Application of the Optical Method in Experimental Cardiology: Action Potential and Intracellular Calcium Concentration Measurement

Marina RONZHINA¹,², Vratislav CMIEL¹,², Oto JANousek¹,², Jana KOLAROvA¹,², Marie NOVAKova²,³, Petr BABULA¹,², Ivo PROV AzNIK¹,²

¹Department of Biomedical Engineering, Faculty of Electrical Engineering and Communication, Brno University of Technology, Brno, Czech Republic
²International Clinical Research Center - Center of Biomedical Engineering, St. Anne's University Hospital Brno, Brno, Czech Republic
³Department of Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic

Corresponding author:
Marina Ronzhina
Department of Biomedical Engineering
Faculty of Electrical Engineering and Communication
Brno University of Technology
Kolejní 4
61200 Brno
Czech Republic
E-mail: ronzhina@feec.vutbr.cz

Short title:
Optical Method in Experimental Cardiology
Summary

It has been shown that, in addition to conventional contact electrode techniques, optical methods using fluorescent dyes can be successfully used for cardiac signal measurement. In this review, the physical and technical fundamentals of the method are described, as well as the properties of the most common systems for measuring action potentials and intracellular calcium concentration. Special attention is paid to summarizing limitations and trends in developing this method.

Key words

Optical method, fluorescent dyes, action potential, calcium concentration

Introduction

Cardiac disorders represent a serious problem of modern society. The most common are myocardial ischemia and concomitant arrhythmias. These disorders result in incorrect electrical and subsequently mechanical activity of the heart and often cause sudden death. Various biological models (isolated cells, tissues, and isolated hearts) are successfully used in experimental cardiology to study changes of cardiac function induced by these disorders. In particular, myocardial ischemia can be modeled using a Langendorff perfusion system by stopping the delivery of perfusate to the isolated heart. Moreover, use of the isolated heart allows experiments with repeated ischemia to investigate the phenomenon of ischemic preconditioning which has been a recent focus of scientific interest (Yellon and Downey 2003).

The most common signals measured in experimental cardiology are electrocardiograms (ECGs), action potentials (APs), and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]). There is a strict relationship between these signals. AP originates in the pacemaker cells of the sinoatrial node and then passes to the other sites of the heart through a conduction system (Bers 2001). APs from particular parts of the heart are of a specific morphology that varies with species and heart rate and is affected by drugs or hormones (Bers 2001). Each phase of AP is characterized by changes in cell membrane permeability to the ions involved in the activation process. Coordinated activation-relaxation alternation of the cell can be explained by AP duration (APD) that is generally almost as long as the Ca\(^{2+}\) transient. Calcium entering the cell membrane activates the myofilaments and induces cardiac muscle contraction. L-type of voltage-gated calcium channels (slow channels) play the most important role in the activation process (mainly in the genesis of plateau phase of
AP). Ca\(^{2+}\) entering into the cell triggers the release of Ca\(^{2+}\) from the sarcoplasmic reticulum, which additionally increases [Ca\(^{2+}\)] (Bers and Perez-Reyes 1999, Takahashi \textit{et al.} 1999). It is known that alterations in the regulation of myocyte [Ca\(^{2+}\)] may cause both mechanical dysfunction and genesis of pro-arrhythmic states: delayed and early afterdepolarizations followed by APD prolongation (Bers 2000, Bers 2002, Clusin 2008). Studies of the ischemic preconditioning effect have shown that systolic [Ca\(^{2+}\)], and Ca\(^{2+}\) transients decline after the induction of ischemia, and transients reappear within a few seconds after reperfusion (Dekker \textit{et al.} 1999). Although numerous studies have been published in this area, the quantitative description of [Ca\(^{2+}\)] and AP changes during repeated ischemia has not been described in literature in detail.

It is necessary to study the correlations between the two above-mentioned signals in order to understand heart physiology. There are different techniques for recording cardiac signals. One technique is recording by an optical method using fluorescent dyes. This approach is still in development. In the present paper, the most important issues about the principles of recording using the optical method are addressed.

