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Exploring Novel Panorama Within Plant-microbe Interface

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ABSTRACT

All plant-microbe interactions are initiated at the level of the cell. Recently, the light microscope has increased in popularity as an investigative tool in plant cell biology despite certain limitations, in part because of the parallel development of some sophisticated techniques like confocal laser scanning, video microscopy, computerized image processing and an ever-increasing array of fluorescent probes that can be applied to living cells.

This review describes some of these approaches and discusses how they are taking us a step closer to viewing the intricate complexity with which plants regulate their own functions down to sub-cellular level.

Keywords: Plant -microbe interaction, microscopic techniques, bio techniques, image processing, living cell, cytology, video microscopy

INTRODUCTION

Microscopic techniques have been of great value in studying plants as well as microbes, their developmental processes and host-microbe interaction. Although, the light microscope does not achieve the resolution provided by the electron microscope, it has recently upsurge in popularity in plant researches because of the parallel development of confocal laser scanning, video microscopy, computerized image processing and an ever-increasing array of fluorescent probes that can be applied to living cells (Heath, 2000; Heyens *et al.* , 2002; Kuzentsov *et al.* , 2001; Zhang *et al.* , 2001; Zizika *et al.* , 2001). The light microscope is now regularly used for detection of gene expression at the cellular level by an *in-situ* hybridization or for the use of visually detectable reporter genes. Electron microscopy has proved increasingly important for the study of plant-pathogen interaction especially after the introduction of immunological, cytochemical, electron microscopy procedures. Nowadays, advances in cellular preservation techniques like high pressure freezing or the use low temperatures have minimized artifacts induced by fixation procedures necessary to prepare tissue for electron microscopy. Video-enhanced contrast microscopy has allowed the recording of real- time events occurring in the living cell.

Immunocytochemical Technique

This technique allows the visualization of specific structures or proteins within the cell. This non-destructive *in-vivo* method has revealed much about fungal cell walls and cytoskeleton (Hardham and Mitchell, 1998). Comparatively late, immunofluorescent microscopy was used to study putative pathogenicity determinants such as secretion of fungal toxins and degradative enzymes (Hardham and Mitchell, 1998). The major shortcomings of the technique is that when the structures inside the cells are to be labeled, permeabilization to the antibody is necessary. Recently, an improved freeze-substitution preparative method to permeabilize whole cells has been described (Bourett *et al.* ,1998). One major advantage of immunofluorescence over the labeling technique is that no fungal transformation system is required e.g., genes induced *in-vivo*, viz. a putative amino-acid transporter (Hahn and Mendgen 1997) and protein probably involved in thiamine biosynthesis (Sohn *et al.* 2000) have been

visualized in haustoria providing interesting information about the role of haustoria in uptake of nutrients from the host cells and biosynthesis of metabolites.

Imaging Technology

1. Conventional Fluorescence Imaging

Conventional epifluorescence microscopy coupled to image analysis has become a powerful tool and has yielded fundamental insights into the functioning of plant cell. The capabilities of this approach have illustrated the regulation of plasmodesmatal function by viruses. Viral movement proteins operate by enlarging the size exclusion limit of plasmodesmata, allowing intercellular transfer of viral nucleic acid (Vaquero *et al.* 1994). This function was largely revealed by monitoring the effect of these proteins on the cell-to-cell transfer of fluorescently labeled dextrans of different sizes. The ability to image and analyze the intercellular transport of fluorescent dyes between cells has led to co-ordinate developmental programs that characterize many aspects of plant development.

This technique suffers from limitations in spatial resolution, quantitative accuracy and image degradation due to the out of focus blur, associated with conventional microscope optics.

2. Simultaneous Ion-imaging and Electro-physiology

An exciting use of this ion-imaging technology is in the simultaneous application of ratio analysis and electro-physiology (Child and Lischka, 1994). Although this technique has not been exploited as such in plant-microbe interaction, yet its indirect implications are broad horizoned. Schroeder and Hagiwara (1990) monitored the effects of ABA on ion channel activities and catabolic Ca^{++} levels in guard cell protoplasts. They found that there was a direct temporal correlation between the openings of Ca^{++} permeable channels and increase of cytosolic Ca^{++} induced by ABA. Such data have been instrumental in developing the model for Ca^{++}dependent signal transduction in ABA action in guard cells.

