Module 7

Cell Biology

Martin Crespi, Béatrice Satiat-Jeunemaitre,

Hanh Trinh and Spencer Brown

Local organiser : Martin Crespi

1. INTRODUCTION

To understand gene function, it is required to characterise the gene products, analyse their expression patterns at tissue, cellular and subcellular levels as well as to study the consequences of functional modifications. This will serve to construct networks of gene products inside the cell and to validate any interaction identified using *in vitro* biochemical approaches. The aim of our module is to introduce students to techniques for the analysis of localisation of gene products in cells from *Medicago truncatula* tissues by combining *in situ* and *in vivo* approaches (i.e. immunolocalisation and monitoring expression of GFP translational fusions, respectively).

We will see, in parallel, different applications of the use of fluorescent probes for labelling *Medicago* cell compartments and these fluorochromes will also serve in cytometry.

We will use various approaches during the proposed experiments :

1. **In fixed cells**, an immunocytolocalisation experiment of selected proteins will be done on : a) "squash" root material and b) thick "whole mount" sections of root nodules. Antibodies recognising Golgi antigens (JIM84), microtubules (anti-tubulin), the endoplasmic reticulum (anti-HDEL) and specific inner cortical cells from the nodule (anti-CA, carbonic anhydrase) will be used on the different samples. Optical and confocal microscopy will serve to analyse the results, coupled with the application of fluorescent probes for labelling specific subcellular compartments.

2. **In living cells**, dynamic analysis of gene expression with constructs carrying fusions of selected proteins to the fluorescent reporter gene GFP will be carried out in a transient expression assay (bombardment of *Medicago truncatula* leaves).

3. In dissociated cells from **transgenic plants** expressing a MAP4-GFP fusion, microtubule reorganisation will be analysed.

4. In nuclei from fresh plant material, we shall use **flow cytometry** and specific fluorochromes to assess nuclear DNA levels. In this way one may simply estimate genome size (using an internal reference) and also screen for changes in ploidy level (by reference to known plants). The same method will also serve to reveal endoreduplication in somatic tissue, notably in nodules where it is a major developmental event.

2. LIGHT, CONFOCAL MICROSCOPY, IMAGE ANALYSIS AND CYTOMETRY : PRINCIPLE OF EQUIPMENT USED DURING THE COURSE

2.1. Microscopy and Imaging

Different microscopes are available for the course at the ISV. In practice, the observations will be made at a conventional microscope before progressing to a **confocal microscope**.

A general scheme of the conventional **wide-field light microscope**, in the epifluorescence mode is given in **Figure 1.** Whether the stand is upright or inverted, "epi-" (="above") signifies that the objective is serving also as condenser for the excitation beam.

The confocal microscope (**Figure 2**) introduces numerical imaging and laser scanning devices. Its principle is to use a pin-hole to block out-of-focus light, thereby improving diffraction-limited resolution in the image plane by two-fold (to ~200 nm) and most significantly in the Z-axis of observation (~500 nm). Our modern confocal microscopes no longer use glass interference filters to select and modulate incoming laser lines, but **Acousto-Optical Tuneable Filters (AOTF)** (**Figure 3**). These add tremendous flexibility and facilitate kinetic studies such as Fluorescent Redistribution After Photobleaching (FRAP). Explanations for image restoration (correction of defects), image enhancement (emphasising certain qualities) and image analysis (extraction, morphometry, granulometry and pulling out statistics) are beyond the available time of this course. Nevertheless, the principles of elementary **numerical filters** (smoothing, contrasting, medians, projections, etc.) will be on display -for those who are unhappy to just press buttons on Photoshop.

Upright light microscopes Polyvar Reichert

These microscopes offer the following modes : Transmission, Differential Interference Contrast (Nomarski), Dark-Field, Epi-fluorescence and Epi-reflectance. Note the choice of infinitely-corrected objectives (dry, oil, ± iris) and their available frontal (working) distances. As for any microscope, check the optical alignment (Koehler) at the beginning. The microscopes are equipped with cameras for photography on film (generally TMAX 100 ASA for black and white or FUJI 400 ASA Provia for fluorescence) or with a digital colour CCD camera (Nikon DXM1200 : 1.3-12 million pixels). **Charge Coupled Device (CCD) cameras** offer a definition that is almost as good as the human eye or film, with advantages of higher sensitivity, different spectral response and simplicity thanks to digitalisation. A matrix of micro-captors receive photons, which are converted into an electronic current then digitalised. Obviously, before any numerical imaging we have to explore data handling. Large images are often unnecessary, and they block networks or software programs. To process an image, a computer uses memory three-fold the file size (input, processing, output tables). Compressed formats are routine, but avoid successive compression of a given image each time it is saved. When doing photography, use the occulars to correct for your own eyes so that thereafter you and the camera will have the same focus.

Upright confocal microscope Leica SP2

Table 1 gives some of the optical choices available. Both Leica microscopes offer spectral analysis, variable spectral slit-widths (choice of colours to detect) and use AOTF elements to modulate laser lines in several microseconds. Although Table 1 is excessively detailed, such a sheet should be prepared in order to optimise objectives, mounting media, resolution and sampling density (pixel size). This upright microscope has an additional UV laser and 5 channels (detectors).

Inverted confocal microscope Leica SP2

This microscope has a transmitted light detector (for DIC) but only 4 channels (See **Table 1)**. The electronic stage allows "mark-and-refind" time-lapse studies of cells.

Upright confocal microscope Sarastro 2000

This confocal microscope is equipped with an argon laser and the excellent ImageSpace 3D software (Molecular Dynamics, Saclay, France) running on Silicon Graphics workstations. We will use a 40x (NA 0.75) long-distance water-dipping objective (Zeiss). Image stacks will typically be acquired with 0.3 μm to 2 μm Z-steps.

2.2. Cytometry

Flow cytometry (**Figure 4**) conveys cell-by-cell quantification of structure or, typically, of fluorescent signals. It benefits from the development of thousands of fluorescent markers for probing cellular functions, physiological states or gene expression. No image is obtained but fluorescent intensities are measured with remarkable ease and precision.

Cytometry implies that the hydrodynamically positioned objects, focused by a sheath fluid, move one-by-one through an optical bench. Sometimes a sorting option allows sampling for examination, for subsequent biochemistry or even culture of isolated cells. The objects such as cells, protoplasts, or isolated organelles may be individually analysed for several parameters at rates of hundreds per second. This multiparametric aspect is central to the power and simplification of procedures, facilitating the study of heterogeneous unpurified samples and use of internal references. Obviously, the major constraint of this technique in plant sciences is that it does not handle tissues, but only individual objects. Cytometers can be large and expensive sorters (our cytometer), or small bench-top analysers. The light sources may be lasers (our cytometer), with or without ultra-violet, or inexpensive mercury lamps. The familiar parameters are Forward Angle Light Scatter (FALS) or Right Angle Light Scatter (RALS) which empirically define the size, morphology or granularity of an object (cell, organelle, debris, etc.) as it flashes past the light beam, and 3-4 fluorescent signals associated with these. Signals may be amplified and converted to intensity values in arbitrary units using both linear and logarithmic scales. Data will be represented as monoparametric histograms (one parameter *versus* frequency), biparametric histograms (also termed cytograms), and multiparametric cluster analyses (usually clarified by colour coding). Data are normally also stored in **listmode** (a huge spreadsheet of values cell-by-cell) so that subpopulations can be defined *a posteriori* with gates (or bitmaps, windows on biparametric graphs) and data can be replayed as often as necessary with different definitions, even once

the sample is finished. This is "virtual purification". **Pulse compensation** is an electronic module allowing the cross-talk between channels due to fluorescence spectral overlap to be corrected, object by object.

