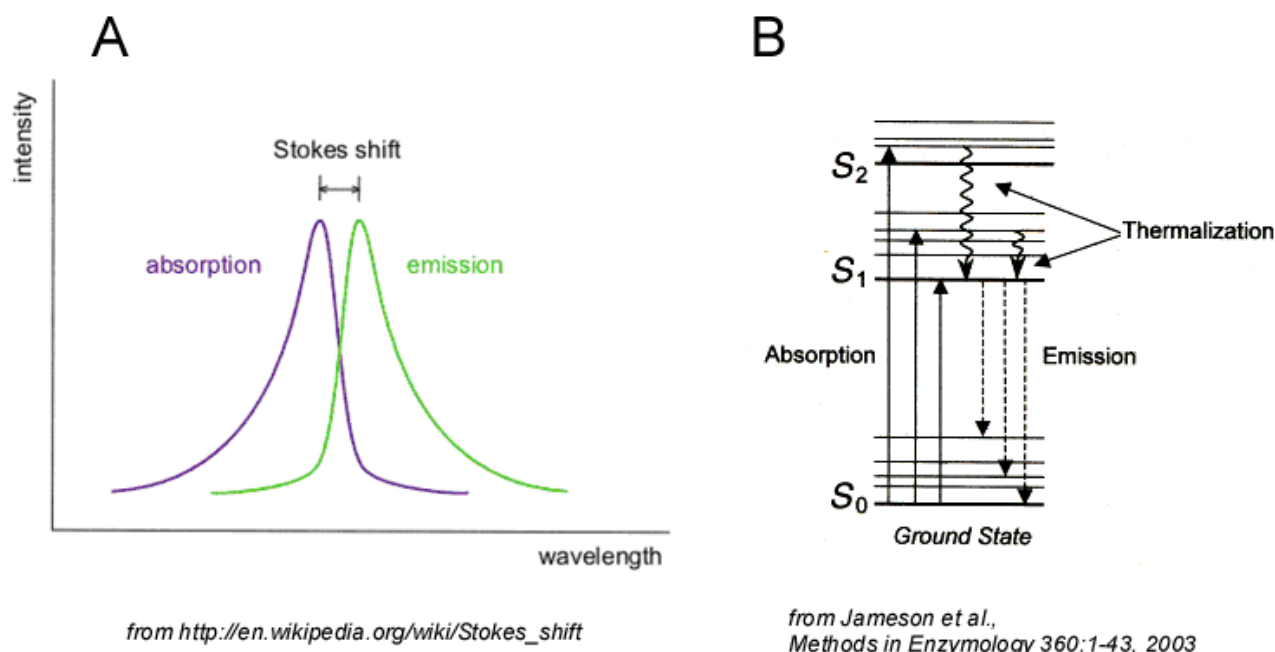


## Fluorescence

The term fluorescence was coined by Stokes circa 1850 to name a phenomenon resulting in the emission of light at longer wavelength than the absorbed light, and his name is still used to describe the wavelength shift (Figure 1A).



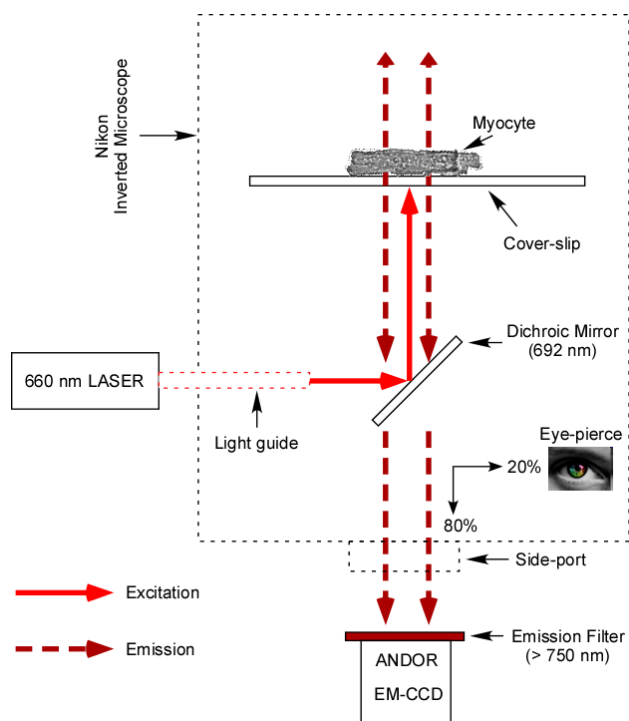
**Figure 1**

The following general rules of fluorescence (see Jameson et al., 2003<sup>1</sup>) are of practical importance:

- 1) *In a pure substance existing in solution in a unique form, the fluorescence spectrum is invariant, remaining the same independent of the excitation wavelength.*
- 2) *The fluorescence spectrum lies at longer wavelengths than the absorption.*
- 3) *The fluorescence spectrum is, to a good approximation, a mirror image of the absorption band of least frequency*

These rules follow from quantum optical considerations depicted in the Perrin-Jablonski diagram (Figure 1B). Note that although the fluorophore may be excited into different singlet state energy levels ( $S_1$ ,  $S_2$ , etc.) rapid thermal relaxation invariably brings the fluorophore to the lowest vibrational level of the first excited electronic state ( $S_1$ ) and emission occurs from that level. This fact explains the independence of the emission spectrum from the excitation wavelength. The fact that ground state fluorophores (at room temperature) are predominantly in the lowest vibrational level of the ground electronic state accounts for the Stokes shift. Finally, the fact that the spacings of the vibrational energy levels of  $S_0$  and  $S_1$  are usually similar accounts for the fact that the emission and the absorption spectra are approximately mirror images.

The presence of appreciable Stokes shift is principally important for practical applications of fluorescence because it allows to separate (strong) excitation light from (weak) emitted fluorescence using appropriate optics. A standard optical configuration includes excitation light source, excitation



**Figure 2**

filter, dichroic mirror, emission filter and the optical sensor (see Figure 2 depicting optical setup for imaging voltage-sensitive dye Di-4-ANBDQBS in isolated myocytes). The right angle between the excitation light and the direction of observation of the emitted light allows for a more complete elimination of exciting light from the observed signal.

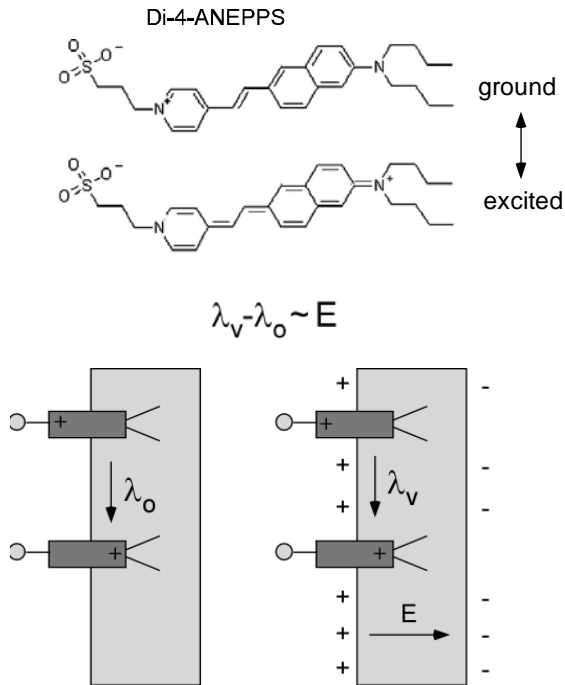
In order for a fluorophore to be used as a reporter of a biological signal in real time, its fluorescence should respond to changes in the measured parameter in a sufficiently fast, reversible and calibratable manner. Changes in the measured biological parameter can alter quantum yield (the ratio between incident and emitted photons), spectral properties, or localization of the fluorophore with respect to certain cellular compartments.

In the EP imaging lab we will use a voltage-sensitive and a calcium-sensitive fluorescent probes for real-time monitoring the action potential and intracellular calcium concentration in cardiac myocytes, and the propagated action potential in whole hearts. The purpose of this document is to provide a brief introduction into relevant fluorescent methods.

## Fast potentiometric probes

### *Sensing mechanism*

The most popular potentiometric probes used in cardiac electrophysiology (e.g., Di-4-ANEPPS) respond to changes in the transmembrane potential ( $V_m$ ) due to electrochromic mechanism (Figure 3, see Ref.<sup>2</sup>). These dyes belong to a structural class of naphthylstyryl. They are amphiphilic membrane staining dyes which usually have a pair of hydrocarbon chains acting as membrane anchors and a hydrophilic group which aligns the chromophore perpendicular to the membrane/aqueous interface. The naphthylstyryl pyridinium chromophore has most of the positive charge localized near the pyridinium nitrogen in the ground state, and near the arylamino nitrogen in the excited state. Thus, a dipole moment of the molecule changes upon excitation by a photon. A change in the voltage across the membrane causes a *spectral shift* resulting from a direct interaction between the field and the ground and excited state dipole moments. Specifically, a decrease in  $V_m$  (corresponding to the event of the action potential) shifts the absorption spectrum to longer wavelength and the emission spectrum to shorter wavelength (the emission spectrum shift is illustrated in Figure 4). Note that the peak fluorescence does not change. In order to track changes in  $V_m$ , fluorescence can be collected either on the short-wavelength wing of the spectrum, or the long-wavelength wing of the spectrum, or both (see Fig. 4). In the latter case, the measurement is *ratiometric* (see below). The sign of the change in fluorescence corresponding to the action potential will depend on the chosen emission wavelength (see Fig. 4, bottom). Observe that an overly broad emission filter capturing the entire emission spectrum may fail to detect voltage-dependent changes in fluorescence, because the