3. Fluorescence Ratio Analysis

Ratio analysis corrects quantitative fluorescence imaging from artifacts in signal strength associated with spatial and temporal variations in sample path length, accessible volume and local indicator concentration (Gilroy, 1997). The approach has been most widely used with plants to monitor ion-levels, principally cytoplasm levels of Ca^{++} and pH because of their fundamental role in signal transduction and ion homeostasis (Bush, 1995). The technique also plays an important role in quantitative analysis of structural dynamics such as actin polymerization (Gilroy, 1997). Generally, radiometric dyes whose fluorescent properties are dependent on the local free concentration of ions (e.g. Ca^{2+}, H^+, Mg^{2+}, $K^{+,}$ Na^+, Cl^-, Zn^{2+}) is used. Their wavelength of fluorescence that increases with ion concentration is monitored and compared with the intensity at a wavelength that is insensitive to or decreases with increasing ion levels.

When a radiometric indicator is unavailable, pseudo-ratio analysis has been applied. In this approach, the signals from two unlinked indicators, one sensitive and one insensitive to the molecule under analysis are used to make the ratio image. The major assumptions for pseudo-ratioings are that the two fluorochromes used redistribute identically and photo-bleach at the same rate. Despite some caveat, the pseudo-ratio technique can be extremely informative e.g., a ratio-able pH indicator for the acidic apoplastic environment is currently unavailable.

However the “single wavelength” pH indicator Cl-NERF can respond to pH in range 4 to 6. In an attempt to use this indicator to image apoplastic pH, the cell-wall space of corn was infiltrated with Cl-NERF and pH insensitive dye Texas Red 3000 (Chiu *et al.,* 1996). Ratio analysis revealed that after 45 min. of gravistimulation, a local acidification (from pH 4.9-4.4) occurred in the elongation zone cells of the upper side of the root. Such analysis is predicted by the acid growth model for tropic response of roots.

4. Fluorescence Resonance Energy Transfer

While ratio analysis increases the quantitative resolution of the fluorescence microscope, FRET increases the resolution of spatial inter-action between cytoplasmic structures.

In FRET analysis, two interacting cell components are labeled with different fluorochromes in such a way that when one, the donor is excited with its appropriate wavelength of light, some part of its emission energy is transferred to the second fluorochrome, the acceptor, which then fluoresce.

The efficiency of the energy transfer (E) falls with the inverse of the 6th power of the distance (R) according to the equation $E = [(1+R/R_o)^6]^{-1}$. R_o is the characteristic distance at which FRET is 50% efficient and depends on the quantum yield of the donor fluorochrome, the absorbency characteristics of the acceptor and the overlap in emission and excitation of both (Tsien *et al.,* 1993). The technique provides the capability to image molecular interactions with super resolution, well beyond the point to point resolution of conventional fluorescence microscopy, e.g., the interaction between plant EF-1 alpha, microtubules and Cam has recently been demonstrated *in-vitro* (Durso and Cyr, 1994) and has highlighted the potential role of

EF-1 alpha as a multifunctional protein in regulating microtubule dynamics (Collings *et al.,* 1994; Durso and Cyr, 1994), actin dynamics (Collings *et al*., 1994), protein synthesis (Merrick,1992), phosphatidylinositol signaling(Yang *et al.*, 1993) and a myriad other cellular functions (Collings *et al.,* 1994; Durso and Cyr, 1994). Critically this approach is applicable to living cells where fluorescent-labeled proteins can be co injected and the dynamics of *in-vivo* interactions could be analyzed with nanometer resolution.

5. Three Dimensional Fluorescence Microscopy

Since cells and tissues are three-dimensional objects, the spatial component of their organization often underlies their function. TDFM has proven highly useful to calculate and remove out of focus information.

a. Computational approach

One approach to solve the problem of out of focus blur is to calculate the contribution of this information in each image of the specimen and then subtract it, leaving only the in focus image. A range of such computational approaches are available (Gilroy, 1997; Scalettar *et al.,* 1996) and can be applied to images of almost any fluorochrome because data are collected using a conventional fluorescence microscope. The newer algorithms also allow the dynamic processes to be observed by reducing the requirement for the time consuming collection of a large, continuous stretch of images for the deblurring calculation (Gilroy, 1997).