Flow cytometer

Our ELITE ESP cytometer (Beckman-Coulter, Roissy, France) has an air-cooled argon laser for 488 nm (40 mW), a helium-neon diode laser at 633 nm (30 mW) and an onerous water-cooled argon laser giving 351 + 364 nm (100 mW), 454, 458, 476, 488, 496, 514 nm. There are six channels: forward light scatter (photodiode) and 5 photomultipliers and a cell-sorting module.

References

- Bergounioux, C., Brown, S.C., and Petit, P.X. (1992) Flow cytometry and plant protoplast cell biology. Physiologia Plant. **85** : 374-386.
- Coba de la Peña T., and Brown S. (2001) In "Plant Cell Biology: A Practical Approach" (eds Hawes C. and Satiat-jeunemaitre B.) Oxford University Press, Oxford.
- Darzynkiewicz, Z. (1994) "Flow cytometry". Methods in cell biology, Vol. 42, Academic Press.
- Hawes, C, and Satiat-Jeunemaître, B (2001) "Plant Cell Biology: A Practical Approach" (2nd. ed.) Oxford University Press, Oxford, e.g. microscopy (Shaw) ; fluorescent probes (Fricker); flow cytometry (Brown) ; GFP (Neuhaus).
- Marie, D., and Brown, S.C. (1993) A cytometric exercise in plant DNA histograms, with 2C values for 70 species. Biology of the Cell **78** : 41-51.
- Mason, WT (1999) Fluorescent and Luminescent Probes for Biological Activity. A practical guide to technology for quantitative real-time analysis. Academic Press, London
- Melamed, M.R., Lindmo, T., and Mendelsohn, M.L. (ed.) (1990). Flow cytometry and Sorting (2nd ed) Wiley-Liss, N.Y.
- Métézeau, P, Miglierina, R, and Ratinaud M-H (1994) La cytométrie en flux : Guide pratique de la préparation à l'analyse des cellules. PULIM, Limoges.
- Pawley, J.B. (1995) Handbook of biological confocal microscopy. 2nd. ed. Plenum Press, N.Y.
- Robinson, J. P., (ed.) (1997) Current Protocols in Cytometry, John Wiley & Sons, N.Y.
- Ronot, X, and Schoëvaërt-Brossault, D (1999) Dynamique de la cellule vivante. Editions INSERM, Paris.

Table 1. MICROSCOPE OBJECTIVES (all infinitely corrected)

Our laser scanning confocal microscopes are **Leica TCS SP2,** from Leica Microsystems (Mannheim, Germany). The UPRIGHT is a DM-RXA2 micros
They each have three air-cooled lasers: an Argon Visible (454+458; 476; 488; 514 nm); two small air-cooled Helium-Neon Green (543 nm) and Red
The UPRIGHT also has a water-cooled Argon UV 351+364 nm laser (Spectra-Physics 2025).
Running on Windows NT4.0, the microscopes are pilotted by Leica TCS Software (version 2.477) with the LCS Software for extended image analysis

Reference ***a priori on...***	**Magnification** **G**	**Numerical Aperture** **NA**	**Immersion**	**Working Distance (µm)**	**Corrected for coverslip (µm)**	**Resolution XY Rd (µm)** [minimal justified pixel size] *	**Resolution Z Rf (µm)**	**Field max (µm)**	**Use**	**DIC**
Leica 11 506 505 *Ipright & Inverted*	HC PL Fluotar **10X**	0,3	no	11000	0 (ou 170)	0,65 [0.28]	4,77 [2.1]	1500	(UV?) Vis	
Leica 11 506 178 *Inverted*	HC PL Apo CS **20X**	0,7	water/glycerine /oil	~170	CORRECTOR 0 or 170	0,28 [0.12]	water ; oil 1.10 ; 1.28 [0.48] [0.56]	750	UV Vis DIC	
Leica ceramic 11 506 155 *Upright*	HCX Apo L U-V-I **40X**	0,8	water	3300	no coverslip dipping objective	0,24 [0.10]	0,82 [0.36]	375	UV Vis DIC	
Leica 11 506 179 *Ipright & Inverted*	HCX PL Apo CS **40X**	Iris 1.25-0.75	oil	100	170	0,16 [0.07]	0,33 [0.14]	375	UV Vis DIC	**Upright : K5 + E** **Inverted : K7+E**
Leica 11 506 131 *Upright*	HCX PL Apo CS **63X**	1,2	water	220	CORRECTOR 140-180	0,16 [0.07]	0,29 [0.13]	238	UV Vis DIC	**Upright : K5 + D** **Inverted : K7+D**
Leica 11 506 180 *Upright*	HCX PL Apo CS **63X**	Iris 1.32-0.60	oil	70	170	0,15 [0.06]	0,29 [0.13]	238	UV Vis DIC	**Upright : K5 + D** **Inverted : K7+D**
Leica 11 506 038 *Inverted*	HCX PL Apo CS **100X**	Iris 1.40-0.70	oil	90	170	0,14 [0.06]	0,24 [0.10]	150	UV Vis DIC	**Inverted : K10+D**

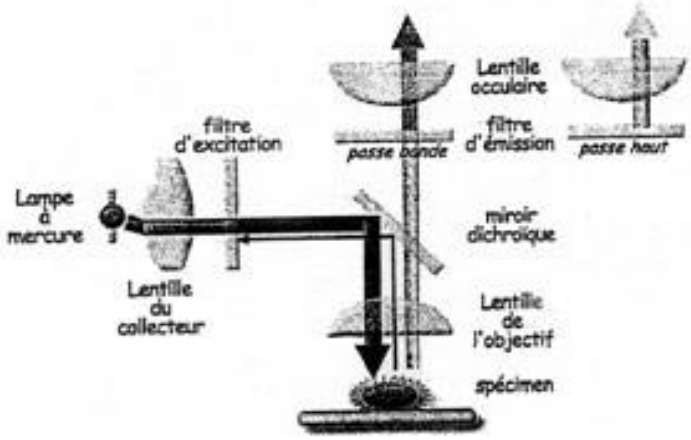
PL = PLan
L = Long working distance
CS = Confocal Scanning