**Principles of cardiac signal recording using fluorescent dyes**

AP and [Ca\(^{2+}\)], can be generally recorded using two methods. The first – conventional – uses microelectrodes to record the signal. This is the gold standard for recording electrical signals on the cellular level (Richardson and Xiao 2010). There is one significant disadvantage to this method: the electrode is inserted into the cell, which leads to the destruction of cell membrane and often to changes in its physiological functions. Moreover, the procedure of electrode application is relatively complicated. Thus, the results of experiments are strongly dependent on the experience and skills of the researcher. The alternative approach is optical recording of the signal. The optical method is based on the fluorescent properties of special chemical compounds, fluorescent dyes. This method allows the non-invasive recording of AP and [Ca\(^{2+}\)], from larger areas of the heart surface. Thus, this method is highly suitable for isolated heart experiments. The spectral characteristics of the dye undergo changes in their molecular structure (for instance, after binding to the cell membrane or accepting calcium ions) (Fast 2005, Salama 2001). Various fluorescent dyes with different properties are commercially available.

The procedure for recording with fluorescent dyes consists of loading the preparation (by injection, perfusion or superfusion; Fast 2005) for a definite time, washing the preparation to remove the unbound dye molecules, and exposing the preparation to the light. A halogen lamp, xenon/mercury arc lamp, high-power
light emitting diode (LED), or laser (Efimov et al. 2004, Fast 2005, Salama 2001, Tritthart 2005) can be used to excite the dye to a higher energy state and provoke light emission. In comparison with the excitation spectrum, the emission spectrum of the dyes generally used for AP measurement is typically shifted (“Stokes shift”) due to the decreased energy by the return to the ground energy state (Salama 2001). Excitation and emission lights must pass through optical filters and/or dichroic mirrors to separate light bands corresponding to the different (excitation or emission) levels of the dye fluorescence. The excitation filter is not necessary for laser illumination, due to the monochromatic character of the laser spectrum. The emitted light can be detected with a fast photodetector. Commonly used photodetectors are photodiodes, photodiode arrays (PDAs), photomultiplier tubes, and charge coupled device (CCD) cameras (Efimov et al. 2004, Laurita and Libbus 2001, Tritthart 2005). The photodetector transforms light to an electrical signal, which can be further digitized and processed with a computer.

Two kinds of fluorescent dyes with different spectral properties are used for optical recording: non-ratiometric (single-wavelength) and ratiometric (dual-wavelength) dyes. The fluorescence of non-ratiometric dyes does not undergo spectral shifting. The variations in membrane potential or \([Ca^{2+}]_i\), only change the intensity of emission light of these dyes. In ratiometric dyes, there are two maxima in emission (“ratio in emission”) or excitation (“ratio in excitation”) fluorescence spectra. In this case, the dye spectra can be measured at two wavelength bands, and the resulting fluorescence intensity can be calculated as a ratio. The fluorescence of the non-ratiometric indicator depends not only on transmembrane potential or \([Ca^{2+}]_i\), but also on other factors. The most important factors are the concentration of the dye, the length of the optical path-way, and the excitation light intensity (Salama 2001, Fukano 2008). These dyes are more sensitive to photobleaching (exponential attenuation of dye fluorescence intensity – drift – in the presence of oxygen with excitation over time) than ratiometric dyes (Salama 2001). However, ratiometric recording of fluorescence can also give incorrect results, as the ratio of photobleaching of two spectra (emission or excitation) is not always the same (Fast 2005, Takahashi et al. 1999). Ratiometric recording helps to eliminate or significantly reduce the motion artifacts that occur in signals recorded on the contracting heart because of changes in the relationship between the preparation and photodetector in time; this can lead to inaccurate measuring of repolarization (Efimov et al. 2004, Laurita and Libbus 2001). However, this method of motion artifact correction produces acceptable results only if the movement of the heart is not large (Tai et al. 2004). Some pharmacological/chemical (Kim et al. 2010, Hayashi et al. 2008, Joung et al. 2009, Laurita
and Singal 2001, Holcomb et al. 2009, Omichi et al. 2004, Wu et al. 2005, Sidorov et al. 2008) and mechanical (Kong et al. 2003) intervention techniques can be used to obtain signals which are free or almost free from motion artifacts.