Loew LM. *Pure & Appl Chem* 1996

**Figure 3**

artifact is by far the greatest problem for voltage-sensitive imaging in beating hearts and cardiac tissues.

### Calibration

For all practical purposes, change in fluorescence ( $\Delta F$ ) of potentiometric dyes such as Di-4-ANEPPS, di-8-ANEPPS, Di-4-ANBDQBS and RH237 is linear with respect to the changes in  $V_m$ . This was shown both in lipid vesicles<sup>5</sup> and cardiac myocytes (e.g., Hardy, 2006<sup>6</sup>). Note however that due to internalization and washout (see below)  $\Delta F$ ,  $F$  or both may change irrespective of the real alterations in the AP amplitude and/or resting potential.<sup>7</sup> Therefore any comparisons of the amplitude of optical APs separated by more than 20–40 minutes (depending on the particular fluorophore) should be interpreted with caution. Still, linearity ensures faithful representation of the AP shape under all circumstances.

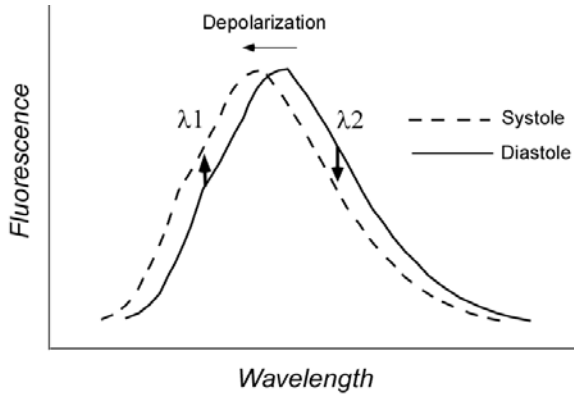
### Confounding factors

Both  $F$  and  $\Delta F$  of voltage-sensitive dyes change with time.<sup>7</sup> Decrease in  $F$  is due to *bleaching* (i.e., chemical transformation of the fluorophore following excitation) and *washout* of the dye. Decrease in  $\Delta F$  at a rate faster than  $F$  (i.e., decrease in  $\Delta F/F$ ) is usually attributed to *internalization*, a process whereby molecules of the dye enter the cell interior.

increase in the short-wavelength part of the spectrum and the decrease in the long-wavelength part of the spectrum will cancel each other. It should be noted also that fast potentiometric probes emit fluorescence even in the absence of any change in electrical field and that this “passive” background fluorescence ( $F$ ) is large as compared to the “active” signal ( $\Delta F$ ). The ratio  $\Delta F/F$  is an important parameter of fast potentiometric probes and a recently introduced probe Di-4-ANBDQBS which we will test in this lab has an improved  $\Delta F/F$  in addition to other valuable features (see Table 1).

### Ratiometry

The fact that the fluorescence spectrum shifts in response to change in  $V_m$  allows for ratiometric measurement of voltage-sensitive fluorescence (see Table 1 and Fig. 4). For example, di-4-ANEPPS signal can be recorded simultaneously at 540 nm and above 610 nm with excitation at 510 nm.<sup>3</sup> The main motivation for ratiometry in cardiac studies is removing motion artifact which should have the same direction in the two recording channels, whereas the changes related to the AP have opposite directions.<sup>4</sup> In addition, ratiometry helps to compensate for dye bleaching, internalization and washout. Note however that motion



**Figure 4**

**this lab**). Photodynamic damage is probably mediated via generation of reactive oxygen species (free radicals). There is some indirect evidence that enzymatically isolated cells are deficient in their ability to maintain normal redox state which might make them more susceptible to photodynamic damage. Indeed, supplementing enzyme catalase into pipette solution alleviates photodynamic damage caused by Di-4-ANEPPS in patch-clamped ventricular myocytes.<sup>8</sup>