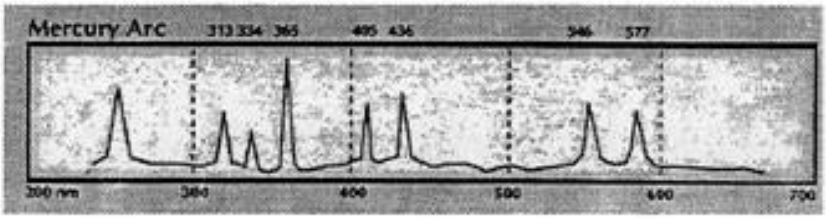
Resolution : for wavelength lambda ; refractive index, n ; Radius of the first Airy disk, r
Lateral Rd = (0.6 * r) / NA **Axial** Rf = 2r / (NA)? (Brakenhoff et al. 1990)
ou = (0.4 * lambda) / NA = (0.45 * lambda) / n(1 - cos alpha) (Leica)
Rd is more sensitive to pinhole size "r" ; Rf is particularly sensitive to Numerical Aperture "NA"

*Shannon's Law, and the Nyquist sampling theorem, show that "the sampling density (pixel size) should be **d**/(2.3) where **d** is the distance between tw

FILTER on Upright & Inverted : block A (UV, DAPI) BP340-380/LP425 & block N2.1 (green, RITC) BP515-560/LP590 ;
for your eyes on Upright : block I3 (blue, FITC) BP450-490/LP515 ; on Inverted block L5 (narrow blue, GFP) BP460-500/BP512-542

Figure 7-1. The wide-field epifluorescence microscope and spectral energies of a high-pressure mercury lamp.





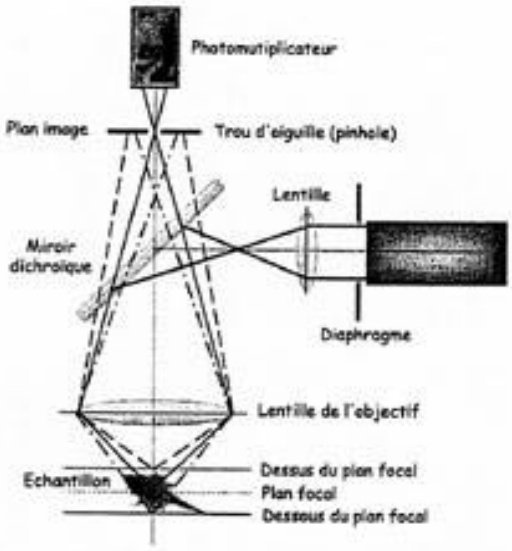


Figure 7-2. The single-photon laser-scanning confocal microscope.

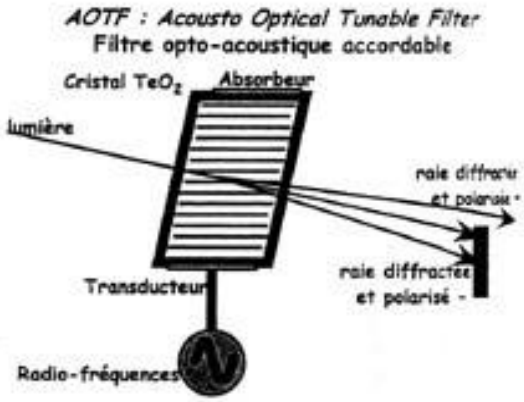


Figure 7-3. A gem for microscopy, the AOTF.

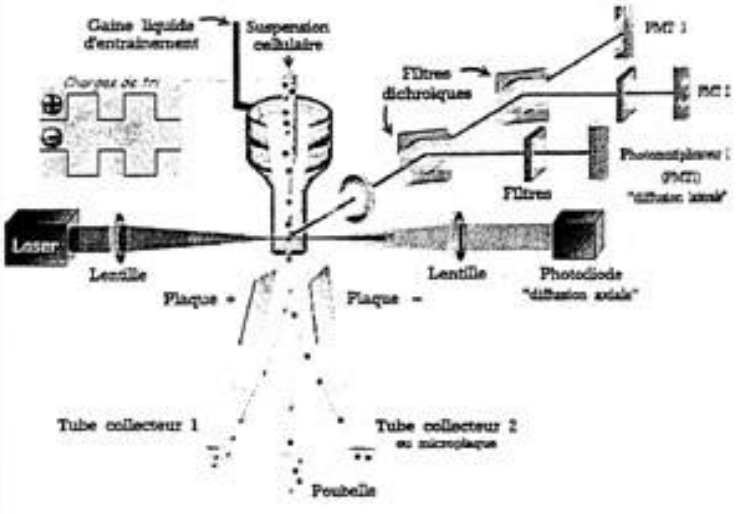


Figure 7-4. Scheme of a sorting flow cytometer.

Figure 1. The epifluorescence microscope. The objective firstly serves as the condenser to illuminate the sample. A set of mobile filters defines the spectral range of the incident light, reflecting this to the sample through a dichroic mirror. The same mirror prevents much of the dispersed light on the return path from reaching the observer (or camera), and a blocking filter on the emission path improves this result. In our example, this filter may be a band-pass (narrow) to selectively target a given fluorochrome or long-pass (generous). The same scheme applies, whether the microscope is upright or inverted.
The light source is generally a high-pressure mercury lamp, with the indicated spectral energies (*www.omegafilters.com*) - characterised by sharp peaks and contrasting with the output of Xenon lamps found on spectrofluorometers, for instance.

Figure 2. The single-photon laser-scanning confocal microscope. The essential principle here is introduction of a "pin-hole" which blocks out-of-focus light (broken lines), letting through to the detectors only signals (solid line) from the focal plane. This aperture should correspond to the first Airy Disk of the given optics. The photomultiplier determines the value for a pixel. By introduction of a raster scanning mirror, an image can be reconstituted (on a plane xy). By electronic displacement of the stage position (in the axis Z), an image stack can be obtained. With several photomultipliers, several channels (colours) may be obtained simultaneously. With several laser lines, divers fluorochromes may be excited.

Figure 3. A gem for microscopy, the AOTF. The Acousto Optical Tuneable Filter allows independent and rapid selection of incident laser lines and modulation of their intensities. A birefringent tellurium oxide crystal is coupled to a piezo-electric transducer. A radio-acoustic frequency compresses and relaxes the crystal, producing a periodic variation in the refractive index of the crystal planes so the system functions as a Bragg diffractometer. The crystal diffracts a specific wavelength, acting as a variable spectral filter. The intensity of transmitted light is proportional to the power of the radio-frequency. A set of frequencies allows modulation of a set of wavelengths (six in the case of our confocal microscopes).

Figure 4. The flow cytometer has three major components
- A stable flow of a sheath liquid focuses the sample suspension.
- An optical bench comprised of one or several light sources and detectors such as photodiodes (for Forward Light Scatter) and photomultipliers with optical filters enables quantification of various fluorescent signals emitted by the object.
- A microprocessor converts the electrical signals into digital form, co-ordinates and represents the data and statistics.

