# Chapter 11
# Flow Cytometric Measurement of Different Physiological Parameters

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## 1 Introduction

A variety of physiological parameters involved in signal transduction pathways, enzymatic activities, ATP production, and many other physiological processes can be analyzed by flow cytometry. Parameters as intracellular pH, membrane potential, calcium concentration, reactive oxygen species (ROS) generation, or glutathione content can be estimated thanks to this technique. One of the principal advantages of flow cytometry is that it allows the measurement of these parameters in living and small cells, usually in real time during the physiological stimulation. Moreover, flow cytometry can detect not only the fluorescence of biochemically specific developed fluorescent dyes but also autofluorescence (such as the chlorophyll *a* fluorescence), which can be measured in parallel and can provide supplementary information about the physiological condition of the measured cells (Franklin et al. 2001).

For the measurement of physiological parameters with fluorescent probes, detailed observations for suitable loading and intracellular distribution of the fluorescent probe must be firstly done by fluorescence microscopy and flow cytometry. Secondly, the effects of different ionophores and inhibitors (specific in each case for the physiological parameter under study) on fluorescence are analyzed by flow cytometry. These observations allow us to verify that the fluorescence behavior of the probe is correct and really dependent on the parameter under study, evaluating

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and discarding possible artifacts. These observations also allow a calibration of the fluorescence and of the range variations of the physiological parameter. Thirdly, physiological effects (and thus, fluorescence variations) induced by different substances (hormones or other biological substances, chemicals) and environmental conditions (abiotic or biotic stress factors) under study are analyzed by flow cytometry. Finally, the recorded data and histograms are analyzed. Cell loading of the probes referred below is accomplished simply by adding the probe directly to the buffer or cell suspension, at different concentrations and incubation times (other loading techniques not discussed here, involve electroporation and microinjection).

Besides the fluorescent dyes already mentioned, an expanding family of fluorescent proteins (FPs), which have become essential tools for studies of cell biology and physiology and that can be detected by flow cytometry analyses, has been developed in the last years. These genetically encoded fluorescent markers can be measured without manipulating the sample and without adding external chemicals to the cell, which represents a great advantage in the analyses of living cells (Day and Davidson 2009). Certain of these fluorescent proteins are calcium, ROS or pH-sensitive fluorescent proteins that have become essential tools for studies of pH homeostasis and cell physiology (Benčina 2013).

# 2 Physiological Parameters

## *2.1 Intracellular pH*

Almost every process of cell metabolism induces or is affected by apoplastic, cytosolic or intraorganellar (luminal) pH changes, including ATP generation, extensibility of the wall, membrane potential, movement of hormones, receptor-ligand interactions, cell growth and proliferation, movement of substances across membranes, etc. In the same way, changes in intracellular pH are a cell response to external applied agents, like hormones, growth factors, biotic or abiotic stress and others. Different cell organelles, as lysosomes, vacuoles or the Golgi apparatus, have different pH than cytosol, which is necessary to accomplish their functions; i.e. almost all vacuolar functions depend either on the acidic pH of the lumen or on the pH gradient across the membrane, and acidification is achieved through the action of the V-$H^+$-ATPase proton pump, located at the membrane (Rodrigues et al. 2013). As well, alkalinization of Golgi pH can cause delayed transport, immature glycosylation, and altered Golgi morphology (Maeda et al. 2008), among others. Therefore, cells have developed different mechanisms for regulating intracellular pH (Smith 1979; Felle 1988; Kurkdjian and Guern 1989; Sakano 2001).

Internal pH can be measured by flow cytometry in single cells or isolated organelles in a generally simple procedure that can be done using any cytometer equipped with a 488-nm argon laser. Fluorescence properties (intensity, emission and excita-

tion spectrum) of several available probes vary depending on the $H^+$ concentration of their environment, because hydrogen ion binding changes the electronic structure of the probe (Haugland et al. 1996). Maximum response of the probe will occur for pH values near its *pKa.* The sensitivity of these probes is around 0.1–0.2 pH units.

On the other hand, the development of genetically encoded fluorescent pH-sensitive sensors (GFP mutants) with a range of *pKa* values, such as Cameleon (Miyawaki et al. 1999), pHluorin (Miesenbock et al. 1998) or pHusion (Gjetting et al. 2012), which offer information about pH dynamics without dye loading or sample manipulation, appears in the last years as a good option for non-invasive intracellular pH detection in those cells that can be transformed with pH-sensing GFPs (Benčina 2013). pH affects the equilibrium between protonated and deprotonated forms of these fluorescent proteins appearing as good pH sensors to detect changes in $pH_i$. An expanding family of pH sensors for apoplastic (Gao et al. 2004), cytosolic (Moseyko and Feldman 2001), mitochondrial (Li and Tsien 2012), Golgi network (Lam et al. 2012) and endoplasmic reticulum (Kneen et al. 1998) $pH_i$ measurement in living cells has been developed. Flow cytometry can easily record the fluorescence emitted by these non-invasive pH indicators.