**Measurement of cardiac AP**

The fluorescence of *voltage-sensitive* (potentiometric) dyes (VSDs) is proportional to transmembrane potential. Some VSDs are characterized by a *fast* (with a time constant <1 µs) and *linear* (within a range of at least ±400 mV) *response* to the membrane potential, which makes them useful for measuring electrical activity in excitable cells (Fast 2005, Cohen 2010). Fast-response dyes interact directly (without molecular motion) with the electric field of membrane by an *electrochromic mechanism* in such a way that their chromophore's electron configuration changes upon excitation. Thus, there is a charge shift induced by changes of membrane potential value (Loew 2010). Emission spectra of electrochromic VSD molecules bound to the cellular membrane shift themselves toward the shorter wavelengths upon membrane depolarization (Knisley 2000, Loew 2001, Loew 2010). This allows the measurement of AP with both non-ratiometric and ratiometric methods (Fig.1). In non-ratiometric setup, AP is proportional to the emission fluorescence measured using high-pass optical filter ('HP' in Fig.1a). In ratiometric setup, AP is proportional to the ratio of emission fluorescence measured using band-pass optical filters at two different wavelength regions ('BP1' and 'BP2' in Fig.1b). In the second option, the wavelengths at the tails of the bell-shaped emission spectra must be chosen by optical filter for recording with a photodetector, because the maximal fluorescence change is in this region (Fig.1b) (Loew 2010, Tritthart 2005). The resulting optical signal recorded with this method contains information about the relative changes of the potential time-course, not about the absolute magnitude of potential. The absolute value of the optical signal depends not only on the value of AP, but also on the density of dye molecules in the area under the photodetector, the degree of dye internalization, and the intensity of excitation light, which in fact differs very widely across the preparation (Fast 2005, Salama 2001). However, optical signals can be calibrated using AP recorded simultaneously with microelectrodes or patch-clamps (Efimov et al. 2004, Tritthart 2005). Another approach is to normalize AP to a voltage range of 100 mV (Salama et al. 2005).
Fig. 1 Spectral characteristics of electrochromic VSDs and possible acquisition systems: a) non-ratiometric setup, b) ratiometric setup (ratio in emission). Ex – excitation spectrum, Em – emission spectrum, HP – high pass optical filter, BP – band pass optical filter. Arrows represent the shift of the emission spectrum by depolarization. Excitation and emission spectra are depicted in black and in grey colors, respectively.

The most commonly used fast VSDs are di-4-ANEPPS and di-8-ANEPPS. Di-4-ANEPPS reaches a time resolution of less than 1ms (Trithhart 2005) and exhibits fluorescence intensity increases of up to 10% per 100 mV (Johnson et al. 1999). For ratiometric recording, emitted light is generally recorded simultaneously in red (at approx. 510-576 nm) and green (at approx. 598-751 nm) regions (Kolářová et al. 2010). These wavelengths produce large fractional changes of fluorescence and low noise (Knisley et al. 2000). The same approach is used for recording with di-8-ANEPPS, the modification of di-4-ANEPPS. Di-8-ANEPPS is characterized by higher stability but more difficult loading than di-4-ANEPPS (Fast 2005). Therefore di-4-ANEPPS is preferred for AP recording in isolated hearts.

The spectral properties of VSDs depend on the environment. Maximal excitation and emission wavelengths can be quite different for VSD bound to the phospholipid bilayer membrane and VSD dissolved in solution (Fast 2005, Čmiel et al. 2010).

Measurement of [Ca^{2+}]_i

Ca^{2+} sensitive dyes (CSDs) are fluorescent indicators with spectra varying in response to the Ca^{2+} acceptance. CSDs can be classified into various groups based on their spectral properties (Takahashi et al. 1999): dyes with excitation in ultraviolet (UV) (e.g. indo1 and fura2) and visible ranges of the light spectrum (blue, green, and red) (e.g. fluo3, calcium green, rhod2); non-ratiometric (e.g. fluo3, fluo4, calcium green dyes, and rhod 2) and ratiometric (e.g. indo1, fura2, and fura red) dyes; dyes with emission in blue (e.g. indo1), green (e.g. fura2, fluo3, indo1, and calcium green), yellow and orange (e.g. rhod2), and red and near infrared (e.g. fura red) wavelengths.
The type of CSD must be taken into consideration when designing the experiment. UV CSDs are not suitable for long-term measurements because of the cytotoxicity of UV light. Besides the cytotoxicity, there are other disadvantages: some cell constituents (for example, pyridine nucleotides) can fluoresce when excited with UV light (Takahashi et al. 1999). Their autofluorescence can be incorrectly detected as the true emission of dye. Moreover, excitation at UV wavelengths requires the use of special UV optical components that are expensive and may lead to a decrease in required signal intensity.