Certain machines include a sorting module. By briefly polarising the undulating stream after analysis, charged droplets (+ or -) will be formed; falling through an electromagnetic field, droplet(s) will deviate with contents to be collected.
Flow cytometry is used to analyse diverse cellular constituents (nucleic acids, proteins, lipids), isolated organelles (nuclei, mitochondria, plastids, chromosomes), and many cellular functions (viability, enzyme activities, reporter genes, pH, membrane potential, ion activities). Typically, the analysis is done at several thousand objects per second.

3. PROCEDURES

3.1. Transient gene expression in *Medicago truncatula* tissues using bombardment

Particle bombardment has become one of the major techniques for the transformation of plant cells, particularly because it is not restricted to specific tissues and cell types. This technique employs high velocity (gold or tungsten) particles to deliver biologically active DNA (coated on those particles) into living plant cells. Even though some tissues may be resistant to particle penetration due to the presence of strong cell walls, by assaying various pressure conditions (particle acceleration) and pre-treatments, efficient transient transformation has been achieved in a wide variety of tissues. Using regenerable tissues, this technique has been widely used on species recalcitrant to *Agrobacterium* transformation for developing stable transgenic plants. In addition, a major use of particle bombardment deals with the functional assay of promoter-reporter gene fusions in transient expression experiments. In addition to analyse promoter function and specificity, this serves to justify and verify constructions before starting the usually long procedure of stable genetic transformation. Recently, also it has been used to assay the *in vivo* localisation of gene products by preparing translational fusions to the reporter gene GFP. This gene has been modified for appropriate expression in plants since it contains a cryptic intron recognised by the plant splicing machinery. In addition, several mutations have been introduced into the GFP to increase its fluorescence and/or induce changes in the emission wavelength (see fluorescent labelling of cells).

During the course we will use the following constructs:

GFP4, a cytoplasmic bright GFP (kindly provided by J. Hasselof, UK)

PTS-YFP, a fusion of the yellow fluorescent protein with a peroxisome targeting signal (kindly provided by Dr. Nam Chua, University of Singapore, Singapore)

GFP-ER, an GFP fused to a HDEL ER retention signal (kindly provided by J. Haseloff, UK)

DEF-GFP, a fusion of a deformylase to the GFP (EMBO J., 19:5916; kindly provided by C. Giglione and T. Meinnel, ISV, France) that labels various organelles.

3.1.1 Plant treatment

M. truncatula leaves detached from greenhouse-plants of around a month are sterilised using Inovchlore 12% for 1 minute. After extensive washing with sterile water (5 times), leaves are pre-treated with mannitol 12% for 5 minutes to plasmolyse cells and increase the transformation frequency. Two leaves are deposited on a Petri dish containing Gibson medium for bombardment with a 650psi rupture disk and a particule GUN PDS100/He (both from BioRad). Bombarded leaves are incubated in the culture chamber for variable times (24 °C, 16 hours photoperiod).

3.1.2 Bombardment

Different materials need to be prepared for bombardment (all consumables are available from BIORAD) :

a. <u>Preparation of particles (microcarrier)</u>

1. Weigh out 60 mg of gold particles in a 1.5 ml eppendorf tube.
2. Add 1 ml of 70% ethanol. Vortex 3-5 minutes.
3. Pellet microparticles by spinning down 5 seconds. Remove supernatant and discard.
4. Repeat the following steps three times : Add 1 ml of water, vortex for 1 minute, spin 2 seconds and discard supernatant.
5. Add 1 ml sterile glycerol 50% and store at 4°C. Microcarriers can be stored for a year in this condition.

b. Coating DNA onto microcarriers

When removing aliquots of microcarriers, it is important **to vortex the tube containing the microcarriers continuously** in order to maximise uniform sampling.
Procedure for six (or two) bombardments:
1. Remove 50 µl (or 17) of microcarriers to a 1.5 ml eppendorf tube.
2. While vigorously vortexing, add 5 µl (or 1.7) DNA at 1 mg/ml.
Then 50 µl (or 17) of $CaCl_2$, 2.5 M
Then 20 µl (or 6.7) of spermidine, 0.1 M
3. Vortex for 2-3 minutes and then allow DNA-coated microcarriers to settle for 1 minute.
4. Spin 2 seconds in a microfuge and discard supernatant.
5. Add 140 µl of 70% ethanol without disturbing the pellet.
6. Spin 2 seconds and discard supernatant.
7. Add 140 µl of 100% ethanol, spin and discard supernatant.
8. Resuspend in 60 µl (or 20) of ethanol 100% and spread 10 µl (1 shot) evenly over the orange macrocarrier. Let it dissecate.

c. Shooting

Open main gas valve and adjust to 200 psi above chosen rupture pressure (e.g. for 650 psi rupture disks : 850 psi). Put power ON and vacuum pump ON.
1. Mount rupture disk (small brown circle) of appropriate pressure (650 psi for *M. truncatula* leaves) on gun-holder and fasten tightly.
2. Put stopping screen and macrocarrier containing the gold particles in the "launch assembly". Take care gold particles must be down! Introduce launch assembly into upper slot.
3. Insert Petri dish with plants/cells/tissues WITHOUT COVER. Close door and secure it.
4. Press pump vacuum (VAC) up to 28mm Hg. Turn VAC/VENT/HOLD button all the way down to HOLD.
5. Press Fire button and hold until rupture disks breaks (noise). Immediately release this button.
6. Let air into the chamber by putting the VAC/VENT/HOLD button in the middle VENT position.
7. At the end of all bombardments, clean chamber, close the main valve and fire repeatedly until no pressure remains in the gas system. Let air into chamber, turn off vacuum pump and put power OFF.

d. Observations

GFP fluorescence can be observed from 6 hours after bombardment depending on promoter strength and particular mutations. Routinely 24 to 48 hours is used to test proper localisation of GFP-fusions (see Observing GFP). GFP fluorescence is stable for 4 to 7 days depending on the stability of the fusion protein and promoter strength. During the course, samples will be observed 48 hours after bombardment. GFP excitation is carried out at 488 nm and the emission peak at 510 nm. Image stacks are acquired with 0.2 µm to 2 µm Z steps using confocal microscopy or under epifluorescence microscopy (see above).

References
- Sanford, T. (1988) The biolistic process. *TibTech.* 6, 299-302.
- Christou, P. (1992) Genetic transformation of crop plants using microprojectile bombardment. *Plant J.,* 2 : 275-281
- Hawes, C, and Satiat-Jeunemaître, B (2001) "Plant Cell Biology: A Practical Approach" (2nd. ed.) Oxford University Press, Oxford. GFP Chapter by Neuhaus G.
- Tsien, R (1998) The green fluorescent protein. *Ann Rev Biochem* 67 : 509-544.