#### 2.1.1 Measurement of Intracellular pH by Fluorescent Probes

Two of the most used pH sensitive probes to measure cytosolic pH are the weak acids BCECF (2′,7′-bis-(2 carboxyethyl)-5-(and 6) carboxyfluorescein acetoxymethyl ester) and SNARF1 (Semi naphtho rhoda fluorine acetoxymethyl ester), which have protonated and free base forms with different emission spectra that allow to get a signal proportional to $pH_i$ (Gonugunta et al. 2008). Both have *pK* values close to 7.0, although SNARF-1 is a more sensitive indicator than BCECF in the physiological range (Robinson et al. 1997). Covalent binding with an acetoxymethyl (AM) residue allows probes to be permeable across biological membranes. Acetoxymethyl-ester forms of these probes are commercially available. Once into the cell, cellular esterases remove acetoxymethyl residue, and the probes become negatively charged, and thereby membrane impermeable, trapped into the cell or into a cell compartment.

BCECF is optimally excited at 488 nm, and its maximal fluorescence emission is 520 nm. Its *pKa* is 6.98, and thereby it is very suitable for the study of cytosolic pH (6.5–7.5). In case of acidification, BCECF becomes more protonated, and its fluorescence intensity decreases. Cellular alkalinization induces an increase in intensity. BCECF is a fast-response probe, allowing kinetic studies of pH changes in real time. One example of the use of BCECF and flow cytometry in plant material is shown in Giglioli-Givarc'h et al. (1996), where activation of a phosphoenolpyruvate kinase after cytosolic alkalinization in *Digitaria sanguinalis* protoplasts is characterized.

SNARF1 is excited at 488 nm and is a 'ratiometric dye', that is, its emission maximum shifts upon pH changes in the microenvironment. The protonated form of the fluorescent probe has a maximum emission at 540 nm, and the maximum of the deprotonated form is at 630 nm. It is possible to record continuously the fluorescence intensity at both wavelengths by flow cytometry, using two detectors. In fact, the ratio of both fluorescence intensities is a very reliable and specific measure, because it discards fluorescence intensity variations induced by several unspecific factors, like differential individual loading among cells (Haugland et al. 1996).

Some ionophores and substances used in the validation and calibration of pH fluorescent probes are:

- Nigericine induces a permeabilization of cell membrane to proton ions, so the extracellular and intracellular proton concentrations make equal, if extracellular and intracellular $K^+$ concentrations are the same.
- Propionic is a weak acid that induces intracellular acidification. $NH_4Cl$ is a weak base that induces intracellular alkalinization. Both are used for monitoring changes on fluorescence intensity.

The 'null point method' is used for calibrating and converting fluorescence intensity values in pH units. Dye-loaded cells are incubated in a series of buffers at different pHs in presence of Nigericine. Intracellular pH equals extracellular pH, and a direct correspondence between known intracellular pH and fluorescence intensity is established (Haugland et al. 1996).

Regarding luminal pH, vacuolar lumen acidity has been assessed by Rodrigues et al. (2013) with two pH-sensitive probes, Acridine Orange (AO) and LysoSensor Green DND-189, by staining the vacuoles with 30 μM AO or 5 μM LysoSensor Green and incubating them for 10 min in the dark at room temperature.

### 2.1.2 Measurement of Intracellular pH by Fluorescent Proteins (FPs)

Different fluorescent proteins, such as pHlourins or Pt-GFP, have been increasingly used in the last years facilitating the detection of cytoplasmic pH in plants (Swanson et al. 2011). The ability of flow cytometry to do rapid analysis and acquisition of multiparameter data at the single-cell level for each cell in a population makes this technique very appropriate to detect the pH sensors based on fluorescent proteins (FPs). This is the case of root and leaf intracellular pH measurements in *Arabidopsis* under abiotic stress (Gao et al. 2004; Schulte et al. 2006) or under growth monitoring (Monshausen et al. 2007).

As previously showed by Valkonen et al. in 2013 for pHluorin detection, a ratiometric flow cytometer equipped with 405- and 488-nm light paths for the dual excitation of all pHluorin-based ratiometric pH probes can be used for pHi analyses. Green fluorescence is detected after excitation with 488-nm and 405-nm laser light, and the ratios of these two fluorescence parameters (F405-nm and F488-nm) are calculated for every cell. Finally, to correlate ratios with pH, a calibration curve that links fluorescence intensity ratios to pH is generated (Benčina 2013).