Relative changes in \([\text{Ca}^{2+}]_i\) can be measured using non-ratiometric dyes with non-shifting excitation and emission spectra of calcium-saturated dye molecules, which only change their emission fluorescence intensity (towards larger values) upon binding to calcium (Fig.2a). Fluo3 and fluo4 are often chosen for \([\text{Ca}^{2+}]_i\) measurement. These dyes have similar structures and similar emission spectra. After binding to calcium, changes of fluo3 emission can reach 40 (Johnson et al. 1999) or even 100 (Gee et al. 1999) times more than calcium-free dye. The response of fluo4, however, is greater: intensity of fluo4 emission is up to twice that of fluo3 (Johnson et al. 1999). Thus, lower fluo4 concentrations can be used to generate an optical signal with the same intensity (Gee et al. 1999). Fluo3 is more phototoxic to cells than fluo4 (Gee et al. 1999) and some other non-UV dyes (Silei et al. 2000).

**Fig. 2** Spectral characteristics of CSDs and corresponding acquisition systems: a) non-ratiometric setup, b) emission ratiometric setup, and c) excitation ratiometric setup. Ex – excitation spectra, Em – emission spectra, BP – band pass optical filter, Ca bound – \(\text{Ca}^{2+}\)-bound dye molecules, Ca free – \(\text{Ca}^{2+}\)-free dye molecules. Excitation and emission spectra are depicted in black and in grey colors, respectively.

*Ratiometric* measurement of \([\text{Ca}^{2+}]_i\) allows better results as well as ratiometric AP measurement. There are two kinds of dyes: ratiometric in emission (Fig.2b) and in excitation (Fig.2c). The former (e.g. indo1) is
often achieved using one light source (due to overlapped excitation spectra of Ca$^{2+}$-bound and Ca$^{2+}$-free dye molecules) and two photodetectors that allow truly simultaneous recordings. Measuring with the latter (fura 2, fura red) requires the switching of excitation filters (for excitation of Ca$^{2+}$-bound and Ca$^{2+}$-free dye molecules) and the synchronization of emission light detection (due to overlapped emission spectra of Ca$^{2+}$-bound and Ca$^{2+}$-free dye molecules). Another ratiometric method is the use of two non-ratiometric dyes to obtain their ratio (Simpson 1999).

CSD can be calibrated on the basis of values of dissociation constant, background fluorescence, and maximal and minimal fluorescence (or ratio fluorescence for ratiometric method), which are measured in Ca$^{2+}$ saturated and Ca$^{2+}$ free dye (Simpson 1999).

**Simultaneous measurement of AP and [Ca$^{2+}$]$_i$**

The simultaneous measurement of [Ca$^{2+}$]$_i$, using CSDs and AP using patch-clamp or voltage-clamp is often used. In these experiments, a wide spectrum of CSDs can be applied: indo1 (Lee et al. 1988, Dekker et al. 1999, White et al. 1993, Bouchard et al. 1995, Van Borren et al. 2007, Armoundas 2009), rhod2 (Bouchard et al. 1995), fura2 (Linz and Meyer 1998, Goldhaber et al. 2005, Hintz et al. 2002), fluo3 (Ghais et al. 2008, Walden et al. 2009), and fluo4 (Bokenes et al. 2008). This experimental setup is especially appropriate if the cardiac cells or tissues (not the whole organ) are chosen as the model. For the whole heart experiments, one of the signals (either electrical activity or calcium concentration) is recorded by system of electrodes. The plate with electrodes is placed on the heart surface or the heart is placed on an array; however, usually the transparency of the plate is limited and thus the optical recording of the other signal is really difficult. Therefore the simultaneous optical measurement is believed to be more appropriate.