***New voltage-sensitive probe Di-4-ANBDQBS***

The important difference is that the absorbance and emission spectra of Di-4-ANBDQBS are shifted to longer wavelengths as compared to its predecessors such as Di-4-ANEPPS and Di-8-ANEPPS (see Table 1). This provides a better penetration of the excitation light into myocardial tissue in the presence of blood, and a better ability to collect fluorescence from deeper layers of myocardium. The new probe demonstrated other beneficial properties, such as increased dF/F (~15% in whole hearts) and slower time course of internalization as compared to standard potentiometric probes.<sup>7</sup> **In this lab we will use Di-4-ANBDQBS for tracking the AP in isolated ventricular cardiomyocytes.**

**Table 1**

Dye	Excitation range	Emission range	$\Delta F/F$ (%)	$\lambda_{excitation}$ (nm)	$\lambda_{emission 1}$ (nm)	$\lambda_{emission 2}$ (nm)	Refs.
Di-4-ANEPPS	Blue-green	Orange-red	8-9	488 510±5	525-555 540±10	650-700 >610	Knisley 2000 <sup>4</sup> Poelzing 2007 <sup>3</sup> Matyukas 2007 <sup>7</sup>
Di-8-ANEPPS	Blue-green	Orange-red	7.5	460-500	540-580	600-640	Hardy 2006 <sup>6</sup>
RH-237	Green	Near IR	2-3	488 520±20	619-641 >715	751-772 N/A	Kong 2000 <sup>9</sup> Choi 2000 <sup>10</sup>
Di-4-ANBDQBS	Red	Near IR	10-15	630-670	>720	N/A	Matyukas 2007 <sup>7</sup>

The hydrophilic “head” of voltage-sensitive dyes which sticks out into the extracellular water phase makes it very difficult for the dye to flip across the lipid bilayer. Yet, such a flip-over takes place at a slow rate which is dye- and species-dependent.<sup>7</sup> If the dye lines the inner and the outer leaflets of the sarcolemma, the response to voltage will cancel out. Because of internalization, attempts to improve signal by excessive loading with voltage-sensitive dye sometimes lead to very bright background (F) without any appreciable voltage-sensitive signal ( $\Delta F$ ).

*Photodynamic damage* refers to toxic effects observed in the presence of dye and excitation light. With voltage-sensitive dyes, such effects appear to be more prominent in enzymatically isolated cells,<sup>8</sup> although there may be adverse effects in whole hearts (**that is what we will investigate in the whole heart part of**

## Calcium-sensitive fluorescent dyes

It is worth noting that almost all currently used calcium-sensitive dyes were designed in the laboratory of Dr. Roger Tsien who received Nobel Prize in 2008 for his crucial contribution in the understanding and development of the green fluorescent protein, GFP. Shall we say that his prior contributions to the world of calcium sensors are equally remarkable?

### *Sensing mechanism*

Ca-sensitive fluorophores change their fluorescence properties upon binding  $\text{Ca}^{2+}$ . Table 2 lists the useful changes in fluorescence and excitation/emission configurations of some popular Ca fluorophores. Dye Fura-2 exhibits a shift in excitation spectrum which permits ratiometric measurements using excitation at two alternating wavelengths. Dye indo-1 exhibits a shift in emission spectrum (see Figure 5) permitting ratiometry using two emission wavelengths. Ratiometry is advantageous because it compensates for variations in signal due to differences in dye concentration, light path and heterogeneous staining. Note however that both fura-2 and indo-1 require UV excitation (see Table 2). Fluor-3 and rhod-2 (see Fig. 5) do not have any appreciable shift either in excitation or emission and thus do not offer an opportunity for ratiometric measurements. However, these dyes are excited in visible range (blue or green) which has several advantages: decreased phototoxicity, better penetration of excitation light into multicellular preparations, lesser contamination from tissue autofluorescence (predominantly emitted by mitochondrial NADH and NADPH<sup>11</sup>). Note however that using two-photon excitation whereby energy of two near-simultaneous double-wavelength photons is combined to excite the fluorophore<sup>12</sup> eliminates some of the problems associated with excitation spectrum in UV range.