## 2.2 Cytosolic $Ca^{2+}$ Concentration

Calcium concentration is a critical factor in the control of many cellular responses, being usually among the most rapid responses, in the range of nanoseconds, in plant cells. Calcium is a second messenger for a broad variety of stimuli, regulating metabolism and gene expression. Tuteja and Mahajan (2007), Riveras et al. (2015), and Edel et al. (2017) have recently reviewed the role of calcium as second messenger in different signaling pathways of plants.

Knowing the changes of intracellular calcium concentrations is important when the response of plant cells to stress factors is being studied, and fast and sensitive techniques are necessary for accurate measurements of this parameter. Continuous monitoring of thousands of cells is now possible thanks to the new generation of flow cytometers, which provide a new method for dynamic $Ca^{2+}$ measurements of the entire population (Vines et al. 2010).

Grynkiewicz et al. (1985) and Haugland (2003) have described several fluorescent probes for measuring cytosolic $Ca^{2+}$ changes. A nice review about the characteristics and use of high-affinity (Calcium-Green-1, Fluo-3, Fluo-4, Fura-2, Indo-1, Oregon Green 488 BAPTA, $Ca^{2+}$ Yellow, $Ca^{2+}$ Orange, $Ca^{2+}$ Crimson, and X-Rhod/Rhod-2), and low-affinity (Mag-Fura-2, Mag-Fluo-4, Mag-Indo-1, Mag-Fura-5, Mag-Fura-Red, Fura-2-ff, Fluo-5N, Oregon Green BAPTA-5N, Rhod-5N, Rhod-FF, X-rhod-5F, X-rhod-FF) calcium indicators has been also published by Paredes et al. in 2008.

Examples of specific dyes excited in the visible range of the spectrum are Calcium-Green 2 and Fluo-1. An 80-fold increase in the fluorescence intensity of Fluo-1 can be recorded upon binding to $Ca^{2+}$. Zottini and Zannoni (1993) also reported the first measurement of [$Ca^{2+}$] in plant mitochondria using the fluorescent $Ca^{2+}$ indicator fura-2/AM (Tsien 1981), which can be successfully trapped into the matrix of mitochondria. As well, Huang et al. (1997) uses also Fura-2 to detect increases in cytosolic $Ca^{2+}$ in parsley mesophyll senescent cells. Subbaiah et al. (1998) investigated the relationship between mitochondrial and cytosolic $Ca^{2+}$ changes in anoxic maize cells using the positive charged dye Rhod-2 AM, which has a dissociation constant ($K_d$) of 570 nm for $Ca^{2+}$ and can be accumulated within the matrix of the mitochondria. The non-ratiometric dyes Fluo-3 with a $K_d$ of ~390 nm (Minta et al. 1989) and Fluo-4, which binds to calcium with similar affinity but with a substantially higher fluorescence output (Gee et al. 2000), allow the flow cytometric measurement of calcium on instruments that are not equipped with a UV light source (June and Moore 2004). However, due to the difficulties on calibration, June and Moore (2004) recommended the combined use of Fluo-3 and Fura Red. One of the most suitable fluorescent probes for the study of calcium by flow cytometry is Indo-1. This is a ratiometric dye, excited in the ultraviolet (338 nm), and its emission spectra shifts following calcium binding (maximum emission for Indo-1 in the absence of $Ca^{2+}$ is 490 nm, and 405 nm if bound to $Ca^{2+}$). Ratio measurements (405/490) allow accurate quantifying of $Ca^{2+}$ concentrations by flow cytometry. Indo-1 is a fast response-dye, and its $K_d$ is 230 nM. Loading can be

performed simply by addition of the acetoxymethyl ester form of Indo-1 in the extracellular medium. Darjania et al. (1993) have measured calcium concentration in *Vicia faba* protoplasts using indo-1 and fluorometry. Bush and Jones (1987, 1990) have developed a methodology for measuring calcium changes in aleurone protoplasts by fluorometry, using this dye, as well as Allen et al. (1999) in *Arabidopsis* guard cells.