For studying the relationship between AP and extra- or intra-cellular Ca$^{2+}$ fluxes, simultaneous measurement using VSD and CSD can be used. The optical properties of these two (or more) indicators determine the choice of the dyes and optics. Three possible combinations (Canepari et al. 2010) of two indicators with different spectral properties are shown in Fig.3.
Fig.3 Possible combinations of two indicators for simultaneous optical measurement (non-ratiometric measurement) of AP and [Ca\textsuperscript{2+}]. Ex – excitation spectra, Em – emission spectra. Excitation and emission spectra are depicted in black and in grey colors, respectively.

Two signals can be measured either using the same light source and detector (switching the excitation filters and synchronizing emission detection) or using two sources and two detectors (truly simultaneous measurement) if both excitation and emission spectra are well-separated (e.g. di-4-ANEPPS and indo1) (Fig.3a). With overlapping excitation spectra and separated emission spectra (e.g. RH-237 and rhod2) (Fig.3b), the dyes can be excited by one light source at the same wavelength. Emission can then be detected by one (with switching emission filters) or two detectors (truly simultaneous recording). Overlapping emission spectra (e.g. di-8-ANEPPS and fura red) (Fig.3c) do not allow truly simultaneous measurement of two signals despite the fact that high speed switching of the excitation filters is possible (in this case, excitation by one light source is also possible). Note that the spectra of the single-wavelength dyes (or one spectrum from each spectral pair of the two-wavelength dyes) are utilized for illustration in Fig.3. This can be more difficult for ratiometric measurement when dyes (one or both) have two excitation/emission spectra. An example of the combination of fluorescent dyes RH-237 and rhod2 (see below) for simultaneous measurement and properties of appropriate acquisition system are shown in Fig.4. In this case, excitation can be also achieved using a light source with a wider spectrum (approx. 530 ± 25 nm). This recording setup corresponds to the combination in Fig.3b.
**Fig.4** Spectra of RH-237 and rhod2 and recommended spectral properties of recording system. Ex, Em – excitation and emission spectrum, BP – band pass optical filter, HP – high pass optical filter.

*Fast* VSDs, such as dyes from ANEPPS (di-4-ANEPPS and di-8-ANEPPS) and RH (RH-237 and RH-414) groups, are generally chosen for AP measurement and are characterized by broad excitation and emission spectra in visible regions. This fact significantly complicates the choice of the appropriate CSD (Canepari *et al.* 2010). CSDs with narrow spectra in a visible range can be combined with VSDs with a large Stokes shift. VSDs with a small Stokes shift are often used with CSDs excited at frequencies in the UV band (see above). Spectral overlap of VSD and CSD emission spectra can lead to errors in the interpretation of measured signals. When using rhod2 and RH-237 (Fig.4), errors of $[\text{Ca}^{2+}]_i$ and AP values occur about 4% and 4.5%, respectively (Holcomb *et al.* 2009). When di-4-ANEPPS and fluo4 are combined, the error of measured fluo4 intensity reaches up to 10%, but can be reduced by selecting a suitable cutoff wavelength and controlling both dye intensities (Johnson *et al.* 1999).