3.2. Immunocytolocalisation by light microscopy

Immunocytochemistry encompasses labelling techniques with antibodies to detect and localise *in situ* specific antigens inside tissues and cells. The part of an antigen recognised by an immunoglobulin is termed epitope. To access epitopes, several barriers have to be crossed by the antibodies including the cell wall and plasma membrane. Moreover, cuticles on epidermal cells act as a further barrier. Two approaches are proposed in the course to overcome these problems : cell permeabilisation and sectioning of fixed tissues. The immunostaining procedure is performed through two successive immunoreactions, the antigen being first detected by the specific antibody, and this primary antibody itself being recognised by a second antibody to which is attached a visual marker. The antibody-antigen complex is held together by multiple non-covalent bonds (hydrogen, van der Waals, hydrophobic) .

In this course, as first antibodies, we will use both monoclonal and polyclonal antibodies. Monoclonal antibodies do not contain contaminant Igs and are specific for individual epitopes. Polyclonal antibodies recognise different epitopes on the same antigen. Therefore, the signal is likely to be strong. Polyclonal antibodies often need to be purified prior to use and are often source of non-specific binding. Secondary antibodies will be labelled with two different fluorochromes (Cyanine 3 (Cy3) and Fluorescein-isothiocyanate (FITC)). For information on the constraints and advantages of various fluorochromes used simply as tags on an antibody, consult *www.probes.com*

Primary antibodies :

- JIM84, recognising the Golgi apparatus (monoclonal, rat IgM)
- anti-tubulin, recognising microtubules (monoclonal, mouse IgG)
- anti-HDEL, recognising the endoplasmic reticulum (HDEL, signal tetrapeptide for ER retention ; monoclonal, mouse IgG)
- anti-CA, recognising specific inner cortical cells from the nodule (CA : carbonic anhydrase gene coding for nodule specific enzyme dealing with gas exchanges in the nodule inner cortical cells ; polyclonal, rabbit).

Secondary antibodies :

- Cy3 labelled-anti-rabbit/rat/mouse antibodies
- FITC labelled anti-rabbit/rat/mouse antibodies

3. 2. 1. Immunofluorescence on *Medicago truncatula* root squashes

This approach consists of partially digesting (with hydrolytic enzymes) and squashing the root apex tissue onto slides to release individual cells. This allows antibodies penetration within the tissue. A similar approach could be done for hypocotyls and, after calibration, for other "squashable" tissues (e.g. nodules). The yield of intact individual cells may depend on digestion times and enzyme cocktail.

a) Preparation of the biological material : fixation, partial digestion, permeabilisation

1. Excise 2-3 mm root apices in 4% PFA (paraformaldehyde) in modified PBS buffer and place the specimens in fixative in glass vials.
2. Fix material for 1h in fixative with gentle agitation ; a small amount of the Brij35 surfactant in the fixative may enhance fixative penetration. Otherwise, vacuum infiltration of fixative may be used.
3. Wash thoroughly the fixed tissue with buffer (5 x 10 min).
4. Perform a partial digestion of the cell wall by incubating the tissues in the enzyme solution for 12 to 16 min (the time may vary with room temperature and the nature of the tissue). Wash with buffer (2 x 10 min).

§ different cocktails of enzyme solutions may be tried according to the tissue type and cell wall composition. See annexes.

§ If cells are damaged, it is probably due to a too long a digestion and/or a too violent squash. Conversely, if the material is difficult to squash, leave the specimens one or two minutes longer in the enzyme solution.

5. Place one piece of root per well of a multiwell slide, in a few microliters of buffer, and tap gently with a glass rod. Remnants of root may be transferred to another well and squashed again. Observe the preparation with bright field light microscope. Intact cells should be released from the tissue, and float in the moistened squashing area.

§ In many cases, fixed cells have to be attached to a support before proceeding with the immunostaining. It is best to use chemically treated slides, coated with Vectabond.

§ Multiwell slides are convenient for immunostaining experiments as each well may be used for a separate staining condition. Alternatively, the whole immunostaining protocol may be performed on coated coverslips.

6. Let the preparations dry partially or completely. Place the slides into a moist chamber.
7. Permeability of the membrane is subsequently achieved by covering specimens with drops of 0.5% Triton X-100 for 20 min. From now on, slides must never dry.
8. Wash for 5 min with drops of buffer.

b) Immunoreaction

9. Add 20 µl of blocking buffer (washing buffer + 1% BSA) on each well for 10 min to mask non-specific binding sites
10. Incubate in primary antibody for 1 h at room temperature (or at 4 °C overnight).
11. Wash (with washing buffer) 5 x 10 min. To optimise the blockage of unspecific binding sites, fish gelatin 1% is added to the washing buffer.
12. Incubate in secondary antibody for 1 h at room temperature.
13. Wash with washing buffer, 5 x 10 min.

c) Counterstaining

14. Optionally stain with Hoechst 33342, or DAPI or propidium iodide (see below) to counterstain the nuclei, and wash once 10 min with washing buffer.
Pretreatment with 50 μg/ml RNase will remove ribosomal RNA to make propidium iodide staining of nuclei specific.

d) Mounting and Visualising

15. Mount in Citifluor or other antifade mounting agent.
16. Seal a coverslip on the slide with nail varnish.
17. Observe with filters :
for UV excited dyes (Hoechst, DAPI), U1 excitation 330-380 nm, emission >420 nm
for blue excited dyes (FITC, Alexa488), broad B1 ex 450-495, em >520 nm
or the more specific narrow B4 ex 475-495, em 520-560 nm
for green excited dyes (Cy3, TRITC, Propidium iodide), G1 ex 540-550, em >590 nm

3.2.2. *Medicago* root sections of LR white-embedded material

To have better access to epitopes and identify cell types expressing a particular antigen, resin-embedded material is used. The advantage of the LR-white resin (other resins are available) is that it can be used for morphological studies in optical microscopy, for immunolocalisation at light and especially electron microscopy level. Demonstration of LR white embedding will be presented during the course, immunostaining will be performed on ready-made sections.

a) Fixation, embedding and sectioning

1. Pre-staining specimens in a dye such as 1% aqueous Ruthenium Red helps with subsequent location of material in solutions and resin mixtures.
2. Preparation of the fixative: PFA 3%, glutaraldehyde 1% in Sodium cacodylate 0.1M.
Add 0.3g paraformaldehyde powder to 60 ml DW and heat to 70°C. Slowly add a few microliters of NaOH 8N until all powder dissolve. Add together 50 ml 0.2 M buffer, 4 ml EM grade 25% glutaraldehyde. Complete to 100 ml with ultrapure water.
3. Fixation : Whenever possible, excise a small [1-3 mm^3] portion of tissue under fixative, transfer to fresh fixative in a vial and agitate or rotate gently. Fix for approximately 1 h at room temperature. Optimum fixation times will vary with different tissues. If tissue does not wet or floats on the surface of the fixative penetration can be aided by the addition of a surfactant [eg Brij 35] or by fixing under vacuum. Cell suspensions and protoplasts can be fixed in small centrifuge tubes and spun down in between treatments. For the fixation of protoplasts add suitable osmoticum (e.g. 0.4 M mannitol) to the primary fixative.
4. Wash three times 10 min in buffer.
5. Dehydrate in 10% and 20% ethanol, 10 min each step, at room temperature
30% ethanol at 0 °C 1h., 50%, 75%, 90% ethanol 100% ethanol, 100% ethanol, at –20 °C, 1 h each step.