However, as previously commented for intracellular pH measurement, the non-invasive detection of cytosolic and intraorganellar calcium has been increasingly used in the last years, displacing the use of fluorescent dyes in flow cytometry. The use of bioluminescent protein aequorin, a genetically encoded $Ca^{2+}$ sensor with three calcium-binding sites, as well as the fusion of aequorin with GFP (green fluorescent protein) or other photoproteins, highly improved the measurement of mitochondrial, chloroplastic and endoplasmic reticulum [$Ca^{2+}$], among others (recently reviewed in Bakayan et al. 2017). As well, the recent development of genetically encoded fluorescent indicators, in which $Ca^{2+}$ modifies the fluorescence of a circularly permutated GFP (camgaroos and pericams) or stimulates the reversible association of two GFP mutants of different colors (cameleons), has allowed a faster, more accurate and non-invasive measurement of intracellular calcium (Demaurex and Frieden 2003). The genetically encoded fluorescent $Ca^{2+}$ indicators Yellow Cameleons (YCs), which have cyan and yellow fluorescent proteins (CFP and YFP), have been successfully used in the last years to measure [$Ca^{2+}$] in guard cells in response to abscisic acid and methyl jasmonate, in roots to analyze responses to salt, hormones, membrane hyperpolarization, or mechanical stimulation, and in leaves to detect calcium in response to extra-cellular ATP, touch, cold, and hydrogen peroxide (Kudla et al. 2010; Swanson et al. 2011; Bonza et al. 2013; Martí et al. 2013; Behera et al. 2015; Loro et al. 2016). Recently, Doucette and collaborators (2016) recommended the use of flow cytometry in the detection of these genetically encoded sensors, based on Förster resonance energy transfer (FRET) between fluorescent proteins (FPs), to avoid heterogeneity in the FRET ratio and the variability of microscopic methods. The use of a cytometer with laser capable of exciting CFP (cyan fluorescent protein) can allow the measurement of CFP-YFP FRET, as that from yellow cameleons (YC). Intermolecular and intramolecular FRET can be measured through flow cytometry, as previously demonstrated by Dye (2005), Adachi and Tsubata (2008), and Doucette et al. (2016). Although different limitations are still in discussion to be overcome with this method (i.e. accurate FRET ratio measurements can only be made for cells expressing relatively high levels of the reporter, and can only provide information about population average behavior, not the relationship between different parameters in a single cell), Doucette et al. (2016) suggest that multiplexed cytometric analysis of intramolecular FP FRET signals could be successfully used to investigate signal transduction cascades, ion fluxes, and metabolism, or to screen compound collections.

Some ionophores and other substances used for validation and calibration are Ionomycin, which increases the permeability of biological membranes to calcium allowing the concentration-dependent flux of this ion across membranes, and has a higher affinity by calcium at neutral and alkaline pH; Ionophore 4-bromo-A23187,

which also binds to $Ca^{2+}$ but its affinity is higher at acidic pH; $CaCl_2$, which induces a massive entry of $Ca^{2+}$ into the cell; $MnCl_2$, which induces fluorescence quenching of all calcium-specific probes; and some chelator agents like BAPTA [1,2-bis(2-aminophenoxy), ethane-N,N,N′,N′-tetraacetic acid] and EGTA [ethylene glycol bis (β-aminoethyl ether)], which bind free calcium ion and are used, in presence of ionophore, for reducing or regulating extracellular (or even intracellular) free-$Ca^{2+}$ concentrations.

Easily reproducible protocols for flow cytometric measurements of intracellular $Ca^{2+}$ concentrations can be found in June and Moore (2004), Vines et al. (2010), Posey et al. (2015), and Doucette et al. (2016).

## *2.3 Reactive Oxygen Species Generation*

The incomplete reduction of the molecular oxygen in plants originates molecules largely known as Reactive Oxygen Species (ROS) and their derivatives. Among the derivatives we can distinguish three types (Das and Roychoudhury 2014):

- Non-radicals such as singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), and ozone ($O_3$);
- Free radicals characterized by one or more unpaired electrons e.g. alkoxyl ($RO^{\cdot}$), superoxide ($O_2^{\cdot-}$), peroxyl ($ROO^{\cdot}$), hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$);
- Peroxynitrite ($ONOO^-$), which derivates from the reaction of superoxide and nitric oxide ($^{\cdot}NO$), acting both as reactive nitrogen species (RNS) and ROS.

In plants, ROS play a pivotal role in several biological processes such as plant development, plant signaling, stress response, etc. (Sharma et al. 2012).

ROS can be generated in several cell compartments and organelles, especially in those characterized by high electron transport rates (e.g. mitochondria, chloroplasts and peroxisomes) (Apel and Hirt 2004). Their accumulation could induce several damages to those organelles but plants possess ROS-scavenging enzymatic and non-enzymatic mechanisms that in normal growing conditions are able to prevent ROS-mediated toxicity (Ortega-Villasante et al. 2005; Demidchik 2015; Petrov et al. 2015). During stress conditions, these defense mechanisms could be overridden or inhibited resulting in ROS accumulation that, as a consequence, could induce cell death through protein degradation/denaturation, lipid peroxidation and nucleotides degradation (Ortega-Villasante et al. 2005; Demidchik 2015; Petrov et al. 2015).