There are a large number of published reports about simultaneous AP and $[\text{Ca}^{2+}]_i$ measurements. Studies with several combinations of VSDs and CSDs performed by various authors during the last two decades are summarized in Table 1. The table does not include all known (because of the large quantity of available and newly synthesized dyes) but only commonly used dyes combinations and properties of corresponding recording systems.
**Tab. 1** Description of the systems for simultaneous measurements of AP and [Ca\(^{2+}\)]. GP – guinea pig, SD – Sprague-Dawley rat, NZ – New Zealand rabbit, LV, RV – left and right ventricle, AP – action potential, [Ca\(^{2+}\)] – intracellular calcium ion concentration, Ex, Em – excitation and emission spectrum, Ex1, Ex2 and Em1, Em2 – excitation and emission spectra for ratio in excitation and emission, nRM, RM – non-ratiometric and ratiometric method.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Model</th>
<th>Signal</th>
<th>Dye and spectral maxima*</th>
<th>Spectral parameters of optical system</th>
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<td>Species / organ</td>
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<td>Light source / excitation filters</td>
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<tr>
<td>Choi and Salama 2000</td>
<td>GP / isolated heart</td>
<td>AP</td>
<td>RH-237</td>
<td>100W halogen lamp / 520nm ± 20nm</td>
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<td>[Ca(^{2+})]</td>
<td>506nm / 687nm**</td>
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<td>Rhod2</td>
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<td>552nm / 581nm</td>
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<tr>
<td>Laurita and Singal 2001</td>
<td>GP / LV</td>
<td>AP</td>
<td>Di-4-ANEPPS</td>
<td>180W halogen lamp / 515 ± 5nm</td>
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<td></td>
<td></td>
<td>[Ca(^{2+})]</td>
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<td>475nm / 560nm,620nm</td>
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<td>Kirk et al. 2003</td>
<td>SD / isolated atrial myocyte</td>
<td>AP</td>
<td>Di-8-ANEPPS</td>
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<td>450nm,510nm / 605nm</td>
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<td>Fluo 4</td>
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<td>494nm / 506nm</td>
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<td>Kong et al. 2003</td>
<td>NZ / isolated heart</td>
<td>AP</td>
<td>RH-237</td>
<td>Argon laser 488nm (22nm resolution)**</td>
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<td>Oregon Green BAPTA 1</td>
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<td>Omichi et al. 2004</td>
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<td>RH-237</td>
<td>Laser 532nm</td>
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<td>552nm / 581nm</td>
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* The values of maximum of the dye spectra are taken from dye specification (Molecular Probes, USA)
** For RH-237, only maxima of the dye spectra measured in methanol are available from specification
*** Ratiometric method was used to calculate AP and [Ca\(^{2+}\)].

Table 1 shows the studies with typical combinations of fluorescent dyes. Studies by other authors are generally based on the methods described in Table 1. Most authors use the same experimental setup proposed by Choi and Salama in 2000 (Fast 2005, Saba *et al.* 2008) with excitation using a halogen lamp,
and by Omichi and co-authors in 2004 (Kim et al. 2010, Joung et al. 2009, Holcomb et al. 2009, Wu et al. 2005, Chou et al. 2007, Sidorov et al. 2008) with a laser as an excitation light source. The authors of these studies prefer RH-237 and rhod2 for recording AP and \([\text{Ca}^{2+}]_i\), respectively (Fig. 4). The fluorescence of RH-237 achieves its maximum at a longer wavelength than that of di-4-ANEPPS. This allows better separation of VSD and CSD emission spectra measured simultaneously.

It is obvious from Table 1 that simultaneous signals are more often measured using non-ratiometric methods using dual-wavelength dyes (such as di-4-ANEPPS, di-8-ANEPPS, RH-237, and indo-1). It is probably due to complex and rather expensive recording systems and complications in the selection of two suitable dyes with well separated spectra. Three photodetectors or a system for switching emission or excitation filters should be integrated for ratiometric measurement of the first signal and non-ratiometric measurement of the second signal. For ratiometric measurement of both signals, it is necessary to use four detectors or a switching system/systems. The cost of such a system is especially high when CCD cameras are chosen for multimodal imaging of cardiac activity, as has been the trend in the last decade (Holcomb et al. 2009). Switching emission or excitation filters is usually performed mechanically, which is too slow for AP recording. Generally, there is no the best, universal experimental setup. For each individual experiment, factors discussed above must be taken into consideration for choosing the dye combination and recording system.

**Potentials for and alternatives to the method**

As the previous overview shows, the optical recording of various cardiac signals has both advantages and disadvantages in comparison with the conventional approach. The limited scale of the application of the optical method is in general determined by the properties of available fluorescent dyes (namely toxicity, sensitivity, internalization, and photobleaching) and by the limitations of commonly used recording devices. Studies of the effects of di-4-ANEPPS on guinea pig and rabbit isolated hearts show that loading the hearts with this dye leads to heart rate slowing and to partial blocks in the atrio-ventricular node (Novakova 2008). The changes in characteristics of electrograms measured in the rat isolated hearts during staining with this dye were of a minor extent and disappeared during the washout phase of the experiment (Fialová 2010, Fialová 2011). Thus, the influence of the dye on the myocardium itself and on its electrophysiological properties varies with animal species. This must be taken into account when designing an experiment. One
possible approach to limit the effect of the dye is to decrease its concentration or to shorten the excitation phase of the experiment. This can be done using modified dyes with improved characteristics, as mentioned above. Another approach is loading by perfusion with a low-concentrate dye solution instead of injecting the dye into the preparation in a high concentration; this allows a lower concentration of the dye without decreasing the output signal quality (Nováková 2000). Shortening the excitation time generally leads to an increase in light intensity. However, this increases the photobleaching that distorts the signal. It is especially significant in long-term experiments. Thus, the use of fluorescent dyes (VSD and CSD) must be validated under experimental conditions to determine the suitable dye concentration, time of loading and washout, light intensity, and other factors important for correct and effective recording.