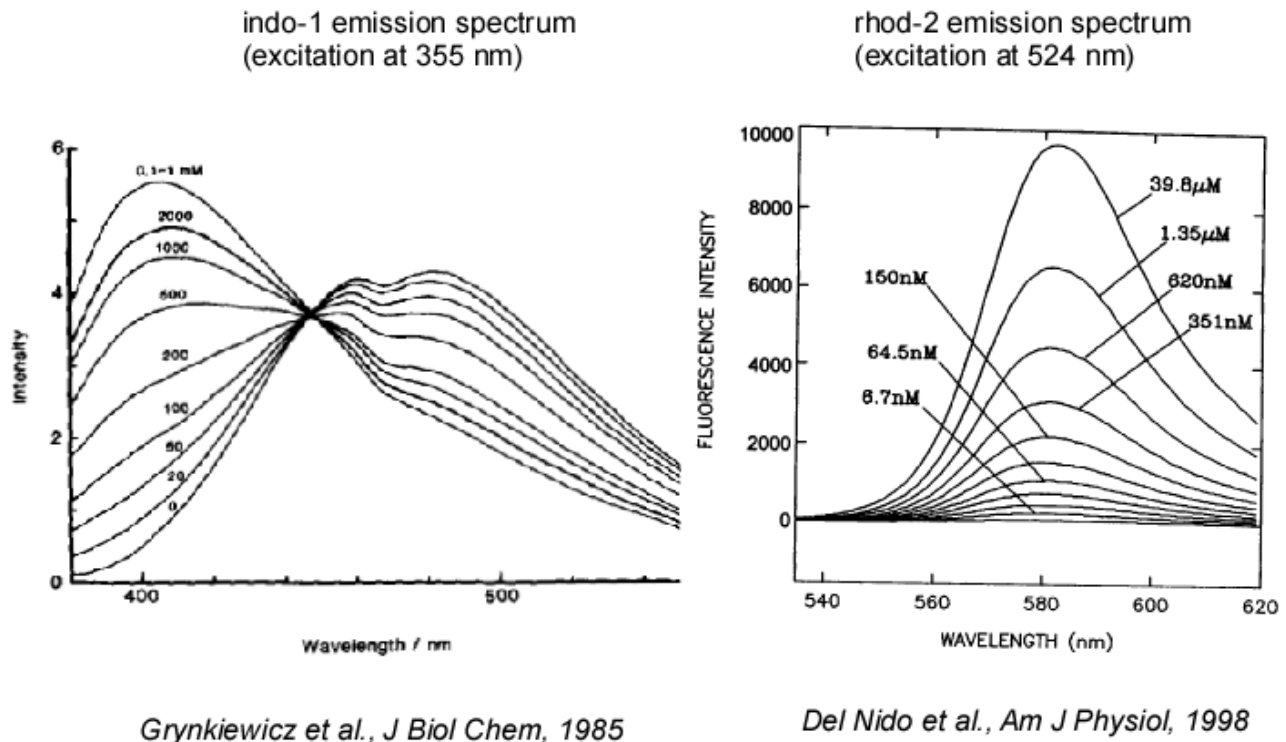
**Table 2**

Dye	Ca-dependent change in spectrum	$K_d$ ( $\mu\text{M}$ )	Excitation		Emission		Refs.
			$\lambda_{\text{excitation 1}}$ (nm)	$\lambda_{\text{excitation 2}}$ (nm)	$\lambda_{\text{emission 1}}$ (nm)	$\lambda_{\text{emission 2}}$ (nm)	
Fura-2	Shift in absorption spectrum	0.135 0.224	340	380	510	N/A	Grynkewicz 1985 <sup>13</sup>
Indo-2	Shift in emission spectrum	0.25	340	N/A	400	490	Grynkewicz 1985 <sup>13</sup>
Fluor-3	Increase in fluorescence	0.4 0.864	506	N/A	526	N/A	Minta, 1989 <sup>14</sup> Yao, 1998 <sup>15</sup>
Rhod-2	Increase in fluorescence	0.5 1.0	524	N/A	589	N/A	Minta, 1989 <sup>14</sup> Stamm, 2003 <sup>16</sup>

### *Confounding factors*

General problems of bleaching and possible photodynamic damage resulting from too high intensity of excitation apply to Ca fluorophores but usually are not a matter of a major practical concern.

*Washout.* Ca fluorophores are actively extruded from the cell which results in a decrease of fluorescence over time. This process can be slowed down by organic anion transport blocker probenidol.