Several ways have been developed for characterizing and quantifying ROS and their radical scavenging enzymes (Cakmak and Marschner 1992; Kruk et al. 2005; Jambunathan 2010). Among them, spectrophotometric methods and in situ localization using dyes are among the most historically used (Zhou et al. 2006; Ortega-Villasante et al. 2016). In the last decades, the use of flow cytometry is quickly growing and several specific probes have been developed for selective ROS detection (O'Brien et al. 1997; Walrand et al. 2003; Eruslanov and Kusmartsev 2010).

For singlet oxygen detection both Singlet Oxygen Sensor Green reagent (SOSG) and trans-1-(2-Methoxyvinyl)pyrene are the most widely used probes (Driever et al. 2009; Flors et al. 2006; Tang et al. 2009; Thompson et al. 1986a). Both are extremely selective for singlet oxygen but whereas the SOSG reagent is the most used for singlet oxygen detection, the trans-1-(2-Methoxyvinyl) pyrene is extremely sensitive allowing the detection of picomole quantities of this ROS (Posner et al. 1984; Thompson et al. 1986b). The SOSG, before reacting with singlet oxygen, is characterized by a slightly blue fluorescence with excitation peaks at 372 and 393 nm, and emission peaks at 395 and 416 nm. Once exposed to singlet oxygen, it emits a green fluorescence with a maximum of excitation/emission around 504/525 nm (Wiederschain 2011). Care should be taken during the experiments, since the fluorescence could quickly degrade in some solutions and alkaline pH could stimulate green fluorescence in absence of the ROS (Burns et al. 2012). Therefore, it is really important to have a control in order to correlate the intensity of the green fluorescence with singlet oxygen concentration. Moreover, could be useful to have a positive control, using chemicals such as Hypericin, to induce in health cells and/or tissues singlet oxygen production (Thomas et al. 1992; Triantaphylidès and Havaux 2009).

Concerning the applications of these probes in plant science, Pattanayak et al. (2012) used the SOSG probe to demonstrate that the accelerated cell death 2 protein (ACD2) localizes dynamically during infection to protect cells from pro-death mobile substrate molecules, some of which may originate in chloroplasts, but have major effects on mitochondria. Moreover, Wang et al. (2015) used this probe to demonstrate that the enhanced transcription of CYP38, mediated by ROS, increases plant tolerance to high light stress.

Superoxide radical has been implicated in the plant response to a wide number of stress conditions (Alscher et al. 2002; Mittler 2002; Wang et al. 2003). Since its lifetime is extremely short (in the order of nanoseconds) it generally induces peroxidation only when it is produced in close proximity to their targets (Georgiou et al. 2008). Superoxide radical detection could be achieved using the Dihydroethidium and its cationic derivative known as red mitochondrial superoxide indicator (Mito SOX™). Both chemicals are extensively used in superoxide detection even if Mito SOX was designed for a highly selective detection of superoxide in the mitochondria of living cells (Wiederschain 2011).

Dihydroethidium, also called hydroethidine, is characterized by a blue-fluorescence in the cytosol, once it intercalates within the cell's DNA it oxidizes staining the organelles (nuclei, mitochondria, etc.) with a bright red fluorescence. Moreover, when intracellular peroxidases, in combination with reactive oxygen species such as superoxide, catalyze the oxidative reaction, a highly red fluorescent product should be observed due to ethidium production.

This compound stains the cytoplasm of living cells in blue with an excitation/emission at 370/420 nm and chromatin of living cells in red with an excitation/emission at 535/610 nm (Wiederschain 2011).

As previously said, Mito SOX™ is extremely selective for mitochondria. Therefore, it should be the first choice if a selective localization of superoxide

radical is needed. The high selectivity of this chemical is due to the cationic triphenylphosphonium substituent, which is responsible for the uptake of the probe in actively respiring mitochondria. Superoxide-driven Mito SOX oxidation leads to the production of 2-hydroxyethidium that exhibits a fluorescent excitation peak at 400 nm, which is absent in the excitation spectrum of the ethidium oxidation product generated by others ROS, thus conferring to this chemical its high selectivity (Wiederschain 2011).