The optical mapping of AP and $[\text{Ca}^{2+}]_i$ helps to study the development of various pathological phenomena such as ischemia and arrhythmia. This knowledge plays an important role in developing new methods for the diagnosis, prevention, and treatment of cardiac diseases. For two-dimensional (2D) recording of AP from a broader area of the preparation, a very sensitive ultraspeed camera with satisfactory time and spatial resolution is necessary. PDAs have good properties for 2D recordings of cardiac signals (Fast 2005), but their spatial resolution is usually not enough. A relatively new approach – electron multiplying CCD (EMCCD) technology – can be chosen in this case. An innovative digital scientific detector – EMCCD camera – was introduced to the imaging community in early 2000. EMCCD is a quantitative digital camera technology capable of single photon event detection; its quantum efficiency is as high as 90-95% due to the electron multiplying structure built into the sensor (Toescu and Graham 2010). An EMCCD camera combined with switched LEDs and new multiband filters (excitation and/or emission) can be a very suitable tool, not only for recording AP or $[\text{Ca}^{2+}]_i$ (Svreck et al. 2009), but also for the simultaneous ratiometric recording of both these signals (Lee et al. 2011).

Electrical properties of the heart in physiology and pathology can be also very effectively investigated using three-dimensional (3D), or panoramic, optical mapping. This approach enables capturing the image of a cardiac preparation (in most cases a perfused isolated heart) from three points of view. Recorded data are further used for reconstruction of heart surface features. A PDA, CCD, or EMCCD camera combined with LED illumination and surface reconstruction techniques can be very suitable for high-speed high-resolution 3D recordings of signals on a preparation loaded with fluorescent dyes (Kay et al. 2004, Evertson et al. 2008, Qu et al. 2007). In this method, some excitation-contraction coupler must be added to the perfusate
solution to eliminate motion artifacts. Panoramic optical recording has a very high potential especially due to new available imaging systems with improved characteristics in terms of speed and temporal and spatial resolution.

For 2D and especially 3D imaging of cardiac activity, *penetration* is one of the crucial factors affecting the quality of recorded data. Therefore, *two (or more) photon excitation* techniques, widely used in neurology (Toescu and Graham 2010), should be very useful in such studies. In this case, a preparation pre-loaded with fluorescent dye (VSD or CSD) is excited with light (often high-intensity laser) at a wavelength approximately double what is generally required to fluorophore excitation. In addition to a greater depth of penetration, this approach brings improvements such as better axial resolution, less photodamage, and less toxicity (Toescu and Graham 2010). Two-photon excitation microscopy allows imaging of living tissue up to a depth of about 1 mm (Denk et al. 1990). The method, however, has one disadvantage: it requires a complete and expensive acquisition system. When applying this method to measurements on the intact heart, complete modification of the optical system is necessary. The second approach enabling higher penetration is the use of *long wavelength* (relative to excitation and emission spectra) *VSDs*. Greater depth penetration in the tissue, reduced light scattering, enhanced sensitivity to the voltage, and larger Stokes shift of the dyes make it possible to effectively record AP from deeper layers in the preparation (Salama et al. 2005). Shifting of excitation and emission spectra of long wavelength dyes towards longer wavelengths than routinely used VSDs avoids problems with overlap between the spectra of two dyes by simultaneously recording AP and [Ca$^{2+}$].