Although highly successful in resolving 3-D data, the computational approach has received much less attention by plant biologists than a purely optical way to section specimens, the confocal microscope.

b. Confocal microscopy

With the confocal microscopy, an object is viewed through a pinhole (slit) set at a focal point in the optical path. Light from the focal plane of the specimen passes through the pinhole to a photo-multiplier detector, whereas light from regions outside the focal plane is largely blocked. Thus, the microscope collects images that are essentially only from the focal plane of the microscope objective. In the confocal laser-scanning microscope, illumination is provided by the well-defined wavelength and intense collimated beam of light offered by a laser. The illuminating beam is focused to a spot on the specimen and the resultant in focus fluorescence emission passes through the confocal aperture to the photo-multiplier detectors. A point-by-point map of the fluorescence intensity in the focal plane of the specimen is constructed by moving the laser across the sample. By taking sequential optical sections at the known two-axis positions, a full-3-D-view of cell can be calculated. Since, no physical sectioning is required for 3-D-data, sample can be a living cell. LSCM is particularly advantageous for examining host-pathogen interactions where the fungus is embedded deep within the host tissue. GFP is chromophore suitable to fully exploit LSCM in the study of biological processes *in-vivo.* Dual labeling with multiple GFPs compatible with the excitation range of the laser used for confocal microscopy is now possible, allowing the observation of two different structures or proteins in the same sample (Chiu *et al.,* 1996).

c. Two photon microscopy

The two-photon microscopy approach uses a mode-locked laser driven by a high power argon laser to deliver sub pico-second bursts of illumination at a frequency of about 100 mHz. The wavelength of the excitation beam is twice that normally required to excite the fluorochrome in question. Two photons of this longer wavelength laser light are simultaneously absorbed by the fluorochrome and deliver equivalent excitation energy to one photon of the normal, shorter excitation light. Long wavelength light is much more penetrating, leading to a capacity for deeper optical sections into tissues with fewer aberrations and image degradation. With this approach, the problem of out of focus illumination and photo-toxicity are reduced because excitation is limited to very small volume at the focal plane and it drops off very rapidly away from this point (Gilroy, 1997).

Fluorescent Indicators

The production of new fluorochromes and indicators has provided a significant driving force for the current advances in cell biology and microscopy. These fluorochromes are available as a range of reactive derivatizing reagents making selective labeling of cell components, environment sensitive fluorophores for range of ions (Haugland, 1992; Read *et al.* 1992; 1993) and hydrophilic and hydrophobic environments (Haugland, 1992).

Fluorescent Probes

New fluorescent dyes are increasingly available that bind specifically to cellular components such as nucleic acids, membranes or specific organelles. Dyes can be used *in-vivo,* and are compatible with confocal or standard fluorescent microscopy. Some fluorescent dyes are sensitive to reactive oxygen species, enzyme activities, pH, and ions and can therefore be used to follow physiological changes inside cells (Heath, 2000). There are dyes that can be internalized by fungal cells. Few of them can only bind to its intracellular target after techniques such as microinjection or electroporation (Jackson and Hardham, 1998).

Fluorescent Analog Cytochemistry

In FAC, a fluorescently labeled protein is introduced into the cell, which incorporates into the pool of normal cellular protein, and functions in normal cellular processes. This fluorescent analog approach has been coupled to high resolution, time lapse DIC video microscopy to directly demonstrate the dynamic regulation of pre-prophase microtubule function and mitotic progression by Ca^{2+} levels (Zhang *et al.*, 1990) and cyclin related kinases (Hush *et al.*, 1996) in dividing cells of *Tradescantia* hairs.

Protein Biosensors

The elegance of the protein biosensor approach is that it capitalizes on the inherent specificity of the tagged proteins to produce a highly selective probe. Protein biosensors are proteins that are tagged with an environmentally sensitive fluorophore.

