S, Oldroyd G, Holsters M: Calcium spiking patterns and the role of the calcium/calmodulin-dependent kinase CCaMK in lateral root base nodulation of *Sesbania rostrata*. *Plant Cell* 2009, 21:1526-1540.
 40. ** Saito K, Hatsuai N, Horikawa K, Kobayashi K, Matsu-ura T, Mikoshiba K, Nagai T: Auto-luminescent genetically-encoded ratiometric indicator for real-time Ca²⁺ imaging at the single cell level. *PLoS ONE* 2010, 5:e9935.
One step on from FRET is BRET, bioluminescence resonance energy transfer. With no need for an external laser light source it is free from phototoxicity and (importantly for plants) autofluorescence. This permits high signal-noise ratios and possibly analysis deeper inside intact tissues. Bioluminescence alone gives a weak signal, but linking it to the enhanced FPs and the well known cameleon calcium-sensing framework has yielded a sensitive, bright, ratiometric quantitative biosensor with broad dynamic range named BRAC.
 41. * Dittmer PJ, Miranda JG, Gorski JA, Palmer AE: Genetically-encoded sensors to elucidate spatial distribution of cellular zinc. *J Biol Chem* 2009, 284:16289-16297.
There have been many zinc sensors based on zinc-binding proteins, but this makes use of a short zinc-finger motif between the familiar CFP and

YFP FRET pair. Mutagenesis of the zinc-finger improve properties and the cassette is provided with targeting signals for a number of subcellular compartments, cytoplasm, plasma membrane and mitochondria. A dynamic picture of zinc homeostasis and stimulus-induced fluxes is generated for brain cells.

42. * Maret W: Fluorescent probes for the structure and function of metallothionein. *J Chromatog B* 2009, 877:3378-3383.
Plants have a bunch of metallothioneins. This paper examines how fluorescent probes are used to describe metal binding, in particular zinc binding over four orders of magnitude of concentration. The same reporters could be harnessed to report on plant zinc signals.
43. Davis JP, Rall JA, Reiser PJ, Smillie LB, Tikunova SB: Engineering competitive magnesium binding into the first EF-hand of skeletal Troponin C. *J Biol Chem* 2002, 277:49716-49726.
44. Helmersson A, von Arnold S, Bozhkov PV: The level of free intracellular zinc mediates programmed cell death/cell survival decisions in plant embryos. *Plant Physiol* 2008, 147:1158-1167.
45. Lalonde S, Ehrhardt DW, Frommer WB: Shining light on signalling and metabolic networks by genetically encoded biosensors. *Curr Opin Plant Biol* 2005, 8:574-581.
46. ** Okumoto S, Takanaga H, Frommer WB: Quantitative imaging for discovery and assembly of the metabolite-regulome. *New Phytol* 2008, 180:271-295.
Everything you need to know about quantitative imaging of FRET biosensors for metabolites and metabolic regulation. Well written and accessible.
47. * Meyer AJ, Brach T, Marty L, Kreye S, Rouhier N, Jacquot J-P, Hell R: Redox-sensitive GFP in *Arabidopsis thaliana* is a quantitative biosensor for the redox potential of the cellular glutathione redox buffer. *Plant J* 2007, 52:973-986.
Redox control and the role of the glutathione pools in plants are reported using cytosol- and endoplasmic reticulum-targeted roGFP.
48. * Schwarzlander M, Fricker MD, Sweetlove LJ: Monitoring the *in vivo* state of plant mitochondria: effect of respiratory inhibitors, abiotic stress and assessment of recovery from oxidative challenge. *Biochim Biophys Acta* 2009, 1787:468-475.
Reactive oxygen species are linked to almost all environmental and biotic stress responses in plants. This paper shows that the redox-sensitive biosensor can help understand the cross-talk and the role of both redox status and mitochondria in signalling. Responses to a range of challenges are recorded. Without targeted biosensors this level of detail about mitochondrial function would remain hidden.
49. Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY: New biarsenical ligands and tetracysteine motifs for protein labelling in vitro and in vivo: synthesis and biological applications. *J Am Chem Soc* 2002, 124:6063-6076.
50. ** Petersson SV, Johansson AI, Kowalczyk M, Makoveychuk A, Wang JW, Moritz T, Grebe M, Benfey PN, Sandberg G, Ljung K: An auxin gradient and maximum in the *Arabidopsis* root apex shown by high-resolution cell-specific analysis of IAA distribution and synthesis. *Plant*

Cell 2009, 21:1659-1668.

Not a biosensor paper, but if illustration were needed of the value of biosensors compare the effort required to achieve cell-type and tissue level quantitation of an analyte (here auxin) by more classical analytical technologies and the work with cameleons above. The effort and dedication, the use of genetically-marked cell files are all admirable and there is no question about quantitation, but dynamic biosensors are clearly important for the field to move forward further.

51. Kramer EM: Computer models of auxin transport: a review and commentary. *J Exp Bot* 2008, 59:45-53.
52. ** Bayer EM, Smith RS, Mandel T, Nakayama N, Sauer M, Prusinkiewicz P, Kuhlemeier C: Integration of transport-based models for phyllotaxis and midvein formation. *Genes Devel* 2009, 23:373-384.
State of the art for promoter-based reporters and a beautiful illustration of the value of biosensor data for mathematical model building, but still without quantitative data on the major determining hormone.
53. * Park S-W, Kaimoyo E, Kumar D, Mosher S, Klessig DF: Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science* 2007, 318:113-116.
Not all plants require the translocation of methyl salicylate, but a good illustration that there are many compounds moving actively in plants at undefined rates and concentrations. We need to know more.
54. * Laothawornkitkul J, Moore JP, Taylor JE, Possell M, Gibson TD, Hewitt CN, Paul ND: Discrimination of plant volatile signatures by an electronic nose: a potential technology for plant pest and disease monitoring. *Environ Science Technol* 2008, 42:8433-8439.
Biosensors are not suited to every analysis or measurement. Plants give off many volatile organic compounds (VOCs) and we are starting to use solid-state sensors to analyse them to our benefit.
55. Zhang F, Ghaffari R, Iliescu D, Hines E, Leeson M, Napier R: Field asymmetric ion mobility spectroscopy based plant disease detection: intelligent systems approach. (In Press)
56. Zhang S, Xia J, Li X: Electrochemical biosensor for detection of adenosine based on structure-switching aptamer and amplification with reporter probe DNA modified Au nanoparticles. *Anal Chem* 2008, 80:8382-8388.