Effectiveness of optical mapping also depends on the *sensitivity* of the fluorescent dye, i.e. on the ability of the dye to respond to the changes in membrane voltage or [Ca$^{2+}$]. The higher the sensitivity of the dye, the more accurate the recording of different ranges of these two values. The response of conventional fluorescent dyes (e.g. VSD di-4-ANEPPS) is small and usually does not exceed 15% (Salama 2001). In addition to modified dyes with enhanced sensitivity (see above), the *fluorescence (or Förster) resonance energy transfer* (FRET) technique significantly improves imaging quality. This technique is based on the interaction between the electronically excited states of two dye molecules (the “donor” and “acceptor” molecule). In a ratiometric FRET system, donor molecules bound to a plasma membrane transfer the excitation to the acceptor molecules, which are redistributed between extracellular and intracellular membrane sites in response to transmembrane voltage changes. The emission of donor and acceptor are inversely related and depend on the
distance between them. At resting membrane potential, the acceptor is predominantly at the extracellular surface of the membrane, leading to significant FRET and enhanced acceptor’s emission; after depolarization, the movement of acceptor molecules to the intracellular membrane surface leads to a FRET decrease and, thus, an increase of the donor's emission (Gonzáles and Tsien 1997). Fluorescence ratio changes induced by depolarization in such a FRET system reach 5-30% per 100 mV in neonatal cardiomyocytes (Gonzáles and Tsien 1997). Various studies using this approach in neurology for recording of AP and [Ca^{2+}], have been published (Bradley et al. 2009, Nadeau et al. 2006, Toescu and Graham 2010, Truong et al. 2001). In experimental cardiology, FRET has been used to study the Ca^{2+}-dependent regulation of Na^+-Ca^{2+} exchanger in transgenic rat neonatal cardiac myocytes (Ottolia et al. 2004) and in transgenic zebrafish heart (Xie et al. 2008), both in vivo (using intact heart) and in vitro (using isolated, spontaneously beating heart). In these studies, animals with linked fluorophores YFP and CFP have been used for expression of Ca^{2+} binding domain of Na^+-Ca^{2+} exchanger. To our knowledge, there is no detailed description of FRET application to visualize cardiac AP in current literature. This is undoubtedly a field worthy of further research.

Cardiac signals recorded optically often contain motion artifacts (see above) that complicate their interpretation. The simultaneous recording of signals by electrical (ECG) and optical (AP and [Ca^{2+}]) methods enables compensation for the loss of information about some parts of the signals distorted by artifacts. Use of pharmacological excitation-contraction uncouplers, which do not provoke changes in the morphology of AP and intracellular calcium transients (such as blebbistatin; Fedorov 2007) might help to suppress contractility in experiments with optical recordings.

New technical approaches and processing methods are thought to be most correct and effective for preventing or removing motion artifacts without mechanical or chemical interventions. The ratiometric technique is widely used for recording AP or [Ca^{2+}]. New studies show the possibilities for applying the excitation ratiometric optical mapping of AP using excitation of di-4-ANNEPS with two LEDs (blue 450 ± 10 nm and cyan 505 ± 15 nm) in alternating phases (Bachtel 2011). This method requires only one photodetector; it is, therefore, simpler and less expensive than emission ratiometric recording of AP. However, the ratiometric approach is difficult to achieve with simultaneous recordings of both signals because of the overlap of available dye spectra and the complexity of the required acquisition system (see above). Moreover, ratiometric recordings do not solve every problem. In the absence of contractility
inhibition, the ratiometric optical signals still contain a mixture of APs corresponding to all cells along the imaging trajectory (Bachtel 2011). New studies of post-processing optical 2D electrical activity recordings show that image registration techniques can be a powerful tool for correcting linear and non-linear motions of the heart (Rohde et al. 2005, Westergaard et al. 2008, Janich et al. 2009). However, this approach has some limitations (motions out of plane and pixels that move out of the imaging field cause registration errors), which can be probably solved by using more intelligent recording systems with multiple cameras (Rohde et al. 2005, Westergaard et al. 2008). Techniques combining different approaches (in terms of experimental setups and off-line processing) for correction of the motion in optical signals perhaps represent greater potential in the future.

There are various alternatives and prospects for using optical recording in cardiac experiments. Close cooperation between experts in cardiac electrophysiology, chemistry, physics, optics, and biomedical engineering must form the basis for designing new approaches and improving current ones.

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