Cid et al. (1996) used dihydroethidium in flow cytometry experiments to evaluate copper toxicity on the membrane system of a marine diatom, observing a time dependent peroxidase activity in response to copper treatment. Moreover, Bradner and Nevalainen (2003) used the dihydroethidium coupled to flow cytometry to evaluate the oxidative status on spores and mycelial growth of antarctic microfungi exposed to prohibitive temperatures.

Concerning $H_2O_2$ quantification, the most used probes in flow cytometry applied to plants are both 2′,7′-dichlorodihydrofluorescin-diacetate ($H_2$DCFDA excitation/emission ~492–495/517–527 nm) and Dihydrorhodamine 123 (DRH123 excitation/emission ~488/560 nm).

DRH123 is an uncharged, non-fluorescent ROS indicator that, once passively diffused across membranes, is oxidized by peroxidase to cationic rhodamine 123, which exhibits green fluorescence, mainly localized in the mitochondrial inner membrane and has a sensitivity to $H_2O_2$ that is extremely higher than $H_2$DCFDA.

On the contrary, $H_2$DCFDA, after diffusion into the cell, is firstly deacetylated to a non-fluorescent compound by cellular esterases and peroxidases and then oxidized by ROS to 2′,7′–dichlorofluorescein (DCF), which is a highly fluorescent chemical and its localization is not limited to mitochondria. The higher is the concentration of $H_2O_2$ the greater is the fluorescent signal emitted by the probe. As reported by Tsuchiya et al. (1994) in animal tissues, the use of $H_2$DCFDA could be coupled with propidium iodide to simultaneously monitor oxidant production and cell injury. This technique could be also applied to plant cells.

As reported by Haugland et al. (1996), the inconvenience of both probes is that they can be oxidized by cytochrome c, and by the oxidative phosphorylation occurring in the mitochondria, making difficult to perform a quantitative estimation of ROS activity and allowing only a comparison of relative fluorescence intensities.

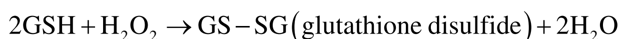
Concerning the potential applications of these probes in plant studies, Cronjé et al. (2004) used $H_2$DCFDA probe to evaluate, in tobacco protoplasts cells, if potentiation of heat-induced Hsp70 mediated by salicylic acid somministration could contribute to a reduction in apoptosis.

Saison et al. (2010), in green algal cultures treated with core–shell copper oxide nanoparticles, observed, using $H_2$DCFDA, an induction of cellular aggregation processes and a ROS-mediated deteriorative effect on chlorophyll by inducing the photoinhibition of photosystem II. Moreover, Joo et al. (2001) used flow cytometry and $H_2$DCFDA to evaluate the role of ROS in mediating the gravitropic response of maize roots trough the induction of auxin production. In particular, they observed that the scavenging of ROS by antioxidants inhibited root gravitropism, concluding that ROS generation is pivotal in root gravitropism.

### 2.4 Glutathione Levels

Glutathione (GSH), and its functionally homologous thiol, is a metabolite essential for several animal and plant functions. Its pivotal role in mammals metabolism has been largely discussed. In fact, it has been demonstrated that the inhibition, in newborn rats, of GSH production caused multi-organ failures followed by a rapid death (Meister 1994).

In plant cells, numerous physiological functions have been attributed to GSH (Rennenberg 1995; Noctor et al. 2012) and one of the most known is its role in protecting cells against damages incurred by free radicals and oxidants. In the past, $H_2O_2$ scavenging was mainly attributed to ascorbic acid, whereas the important role of GSH was les apparent. Nowadays, it is well known that in presence of both, GSH and $H_2O_2$, the enzyme glutathione peroxidase reduces free hydrogen peroxide to water according to the following formula:



$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GS} - \text{SG}\left(\text{glutathione disulfide}\right) + 2\text{H}_2\text{O}$$

Recent studies carried on Arabidopsis knockout mutants, characterized by GSH depletion due to a lack of the first enzyme involved in GSH synthesis, pointed out embryonic death during seed maturation (Cairns et al. 2006) a seedling-lethal phenotype (Pasternak et al. 2008). Moreover, transgenic Arabidopsis plants, where the tomato glutathione S-transferase was highly expressed, were characterized by a high resistance to both, drought and salt stress (Xu et al. 2015). In addition, it has been demonstrated that GSH is involved in various physiological processes, such as the regulation of sulfur assimilation (Maruyama-Nakashita and Ohkama-Ohtsu 2017), formaldehyde metabolism and detoxification (Haslam et al. 2002; Achkor et al. 2003), in defense against biotic stress (Ball et al. 2004; Parisy et al. 2007; Maughan et al. 2010) as well as in plant signaling and development (Noctor et al. 2012).