**Figure 5**

*Distribution of the dye.* Ca sensitive fluorophores are cell-impermeable unless their carboxylate groups are derivatized as acetoxymethyl (AM) esters rendering the indicator permeant to membranes and insensitive to ions. Once inside the cell, these derivatized indicators are hydrolyzed by ubiquitous intracellular esterases, releasing the ion-sensitive polyanionic indicator (see <http://probes.invitrogen.com/handbook/boxes/0428.html>). Cell-impermeable (salt) forms of Ca fluorophores can be loaded in the cytoplasm only via a micropipette attached to the cell. The esterified (AM) forms of fluorophores are effectively trapped inside a membrane-enclosed compartment where the ester groups were cleaved. Thus, AM forms can accumulate in intracellular organelles, such as mitochondria, sarcoplasmic reticulum and lysosomes. The actual distribution between compartments depends on the relative rates of permeation into a given organelle and de-esterification. For example, rhod-2 which has delocalized positive charge in the ester groups of phodamine, tends to accumulate in mitochondria which negative potential with respect to the cytoplasm. This process is enhanced at low temperatures because esterase activity is low and more molecules of fluorophore can enter mitochondria (and other intracellular organelles) before they get de-esterified in the cytoplasm. In fact, cold loading is used for preferential staining of mitochondria in order to track mitochondrial Ca dynamics.<sup>17</sup> Cell-impermeable dyes loaded via a pipette are restricted to cytosol and cannot enter intracellular organelles. Thus, one can use a combination of an cell-permeable and cell-impermeable forms (of the same or different fluorophore) to compare cytosolic and mitochondrial Ca transients.<sup>18</sup> Another approach is to use cold-loading rhod2-AM for tracking mitochondrial Ca transient in combination with warm-loading fluor3-AM for tracking cytosolic Ca transient.<sup>17</sup> Note however that lysosomal accumulation of AM fluorophores may significantly contribute to the background (time-independent) fluorescence with any loading protocol and may complicate estimation of resting Ca level (see Calibration). Using very high resolution confocal or two-photon microscopy may help to sort out fluorescence coming out from

different cellular compartments or organelles. In a macroscopic, wide-field Ca imaging performed in tissues and organs the subcellular source of Ca-sensitive fluorescence can be only indirectly inferred from pharmacologic interventions which are expected to alter Ca content and/or Ca transport in various compartments.<sup>19</sup> Another problem of macroscopic imaging at the organ level is the presence of different cell types. For example, endothelial cells comprising capillary wall are stained with Ca-sensitive fluorophores and contribute to integral fluorescence emitted from the heart surface thus confounding estimates of resting  $[Ca^{2+}]_i$  in the cardiac cells.

*Autofluorescence* is fluorescence emitted by the tissue. The most prominent source of tissue fluorescence is one emitted by mitochondrial NADH and NADPH<sup>11</sup> when excited by UV light. The interference from NAD(P)H fluorescence is a particularly difficult problem when the mitochondrial redox state is altered, e.g. during hypoxia or ischemia. Note also that sometimes components of the optical apparatus (e.g., fiber optics) can emit fluorescence in the range of interest.

*Ca buffering.* It is always true that in order to study a physical system one have to perturb it. It rarely applies as directly as in the case of measuring Ca-sensitive fluorescence. In order for free ions of Ca to be detected, they have to bind to molecules of fluorophore, that is, to lose their free status. Thus concentration of Ca probe has to be carefully selected so that it achieves acceptable brightness of fluorescence but without significant suppression of Ca transient and contractility.

*pH-sensitivity.* Affinity of Ca fluorophores to Ca generally decreases ( $K_d$  increases) in acidic solutions (low pH) which, if not corrected for, can lead to significant errors, for example, underestimation of  $[Ca^{2+}]_i$  when using rhod-2 in ischemic hearts.<sup>16</sup>

### **Calibration**

Calibration of Ca-sensitive fluorescence is always challenging, to a large degree because it is difficult to compensate for confounding factors mentioned above. However, to the extent those factors can be ignored or corrected for, calibration is relatively straightforward. At any instance of time, fluorescence  $F$  emitted by a Ca fluorophore and measured at a fixed excitation and emission wavelengths and optical path is equal to:

$$F = F_b + F_u + F_{bkg} \quad (1)$$

Where  $F_b$  is fluorescence of fluorophore bound to Ca,  $F_u$  is fluorescence of unbound fluorophore, and  $F_{bkg}$  is background fluorescence in the absence of fluorophore (autofluorescence of the cells and optics, etc.)

$F_b$  and  $F_u$  are proportional to the concentrations of bound and unbound fluorophore ( $[dye]_b$  and  $[dye]_u$ ):

$$F_b = gb[dye]_b \quad (2)$$

$$F_u = gu[dye]_u \quad (3)$$

$$[dye]_{total} = [dye]_b + [dye]_u \quad (4)$$

The “gain” coefficient  $g$  absorbs the instrumentation properties such as excitation intensity, path length and the instrumental efficiency of collecting the emitted fluorescence. Coefficients  $b$  and  $u$

reflect photochemical properties (such as extinction coefficient and quantum efficiency) of the bound and unbound molecules of fluorophore, respectively. Let us consider fluorescence in the two limit cases. Minimal level of fluorescence ( $F_{min}$ ) is observed in the absence of Ca, when all molecules of the fluorophore are free:

$$F_{min} = gu[\text{dye}]_{\text{total}} + F_{bkg} \quad (5)$$

Maximal level of fluorescence ( $F_{max}$ ) is observed in the presence of very high, saturating  $[\text{Ca}^{2+}]$ , when all molecules of fluorophore are bound:

$$F_{max} = gb[\text{dye}]_{\text{total}} + F_{bkg} \quad (6)$$

It is useful (as shown below) to consider background-subtracted fluorescence values:

$$F' = F - F_{bkg} \quad (7)$$

$$F'_{min} = F_{min} - F_{bkg} \quad (8)$$

$$F'_{max} = F_{max} - F_{bkg} \quad (9)$$

Note that

$$\beta = F'_{max} / F'_{min} = b/u \quad (10)$$

is the ratio between the fluorescence of the bound and unbound form of the fluorophore which is determined by the photochemical properties of the dye and is constant in the same environment.

In order to bring Ca in the picture, recall that the relationship between the concentrations of free fluorophore, free Ca and bound fluorophore is described by a familiar 1:1 complexation equation:

$$K_d = \frac{[\text{Ca}^{2+}][\text{dye}]_u}{[\text{dye}]_b} \quad (11)$$

Note that  $K_d$  is equal to the concentration of free Ca when 50% of dye is bound.  $K_d$  of popular Ca fluorophores are in the range of 0.1 – 1.0  $\mu\text{M}$ .

Using equations above it is not difficult to derive the Ca calibration function for a single wavelength measurement:

$$[\text{Ca}^{2+}] = K_d \frac{F - F_{min}}{F_{max} - F} = K_d \frac{F' - F'_{min}}{F'_{max} - F'} \quad (12)$$

In order to convert fluorescence level to  $[\text{Ca}^{2+}]$ , one need to know  $K_d$ ,  $F_{min}$  and  $F_{max}$ .  $K_d$  is usually measured *in-vitro* by titrating solutions realistically mimicking cell interior with varying concentrations of  $\text{Ca}^{2+}$ .  $F'_{max}$  can be measured by making cell membrane permeable to Ca (using a Ca



ionophore) and increasing extracellular  $[Ca^{2+}]$  to saturating levels. It is difficult to measure  $F_{min}$  in living systems because it is nearly impossible to achieve complete absence of free  $Ca^{2+}$  ions. Note however that  $F'_{min}$  can be calculated from  $F'_{max}$  knowing  $\beta$  (see Eq. 10). Note also that for dyes with high values of  $\beta$  (e.g.,  $\sim 40$  for fluor-3<sup>14</sup> and  $\sim 100$  for rhod-2<sup>19</sup>)  $F'_{min}$  can be considered  $\approx 0$ . Another approach involves quenching Ca fluorophore by saturating concentration of manganese ions.<sup>20</sup> Knowing the ratio between fluorescence of  $Mn^{2+}$ -saturated dye and  $Ca^{2+}$ -saturated dye from *in-vitro* measurement,  $F'_{max}$  can be calculated from manganese quenching measurement *in-vivo*.

Calibration of a single-wavelength Ca measurement can be much simplified assuming  $F'_{min} \approx 0$  and postulating values for  $K_d$  and resting (diastolic) level of Ca.<sup>21</sup> In that case the following equation can be derived from the equations above:

$$[Ca^{2+}] = \frac{K_d R}{K_d / [Ca^{2+}]_{rest} + 1 - R} \quad (13)$$

where  $R = F/F_{rest}$  and  $F_{rest}$  is fluorescence during diastole.

For a ratiometric measurement at wavelengths  $\lambda_1$  and  $\lambda_2$ , the Ca calibration equation is only slightly different from Eq. 12 above (see Grynkewicz, 1985<sup>13</sup> for detailed explanation):

$$[Ca^{2+}] = K_d \frac{R' - R'_{min}}{R'_{max} - R'} \left( \frac{1}{\beta_{\lambda_2}} \right) \quad (14)$$

where

$$R = \frac{F'_{\lambda_1}}{F'_{\lambda_2}}$$

$$R_{min} = \frac{F'_{min,\lambda_1}}{F'_{min,\lambda_2}}$$

$$R_{max} = \frac{F'_{max,\lambda_1}}{F'_{max,\lambda_2}}$$

## Reference List

1. Jameson DM, Croney JC, and Moens PDJ. Biophotonics. Chapter 1: Fluorescence: Basic Concepts, Practical Aspects, and Some Anecdotes. Mariott G and Parker I. (360), 1-43. 2003. Academic Press. Methods in Enzymology. Abelson JN and Simon MI. 2003.  
Ref Type: Serial (Book, Monograph)