6. Infiltrate with 100% ethanol/LR White mixtures 2:1, 1:1, 1:2 at -20 °C 4 h each step.
LR White at –20 °C. Three changes of 8 h each.
7. Transfer material into embedding moulds, capsules (gelatin or polyurethane) or dishes and polymerise in an O_2-free atmosphere under UV light at -20 °C for 24 h and 0-15 °C for 16-24h.
8. Trim and section at 200 nm. Collect in plastic ring or wire loops, deposit on slides.

b) Immunoreaction

Repeat step 9 to 17 from the previous protocol.

3.2.3. Immunolocalisation on vibratome sections

To avoid relatively time-consuming protocols of resin embedding, an alternative procedure is to use vibratome thick sections. In this case, optical sections under the confocal microscope are required to determine the appropriate location of the antigen. Certain tissues are very difficult to section using these conditions (e.g. roots) and calibration on resin-embedded material is usually required to confirm the observed localisation. However, afterwards this method can be used routinely with large amount of samples (e.g. screening of transgenic plants)

a) Fixation and sectioning

1. Nodules are fixed in PFA 3% (pH: 6.8) for 1.5 h using vacuum infiltration.
2. Fixed tissues are washed 5 x 10 min in MPBS.
3. Samples are introduced through a cushion of 6% agarose (Genaxis, molecular biology grade, in water) previously melted. Melted agarose has been loaded on cut falcon tubes, avoiding as much a possible the formation of bubbles. After 10-15 minutes, samples are embedded in solid agarose.

b) Vibratome sectioning

4. Stick agarose blocks to the vibratome plateform.
5. Make sections of 100 µm (for certain tissues up to 50 microns can be done, less is usually very difficult). Thinner sections have better resolution than thicker ones.
6. Sections are deposited on MPBS (during sectioning) and then treated with MPBS, 1% BSA for 1 hour to block unspecific sites.

c) Immunoreaction

7. Add antibodies in MPBS, 1% BSA and leave them overnight at 4 °C (or 1 hour at room temperature).
8. Wash 5 x 10 min in MPBS, 1% BSA.
9. Add Cy3 antibodies in MPBS, BSA1%, 1h at room temperature.
10. Wash 5 x 10 min in MPBS, 1% BSA.
11. Put sections on slides and observe on the appropriate filter and/or confocal microscope.

References
- Harris, N., Spence, J., and Oparka K.J. (1994) Plant cell biology : a practical approach (ed N. Harris and K.J. Oparka), p. 51, Oxford University Press Oxford.
- Wick, S.M. (1993) In « Antibodies in cell biology. Methods in cell biology » (ed. D.J. Asai), Vol. 37, p. 171. Academic Press, San Diego.
- Ruzin, S.E. (1999). Plant microtechnique and microscopy. Oxford University Press, Oxford.
- Harlow, E., and Lane, D. (1988) Antibodies : a laboratory manual. Cold Spring Harbor Laboratory, New York.
- Hawes, C., and Boevink, P. (1999) In « Protein localization by fluorescence microscopy » : a practical approach (ed. V. Allan), p. 163. Oxford University Press, Oxford.
- Satiat-Jeunemaitre, B., and Hawes, C. (2001) Immunocytochemistry for Light Microscopy. In «Plant cell biology : a practical approach» (eds. Hawes C. and Satiat-Jeunemaitre B.), p. 207, Oxford university Press.
- Hall, J.L., and Hawes, C. (1991) Electron Microscopy of Plant Cells. Academic Press , London.

3. 3. Fluorescent probes for labelling *Medicago* cells

The use of fluorescent probes may be an easy and straightforward way to observe a cell compartment, a cell constituant or a cell function. Good texts are available on the mechanisms involved (Haugland, 1996 ; Mason, 1999 ; Fricker in Hawes and Satiat-Jeunemaître, 2001). Most of the fluorescent dyes indicated below will be available for trials on *Medicago* cells. Their optical characteristics will be on display.

a) Fluorochromes to mark structural parameters *(in bold, available for the course)*

* DNA staining/nucleus : **Propidium iodide**, YOYO, **SYTO16** (vital), chromomycine A3 or mithramycine (bases GC), **bisbenzimide Hoechst 33342**
* Mitochondria: potential sensitive Rhodamine123, **$DiOC_6(3)$**, $DiOC_7(3)$, JC-1, **MitoTracker Red CMXRos** ; nonyl acridine-orange (largely insensitive to potential)
* Membranes *:* FM1-43, **FM4-64** (longer emission to avoid GFP)
* Endoplasmic reticulum : **$DiOC_6(3)$** at higher concentrations
* Vacuoles **:** neutral red, **acridine orange** : accumulate by protonation of the weak bases
* Cell wall : **Calcofluor white M2R**, and, in an unspecific manner on tissue, **propidium iodide** and FM4-64.

b) Fluorochromes to mark parameters reflecting physiological changes in living cells like ion movement, enzyme activity, or membrane permeability

* Viability/mortality test : **Fluorescein di-acetate** (FDA), where free fluorescein is retained behind the intact membrane in living cells; **Propidium iodide**, which is excluded by an intact membrane, and so only labels dead cells
* pH state : carboxy-SNARF-1, BCECF.
* Calcium gradient : Fluo-3, Fluo-4, Fluo-4FF (less affinity), CalciGreen2

c) Fluorochromes used in immunocytochemistry

AMCA, **FITC**, Cy2 (Cyanine2), , **Cy3**, TRITC, lissamine rhodamine, Texas Red, Cy5, and notably the set **Alexa** (350, 430, **488**, 532, 546, **568**, 594, 633, 647, 660, 680)

d) Autofluorescent proteins

Green Fluorescent Protein (**GFP**) from jelly-fish and its numerous variants developed for : better transcription, folding (or "Timer"), auto-oxidation (or anoxia sensitivity), stability (or instability), pH independence (or dependence), calcium sensitivity (cameleon), and of course for spectral variants: BFP, CFP, GFP, YFP, in addition to its cousins from coral species DsRED and DsRED2.