Due to the high importance of this chemical in plant metabolism and defense several techniques have been employed for its quantification. Among them, especially in human and animal field, flow cytometry is one of the most important. Several fluorescent probes have been developed to determine cellular levels of GSH and glutathione S-transferase (GST). But kinetic measurements under saturating substrate conditions is quite difficult because of the high and variable levels of intracellular glutathione, the multitude of glutathione S-transferase isozymes (Hedley and Chow 1994; Vanderven et al. 1994), and because of the reaction of the reagents used for GSH quantification with intracellular thiols other than glutathione (Bakker et al. 1991). Therefore, during GSH and GST quantification, is important to do preliminary experiments testing several dyes under controlled conditions, in which glutathione is depleted, to verify which dye fits better for the experiments purposes (Tauskela et al. 2000).

Among the dyes used for GSH detection, Bimanes was one of the most used although its use was recently substituted by ThiolTracker™ Violet reagent (excitation/emission maxima ~404/526 nm), which reacts with reduced thiols, and is more than tenfold brighter. This compound can cross living cell membranes, becoming cell-impermeable after reacting with cellular thiols (Held 2010). Therefore, living cells staining should be carried out in a thiol free buffer and then they could be immediately observed with a conventional xenon or mercury arc lamps or cells can be fixed with aldehyde before imaging. Although this compound is largely used for GSH detection and quantifications in human cells and other organisms (Rubio et al. 2011; Benson et al. 2015; Deorukhkar et al. 2015), no scientific articles are available regarding its use on plants.

Another probe used for GSH and thiols quantification is represented by the ortho-phthaldialdehyde (OPA) reagent. This is a non-fluorescent compound that, once loaded into the cells, forms two different fluorescent conjugates with both thiols and GSH, which could be observed using an excitation of 350 nm and an emission of 450, 525 and 575 nm for OPT-GSH conjugates and 405 nm for OPT-thiols conjugates. As previously reported by several authors, to get a reliable estimation of GSH content it is extremely important to have a ratiometric measurement of both types of conjugates (Treumer and Valet 1986; Haugland et al. 1996; Coba de la Peña 2001).

The thiol-reactive probes CellTracker™ Green CMFDA (based on 5-chloromethyl fluorescein diacetate) is another interesting probe, largely used in both flow cytometry and laser scanning microscopy, for intracellular GSH and intracellular thiols quantification using visible light with an argon-ion laser (Lilius et al. 1996). The conjugated products obtained from the reaction of this probe with GSH have higher fluorescence than monochlorobimane. After cells loading, the dye is well retained and it can be observed using an excitation/emission of 492/517 nm. This probe is largely used in plant field, both in flow cytometry and confocal microscopy studies (Vivancos et al. 2010; De Simone et al. 2015; Munoz et al. 2016). In particular, Munoz (2014) and Munoz et al. (2016) used this probe coupled to flow cytometry on the microalga *Chlorella vulgaris* to understand the mechanisms of detoxification of various forms of arsenic and dimethylarsenic acid.

Finally, a probe commonly used for GSH quantitation and GST activity is the non-fluorescent cell-permanent monochlorobimane, which once conjugated with thiols emits a strong blue fluorescence (Coleman et al. 1997). This probe could be used in both living cells as well as in tissue homogenates, and the glutathione conjugate of monochlorobimane has absorption/emission maxima ~394/490 nm (Kamencic et al. 2000). The possibility to work with tissue homogenates allows GSH quantification also through fluorescence spectrophotometry. In fact, this technique was used by Meyer et al. (2001) to quantify GSH level in Arabidopsis cells. Moreover, it has been used to analyze tracheary element differentiation in *Zinnia elegans* cells (Weir et al. 2005) and in studies focused on the reversibility of early stages of apoptosis in plant cells (O'Brien et al. 1998).

### *2.5 Membrane Potential*

Electric potential differences across membranes of prokaryotic and eukaryotic cells reflect the differential distribution and activity of ions such as $Na^+$, $Cl^-$, $H^+$ and especially $K^+$ across these biological membranes. Diverse membrane electrogenic pumps generate these ionic gradients, with a contribution from the intrinsic membrane permeability for each ion. Membrane potential plays a major role in the processes involving external stimulation of the cell, photosynthesis, nutrient and ion transport across the membrane, and signal transduction. In eukaryotic cells, major examples are cytoplasmic, mitochondrial (inner membrane) and lysosome membrane potential, negative inside the cell (or inside the organelles) relative to the external medium. In chloroplasts, the thylakoid potential is relatively more positive inside, but here the major electrochemical gradient is due to protons, the lumen being acid and the stroma alkaline. The mean potential values in eukaryotic cells are between −10 and −100 mV. In the mitochondria, the potential values are around −100 mV, and −50 mV in lysosomal membranes (Shechter 1984).