Video Microscopy

It enhances the performance of the light microscope by taking advantage of electronic imaging and image processing. A charge-coupled device (CCD), a video-camera, converts the optical image to an electrical video signal. Some image processing can be controlled through the camera circuitry and even more can be done after the image has been digitized. These enhancements of the image allows the non-destructive visualization, recording and analysis of events in living cells unresolvable by conventional light microscopy (Heath, 2000).

Laser Trapping

Intense laser beams that are concentrated into a spot can generate enough photo-driven force to "capture" small subcellular particles (~100nm to 1µm) that have refractive index different from their surroundings. These optical tweezers, using near IR light that is relatively poorly absorbed by biological material or an argon ion-laser can grasp and manipulate structures in living cells. The potentiality of the technique has been exploited in optically trapping and displacing transvacuolar and cortical cytoplasm strands--indicating tension and elasticity of the cytoplasm, which change during the plant-microbe interaction (Heath, 2000).

Image processing

Digitized images, produced by video microscopy using conventional light microscopes or CLSMs, can be manipulated or analyzed in a number of ways using the wide variety of image analysis software program that are commercially available. Specific parts of the image can be selected for morphometric or densitometric measurements and areas of a specific gray level can be measured, as can the size of specific structures. Different images may also be added, subtracted, divided, multiplied on a pixel. Such manipulations are the basis of ratio-imaging of intracellular levels of ions such as Ca^{2+} during which images are collected alternately after exciting the specimen with a wavelength at which the flourescent dye is sensitive to Ca^{2+} concentration and one at which the same dye is relatively insensitive. The ionic concentration is calculated from the ratio of two images (Heath, 2000).

Aequorin

Many jellyfish show green luminescence upon mechanical stimulation. This light comes from a Ca^{2+}activated photo-protein called aequorin. The aequorin apoprotein combines with a cofactor coelentrazine, to yield a protein that upon binding Ca^{2+} emits a photon of blue light (475 nm). It is loaded into cell by variety of techniques, viz., mechanical, chemical, electrical permeabilization of plasma membrane and microinjection (Callaham and Hepler, 1991; Gilroy, 1997). The luminescence of the protein is then monitored. This aequorin technology allows the non-invassive visualization of signaling events in living tissues. Although signal levels from plants expressing aequorin are generally too low to allow cellular imaging, however, the use of these organelle targeted aequorins, effectively provide subcellular resolution for the technique. This technology reveals the complexity of whole organ and whole plant Ca^{2+}signaling network that may well co-ordinate plant responses to diverse environmental stimuli.

Caged Probes

Using the fluorescence and luminescence technology, one can now literally see the dynamics of the structural, biochemical and regulatory activities of the plant cell. However, some recent advances in optical technology have allowed us to begin to manipulate and control the cell in very regulated ways.

One such approach to manipulating the cytoplasm at the single cell level is through the use of caged compounds. These compounds are molecules whose biological activities have been chemically marked by photolytic "caging" group. Illuminating the caged compounds with UV light, causes the cage to open and the biologically active molecule to be released (Adam and Tsien, 1993). The timing, amplitude and localization of illumination and there fore of caged probe release, are highly controllable.

The use of caged compounds has greatly expanded over the past few years and the development of commercially available caging kits (Molecular Probes, Eugene, OR) will undoubtedly continue to advance this field. Many caged compounds are related to signal transduction including caged neurotransmitters, hormones, nucleotides, ions and peptides. There are now caged fluorochromes, structural proteins and even enzymes. The ability to manipulate and

control cell chemistry on demand with light is proving a powerful approach to dissect the role of putative regulators of plant cell function. Caged phytohormones have been synthesized and used to determine an intracellular site for an ABA receptor in guard cells. The use of caged Ca^{2+} and inositol 1,4,5, triphosphate has revealed the regulatory role of Ca^{2+} in hormonal regulation of stomatal function, growth of pollen tubes, etc.

CONCLUSIONS

The article reviews briefly some of the more complex techniques which have been employed, particularly in fungi, noting their value and the types of information which may be derived from them. The employment of a particular technique in a family or genera leads to data of interest to taxonomist. Fluorescent microscopy techniques, especially with laser confocal microscopy, allow nondestructive observation.

However, a significant goal of all those using these new imaging techniques will continue to be the development of biological systems where response parameters can be viewed in a single living functioning cell.

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