2. Loew LM. Potentiometric dyes: Imaging electrical activity of cell membranes. *Pure & Appl Chem.* 1996;68:1405-1409.
3. Poelzing S, Veeraraghavan R. Heterogeneous ventricular chamber response to hypokalemia and inward rectifier potassium channel blockade underlies bifurcated T wave in guinea pig. *Am J Physiol Heart Circ Physiol.* 2007;292:H3043-H3051.
4. Knisley SB, Justice RK, Kong W, JOHNSON PL. Ratiometry of transmembrane voltage-sensitive fluorescent dye emission in hearts. *Am J Physiol Heart Circ Physiol.* 2000;279:H1421-H1433.
5. Fluhler E, Burnham VG, Loew LM. Spectra, membrane binding, and potentiometric responses of new charge shift probes. *Biochemistry.* 1985;24:5749-5755.
6. Hardy MEL, Lawrence CL, Standen NB, Rodrigo GC. Can optical recordings of membrane potential be used to screen for drug-induced action potential prolongation in single cardiac myocytes? *Journal of Pharmacological and Toxicological Methods.* 2006;54:173-182.
7. Matiukas A, Mitrea BG, Qin M, Pertsov AM, Shvedko AG, Warren MD, Zaitsev AV, Wuskell JP, Wei MD, Watras J, Loew LM. Near-infrared voltage-sensitive fluorescent dyes optimized for optical mapping in bloodperfused myocardium. *Heart Rhythm.* 2007;4:1441-1451.
8. Schaffer P, Ahammer H, Muller W, Koidl B, Windisch H. Di-4-ANEPPS causes photodynamic damage to isolated cardiomyocytes. *Pflugers Arch.* 1994;426:548-551.
9. Kong W and Knisley SB. Effect of diacetyl monoxime on action potential duration in rabbit hearts as revealed by fluorescence emission ratiometry. Proceedings of BMES Conference in China . 2000.

Ref Type: Abstract

10. Choi BR, Salama G. Simultaneous maps of optical action potentials and calcium transients in guinea pig hearts: mechanisms underlying concordant alternans. *Journal of Physiology-London.* 2000;529:171-188.
11. Eng J, Lynch RM, Balaban RS. Nicotinamide adenine dinucleotide fluorescence spectroscopy and imaging of isolated cardiac myocytes. *Biophys J.* 1989;55:621-630.
12. Ragan TM, Huang H, and So PTC. Biphotonics, Part B. Chapter 22: In Vivo and ex Vivo Tissue Applications of Two-Photon Microscopy. Mariott G and Parker I. (361), 481-505. 2003. Academic Press. Methods in Enzymology. Abelson JN and Simon MI.

Ref Type: Serial (Book, Monograph)

13. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985;260:3440-3450.
14. Minta A, Kao JP, Tsien RY. Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem.* 1989;264:8171-8178.

15. Yao A, Su Z, Nonaka A, Zubair I, Spitzer KW, Bridge JH, Muelheims G, Ross J, Jr., Barry WH. Abnormal myocyte Ca<sup>2+</sup> homeostasis in rabbits with pacing-induced heart failure. *Am J Physiol Heart Circ Physiol*. 1998;275:H1441-H1448.
16. Stamm C, Friehs I, Choi YH, Zurakowski D, McGowan FX, del Nido PJ. Cytosolic calcium in the ischemic rabbit heart: assessment by pH- and temperature-adjusted rhod-2 spectrofluorometry. *Cardiovascular Research*. 2003;59:695-704.
17. Trollinger DR, Cascio WE, Lemasters JJ. Mitochondrial Calcium Transients in Adult Rabbit Cardiac Myocytes: Inhibition by Ruthenium Red and Artifacts Caused by Lysosomal Loading of Ca<sup>2+</sup>-Indicating Fluorophores. *Biophys J*. 2000;79:39-50.
18. Maack C, Cortassa S, Aon MA, Ganesan AN, Liu T, O'Rourke B. Elevated Cytosolic Na<sup>+</sup> Decreases Mitochondrial Ca<sup>2+</sup> Uptake During Excitation-Contraction Coupling and Impairs Energetic Adaptation in Cardiac Myocytes. *Circ Res*. 2006;99:172-182.
19. del Nido PJ, Glynn P, Buenaventura P, Salama G, Koretsky AP. Fluorescence measurement of calcium transients in perfused rabbit heart using rhod 2. *Am J Physiol Heart Circ Physiol*. 1998;274:H728-H741.
20. Kao JP, Harootunian AT, Tsien RY. Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J Biol Chem*. 1989;264:8179-8184.
21. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science*. 1993;262:740-744.