References

- Haugland, R.P. (1996) Handbook of fluorescent probes and research chemicals. 6th ed., Molecular Probes Incorp, P.O. Box 22010 Eugene, Oregon, USA or Molecular Probes PoortGebouw, Rijnsburger 10, 2333 AA Leiden. The Netherlands (CD and *www.probes.com*) : offers 5000 fluorochromes, 30000 references and mini review chapters about their properties and application in biology.
- Hawes, C, and Satiat-Jeunemaître, B (2001) "Plant Cell Biology: A Practical Approach" (2nd. ed.) Oxford University Press, Oxford.
- Hovius, R, Vallotton, P, Wohland, T, and Vogel H. (2000) Fluorescence techniques : shedding light on ligand–receptor interactions. *Trends Pharmaceutical Sci* **21** : 266-273 (July)
Ludin B, Matus A (2001) "GFP in motion 2" [CD] *Trends Cell Biol* **11**(5), Elsevier Science (and *www.lis.ch*).
Tsien, R. (1998) The green fluorescent protein. *Ann Rev Biochem* **67** : 509-544.
www.bio.net:80/hypermail/FLUORESCENT-PROTEINS/
www.clontech.com/gfp/
www.qbiogene.com/products/gene-expression/afp.html
www.stratagene.com

3.4. Observing GFP in transgenic *Medicago truncatula* plants

Transgenic plants expressing translational GFP fusions can be used to follow *in vivo* changes in particular subcellular structures labelled with these autofluorescent proteins. MAP4 (microtubule associated protein) has been fused to GFP and this fusion was able to label microtubules *in vivo* in various species. Transgenic *Medicago truncatula* plants expressing MAP4-GFP have been prepared. To obtain dissociated cells from transgenic plants, proliferating callus from leaf explants of T2 progeny plants is transferred onto MSO1 liquid medium (Murashige and Skoog basal medium with Gamborg's vitamins; Sigma, M0404 ; supplemented with 30 g/l sucrose, 250 mg/l casein hydrolysate, 4 mg/l 2-4D) and maintained on a rotary shaker (120 rpm) for one week. After 2-3 h of incubation in water, microtubule organisation is observed in cells of different shapes. In addition, transient expression of translational GFP fusions also serve to monitor at single-cell level subcellular structures.

For the visualisation of GFP fluorescence, see paragraph 3.1.3.

3.5. Cytometric analysis of genomic content and endoreduplication in *M. truncatula* organs

The general principles of flow cytometry and various protocols are given in Coba de la Peña and Brown (2001). During the course, we will see several applications of cytometry studies on *Medicago* cell proliferation, based on the preparation of isolated nuclei (e.g. Roudier et al. (2000) *Plant J.*, 73) which will be obtained by chopping with a razor blade. These assays rely on nuclear staining with a DNA binding fluorochrome (and possibly through incorporation of bromo-deoxyuridine). A monoparametric histogram of nuclear DNA levels in an asynchronous cell population will typically give intensities of 2,000-20,000 nuclei. It is normally characterised by two major peaks of events, at 2C and 4C, superimposed upon intermediary values taken to correspond to S-phase nuclei and possible debris.

The reliability of the measures is evaluated through several essential parameters displayed with the histogrammes, i.e. the **Coefficients of Variation** (CV). An excellent analysis will have CV = 1-2% ; 3% can be routine. This CV says nothing about the reproducibility of the sample nor its representativity.

3.5.1 Genome size determination

The number of chromosomes present in the gametes without replication, constituting the haploid complement, is represented as **n.** The basic chromosome number for a given taxon is represented as **x**, a unit of ploidy. Ploidy is strictly defined by cytogenetic description of the karyotype as the number of copies of the chromosome complement for that species. The quantity of DNA corresponding to the haploid **Complement** is represented as **C**, in arbitrary or in absolute units such as picograms or base pairs. The simple flow cytometric assay of interphasic nuclei yields an estimate of DNA quantity, 2C, rather than chromosome number, 2n. This value C is compared to that of a reference plant of known ploidy (true C) or to published values of C for the given species. Therefore, the DNA ploidy reported from cytometry equates a constant DNA quantity with a complete chromosome complement. This

certainly may not always be true (e.g. an aneuploid specimen having lost one chromosome but gaining another), yet it is a highly advantageous simplification. The terminology "DNA ploidy" is used to respect this logic.

The fluorescence intensities indicated on a histogram are normally in arbitrary units of channel numbers derived from photomultiplier outputs. In order to assess genome size or ploidy of an unknown, this scale must be calibrated with a reference. The simplest calibration is to analyse a reference plant of known ploidy (*e.g.* 2x) of the same species, note the position of its G1 nuclei (2C), and characterise all other samples by the relative position of their G1 peak. However, an internal reference within the preparation is more reliable: for instance, use of a tetraploid reference to screen for diploids.

If the intensity scale is to be calibrated in absolute units of DNA (pg or bp), plants of known genome size or chicken erythrocytes are included as internal standards. They must be fixed and stained along with the unknown. The DNA quantity is then deduced from the fluorescence ratio, R, of the two species of nuclei.

a) Chopping Method for nuclei

1. Place 1-2 cm^2 *Medicago truncatula* leaf (avoiding major vascularisation) or about 150 mg of any tissue or cells on a 90 mm plastic Petri dish. Add half that quantity of *Petunia hybrida* (Hort.) PxPc6 leaf at this stage as our internal standard (2C = 2.85 pg = 2750 Mbp, 41.0% GC)
2. Cover with 500 µl ice-cold modified Galbraith nuclear buffer.
3. Chop with a fine double-edge razor blade, limiting dispersion or drying. Work quickly: 30 sec should suffice.
4. Transfer this to a 48 µm filtrette in an ice-cold cytometer sample tube. Remove filter.
5. Add 1% (w/v) formaldehyde to stabilise unless the cytometric analysis follows immediately.
6. DNA intercalating stains such as propidium iodide require use of RNase: add 10 µl/ml from 1% RNase stock at this stage.
7. Add propidium iodide and wait at least 15 min before cytometric analysis.
8. Note the positions of the 2C and 4C nuclei of *Medicago* and of *Petunia*. Is there the expected linearity for the same species ? Given that *Petunia hybrida* has 2750 Mbp, use the relative positions of the 2C nuclei to deduce the genome size of *Medicago truncatula* (cf. Blondon et al. 1994, *Genome* **37,** 264).

b) Running and reading the cytometer data

Use fluorescent polystyrene beads to check machine alignment and stability. Ideally, DNA related fluorescence should be accumulated on two scales through distinctive linear and logarithmic amplification. The linear scale is intrinsically understood and is necessary for cell-cycle algorithms and genome-size calculations. The logarithmic scale is useful to visualise endoreduplication, multiple ploidy levels or polysomaty on a single range covering 1 to 10,000 (arbitrary units).

Use forward-angle light scatter (FALS) to identify isolated nuclei and to gate out debris, and pulse analysis to eliminate doublets. The typical protocol for cytograms and histograms display will be presented during the demonstration.