Membrane potential changes involve either depolarization (that is, a decrease in transmembrane potential) or hyperpolarization (an increase in the potential difference across the membrane).

Many excellent reviews are available concerning the fluorometric methods and probes developed for estimation of membrane potential, especially in organelles or in cells that are too small to allow the use of microelectrodes (Montana et al. 1989; Gross and Loew 1989; Loew 1993; Haugland et al. 1996; Roy and Hajnóczky 2009; Sabnis 2015).Dyes usually used in flow cytometry are molecules with a single negative or positive net charge, are highly hydrophobic and their partition across the membrane is a function of the Nernst equation:

$$[C]_{in} / [C]_{out} = e^{-nFE/RT}$$

where n = net electric charge of the indicator; [C] = intra and extracellular concentration of the indicator; F = Faraday constant; R = gas constant; T = temperature (°K); E = membrane potential.

These dyes are excited at the visible range of the spectrum, and with slow response to environmental changes in membrane potential ($\Delta\Psi$; Plásek and Sigler 1996). In general, fluorescence dyes' detection by flow cytometry is useful for finding differences in $\Delta\Psi$ within or between populations rather than for assigning specific values of $\Delta\Psi$ (Shapiro 2004).

Oxonol dyes have one net negative electric charge, and they will accumulate principally in the external volume of a negatively charged membrane, a lesser portion of the dye being retained in the internal compartment. A hyperpolarization of the membrane produces dye redistribution to the external medium. These dyes are excluded from the negatively charged inner mitochondrial compartment and their fluorescence reflects mainly the plasmic membrane potential. One example is the Oxonol dye $DiBaC_4(3)$, commonly used in cytometry, because it can be excited at

488 nm. These dyes are very sensitive to the variation of external ionic concentrations.

Cyanine dyes have one net positive electric charge at physiological pH, so their cellular partitioning is the contrary of oxonol dyes. These dyes are also partially accumulated in some organelles with negative inner membrane potential, like mitochondria and endoplasmic reticulum, and they are relatively toxic to cells. The cellular fluorescence intensity reflects membrane potential from the plasma membrane and also mitochondrial and endoplasmic reticulum membranes. This class of dye is the most used in flow cytometry. Two examples are the Carbocyanines $DiOC_6(3)$ and $DiOC_5(3)$. These dyes can undergo quenching when they are at high local concentration and polymerize.

The Carbocyanine dye JC-1 can be used for the study of mitochondrial potential. At low local dye concentration (low potential), the molecule is in the monomeric state with green fluorescence emission (527 nm) when excited at 490 nm. When the mitochondria are hyperpolarized, the local dye concentration increases and it forms polymer conjugates (J-conjugates) with a shifted red fluorescence (590 nm). This property makes possible ratiometric red/green fluorescence measurements in flow cytometry.

Merocyanine dyes undergo molecular reorientations with membrane depolarization, forming fluorescent dimers with altered absorption spectra. Merocyanine 540, principally associated with unsaturated lipids, is a common example.

Finally, we can cite Rhodamine 123. Its incorporation depends on the voltage gradient of the mitochondrial inner membrane, and it is less toxic that Carbocyanine dyes. This dye is used in tests for early modifications of energy metabolism.

Validation of the specificity of the dye fluorescence is done using some ionophores that modify membrane potential:

– Valinomycin facilitates the passages of K+ ions down their concentration gradient across the membrane.
– Gramicidine D makes pores in the membrane, facilitating the free passage of mono and divalent ions, and it is generally used for membrane depolarization.
– Vanadate inhibits the ATPase proteins susceptible to phosphorylation, that are mainly the cytoplasmic membrane ATPases.
– Regarding mitochondrial potential, FCCP (carbonyl cyanide *p*-trifluoro methoxyphenyl hydrazone) induces an increase in mitochondrial membrane permeability for $H^+$ passage, producing depolarisation.
– Nigericine is used to cancel pH gradient, and thereby to hyperpolarize the mitochondrial membrane because there is an interchange that is electrically neutral between $K^+$ and $H^+$ ions.

Examples of calibration curves and flow cytometric determinations of absolute membrane potential are shown in Krasznai et al. (1995). A good protocol to give an estimation of membrane potential ($\Delta\Psi$) by flow cytometry, as a function of the distribution of fluorescent lipophilic dye between cells and surrounding medium is provided in Shapiro (2004).

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