3.5.2 Endoreduplication

Most plant families display developmentally regulated endoreduplication so that tissue is polysomatic. This is remarkably so in *Arabidopsis thaliana* (Gendreau et al., 1998, *Plant J.*, **13** : 221), less so in *Medicago* spp. but very evident in nodules. Notably, the gene *cell cycle switch 52 (ccs52)* has been identified in *Medicago* as a homolog of the anaphase-promoting complex activators involved in mitotic-cyclin degradation (Cebolla et al. 1999 *EMBO J* **18** : 4476). Overexpression of this gene in fission yeast resulted in the arrest of cell division and induced endoreduplication and cell enlargement. In transgenic *Medicago truncatula* plants, downregulated expression of *ccs52* correlated with the decrease of ploidy levels and cell volumes, indicating that *ccs52* may control both endocycling and cell size.

If we repeat the above procedure with nodules using Propidium iodide or Hoechst or DAPI (as base-composition is no longer relevant), omitting the internal standard, we obtain a monoparametric DNA histogram with a series of equidistant peaks indicating each population of nuclei corresponding to ploidy increments 2C, 4C, 8C, 16C. Obviously, the logarithmic scale is useful for handling such signals.

4. TIME SCHEDULES

Day 1 (10:00 a.m. till noon)

1. Bombardment of *M. truncatula* leaf tissues with different GFP constructs.

2. Immunocytolocalisations will be done on *M. truncatula* using :
   a) Squashes of root apices
   b) Vibrating microtome sections.

Day 2 (8:00-10:00 a.m.)

1. Analysis by Confocal imaging of the immunocytolocalisations.
2. Labelling of subcellular compartments with different fluorescent probes.

Day 3 (8:00-12:00)

1. Analysis by Confocal imaging and light microscopy of GFP localisation on living cells from bombarded tissues and dissociated cells from transgenic plants.

2. Cytometry to measure endoreduplication and genomic content in *M. truncatula* (1 hour, rotation will be done between different groups).

Annex 1 : Materials and Solutions

1. Phosphate buffered saline (PBS) x 10 : 4 M NaCl ; 27 mM KCl ; 8 mM Na_2HPO_4 ; 150 mM KH_2PO_4, pH 7.2

2. MPBS : PBS, 10mM MgSO4, 10 mM EGTA. (Modified PBS)
EGTA 100 mM (150ml) : 5,70 g EGTA in 100 ml H_2O, add NaOH 8N till pH=6.8
$MgSO_4$ 100 mM 150ml : 3.69gr in 150ml H_2O,pH=6.8

3. Fixative 4% paraformaldehyde (PFA) in MPBS buffer pH 6.8 :
Heat 0.4 g PFA in 50 ml of distilled water up to 70 °C in a fume cupboard, add a few microliters of 8 N NaOH until PFA dissolves. Add10 ml of $MgSO_4$ 10 mM and 10 ml of EGTA 10 mM in PBS buffer. Adjust to 100 ml with distilled water, check the pH 6.8. In vibratome sections 3% PFA instead of 4% is used. Fixatives are not stable and can be conserved few days in the fridge and several months at -20 °C.

3. Brij 35 (polyoxyethylene 23 lauryl ether, Sigma ref. P-1254)

4. Freshly prepared blocking buffer : MPBS buffer with 1% bovin serum albumin (BSA)

5. Washing buffer : buffer with 0.5-1.0 % fish gelatin (Sigma, ref. G-7765)

6. Nuclear stain (Hoechst 33342, at 3 µg/ml or DAPI or, propidium iodide at 3 µg/ml.

7. Multiwell coated slides. To facilitate cell adhesion, slides should be coated with Vectabond (Vector Laboratories) or similar products according to the manufacturers instructions (the treated slides may be kept for a year). Alternatively, slides may be freshly coated with poly-L-lysine (MW > 300 000, Sigma) : place a 20 µl drop of 5 mg/ml poly-L-lysine on a parafilm sheet and invert slide or coverslip onto the drop. Leave for 1-3 h, rinse in 200 ml distilled water and dry. Heating for 20 min at 60°C improves the adhesive quality of the slides. Use multiwell coated slides.

8. Moist chambers : 10 cm diameter Petri dishes (or similar containers), inlaid with wet filter paper

9. Enzyme solution for squashes : 1% cellulase "Onozuka" R10 (Yakult Honcha Co.); 1% pectinase (Sigma, ref. p0690) in buffer, pH 6.8.

Annex 2. Cytometry Equipment and reagent

- *Nuclear Extraction buffer* modified Galbraith Nuclear Buffer (Galbraith et al. (1983). *Science,* **220,** 1049): 45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM 4-morpholinepropane sulfonate pH 7.0, 0.5% (w/v) Triton X-100 (rather than the original 0.1%). To reduce browning induced by phenols and polyphenols in nodules, for instance, we have added 1% (w/v) polyvinyl pyrrolidone (~10,000 M_r, Sigma P6755).
- *Nuclear stains.* All the common dyes should be treated as health risks. Hoechst bis-benzimide 33342 (*M*r 616) requires UV excitation at ~365 nm, giving a blue emission at 455 nm. It is A-T specific and so can be used to assess AT/GC base composition. It is inexpensive and staining is readable within 5 min. Aqueous stocks of 1 mg/ml may be aliquoted, stored frozen, or kept for weeks at 4°C. DAPI (4',6-diamidino-2-phenylindole ; *M*r 350) has similar qualities, with excitation at 340 nm, emission 465 nm, and is more resistant to photo-quenching in microscopy.

Propidium iodide (M_r 668) with excitation 535 nm, emission 617 nm and ethidium bromide (M_r 394) with excitation 518 nm, emission 605 nm, are intercalating dyes having broad excitation bands in UV and blue-green, compatible with both the UV and visible excitation of all flow cytometers. Aqueous stocks at 1 mg/ml may be stored frozen. Nuclear staining is stable within 10 min but not after several hours. As these dyes also intercalate with double-stranded RNA, a 20 min RNase treatment may be necessary.

- Formaldehyde EM grade 38% (w/v). Store this at room temperature, avoiding direct sunlight. Do not disturb any sediment of polymer that may form (accelerated by cold) and discard the stock once this sediment becomes important as both the fixation and buffering will be severely compromised. Purchase small stocks.
- Ribonuclease A. Use DNase-free RNase (Boehringer 109169, 50 Units/mg). Prepare Tris saline buffer: 12 g/l Tris and 6 g/l NaCl (final concentrations 100 mM), adjusted to pH 7.6 with HCl. Prepare a 1% (w/v) RNase stock in this Tris buffer, boil for 20 min to inactivate any residual DNase, aliquot and freeze.
- Double-edge razor blades (Gillette blue)
- Filtrettes : cut 20x20 mm squares of nylon filter (Nylon Scrynel NYHC 48 µm pore size from ZBF, 8803-Ruschlikon, Switzerland or PolyLabo, Paris). Cut across a 1 ml blue pipette tip (e.g. Treff) in two places : 27 mm from its tip (discard this extremity) and 6 mm from the top. Using the latter as a collar, fix the nylon filter onto the central section. In most situations the assembly may be washed and re-used.