Images of Action Potential Propagation in Heart

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Activation and repolarization across mammalian hearts follow complex three-dimensional pathways that are governed by fiber structure, intercellular coupling, and action potentials (APs) with spatially heterogeneous properties. Voltage-sensitive dyes and imaging techniques offer new insights on how spatiotemporal heterogeneities of APs govern propagation, repolarization, and AV node conduction and help us visualize arrhythmias with previously unattainable details.

The electrical potential across the cell membrane plays a pivotal role in cellular function. In excitable cells, rapid membrane potential changes are the signaling mechanism for cell-to-cell communication and the trigger for important cellular functions, as in excitation-contraction or excitation-secretion coupling. Our knowledge of membrane potentials is based primarily on impalements of cells with glass pipette microelectrodes. In many experimental conditions, the difficulties in maintaining the microelectrode tip in the intracellular space and damage to the cells limit the use of the method. Microelectrode techniques are not applicable to small cells (<3 μm in diameter) nor are they practical in situations requiring the simultaneous monitoring of membrane potential from multiple neighboring cells. Hence, attempts were made in the late 1960s to develop techniques that transduce membrane potential to an optical signal.

Voltage-sensitive dyes

Initial attempts to measure voltage-dependent optical signals in biological systems were based on intrinsic signals (light scattering, birefringence) from isolated nerve cells and skeletal muscle fibers. Intrinsic signals appeared to mimic the time course of the action potential (AP), but their signal-to-noise ratio (S/N) was very poor. The next step was to stain excitable cells with a dye and show that extrinsic optical signals (changes in dye absorption and/or fluorescence) followed changes in membrane potential. Extrinsic signals provided a major improvement in S/N and bolstered the search for voltage-sensitive dyes (10).

The first measurements of optical APs from heart muscle were obtained with merocyanine 540 (M-540) (13). Dyes with larger voltage-dependent responses and less phototoxic damage have now replaced M-540. Figure 1A illustrates an instrument to measure light from a 1-mm light spot focused on a sheet of ventricular muscle. Electrical stimulation of the unstained preparation produces a change in light scattering (ΔI/I) due to the muscle contraction (Fig. 1B, top). After staining the muscle with M-540, the stimulus produced a rapid increase in fluorescence followed by a slower response caused by the movement artifact (Fig. 1B, middle). After bathing the muscle in a Ca2+-free solution to suppress contractions, the fluorescence signal (ΔF/F) had the time course of the cardiac AP (Fig. 1B, bottom). The fluorescence intensity depends on the amount of bound dye (after washout of excess dye), the optical apparatus (i.e., intensity of excitation, filters, and so forth), and the loss of dye over time. In general, “fast” dyes bind tightly to the myocyte membrane and do not seem to be internalized in the cytosol, making it possible to record optical APs for 2–4 h. Their spectral properties depend on the local environment, and only dye molecules bound to excitable membranes can transduce potential changes to an optical response. The larger the fractional absorption and/or fluorescence (ΔF/F) change per AP the greater the voltage “sensitivity” of the dye. These dyes
possess a number of properties that are critical to the interpretation of the signals. The optical response provides a high-fidelity measurement of the shape and kinetics of the cardiac AP but not an actual value of the transmembrane potential. As shown in Fig. 1C (top), fluorescence APs (from a 1-mm diameter spot of ventricle) accurately track the APs recorded with an intracellular microelectrode impaling a cell at the center of the light spot. ΔF/F recorded during a cardiac AP can be calibrated with a microelectrode, but, in most studies, the amplitude of the optical AP is assumed to represent a depolarization of ~100 mV for a “normal-looking” AP. ΔF/F varies with time due to photobleaching and washout of the dye yet is remarkably reproducible from heart to heart and from species to species as long as the heart is “healthy” and the staining procedure is not altered (10). When examined at fast sweep speeds, the upstroke (and downstroke) of optical APs are slower than for microelectrode APs (Fig. 1C, bottom) because optical APs represent the sum of APs from thousands of cells in the patch of tissue viewed by the detector. As a result, optical APs integrate the single-cell APs over the time it takes for the activation wave to propagate over the tissue. However, “fast” dyes are appropriately named because, under voltage-clamp conditions, their
response is as fast as that of a microelectrode (within a microsecond) and, when the excitation beam is ≤ 90 µm, the optical AP upstroke is faster than that of the microelectrode (10). For merocyanine dyes, fluorescence changes vary linearly with potential changes for hyperpolarizing and depolarizing voltage-clamp steps (8, 12). Another key feature is that optical signals follow direct current changes in membrane potential and show no signs of alternating current coupling during long (200–300 ms) voltage-clamp steps or the plateau phase of long AP durations (APDs = 3–5 s). This makes the fast dyes particularly useful when the precise time course of the AP plateau is required to analyze heterogeneities of APDs and refractoriness. For example, a frog ventricular AP can be markedly prolonged by perfusing the heart with a Ringer solution containing 1 mM Ni2+. The dye detects the long APD and the early afterdepolarization (Fig. 1D, top left). Another critical issue is whether pharmacological agents alter the fidelity of dye responses to voltage. Thus far, optical APs recorded under a variety of ionic and pharmacological interventions monitored the same changes in APs as those detected with microelectrodes (Fig. 1D, right). The dyes also resolve the characteristic shape and time course of APs recorded from different regions of the heart, such as the atrium (Fig. 1D, right), ventricle (Fig. 1, B–D, top left) and the sinoatrial (bottom left) node (10, 13). Thus fast dyes track the time course of voltage changes with high accuracy and sufficient S/N to resolve changes of ±1 mV or better, but absolute potential values must be deduced indirectly.

Mechanisms of potentiometric response

The mechanisms responsible for the voltage-dependent optical response of fast dyes remain unknown, yet from a practical point of view they are important for the development of new dyes with higher sensitivity and lower phototoxicity. Several mechanisms have been suggested; one theory is that the dyes are “electrochroic,” that is, they exhibit wavelength shifts in peak fluorescence and/or absorption due to a change in membrane potential. Merocyanine dyes were initially tested because they exhibit large spectral changes as a function of the solvent’s polarity or local environment. This property seemed desirable because potential changes also alter the local environment and might modify the spectral characteristics of the dye. Voltage-dependent spectral changes were measured at high speeds (20 ms/spectrum) and showed that a membrane depolarization changed the amplitude of fluorescence and absorption spectra, with negligible wavelength shifts (±1 nm) (10). The lack of electrochromic shifts suggested that a membrane potential change might 1) displace dye molecules partially “in” and “out” of the membrane, 2) alter the dielectric properties of the membrane around the dye, or 3) promote dimer-to-monomer transitions. In the absence of definitive proof regarding the mechanism, the design of new probes remains largely empirical. At this time, the dye with the most desirable features for cardiac electrophysiology is 1-(3-sulfonatopropyl)-4-[β-[2-(di-n-butylamino)-6-naphthylvinyl]-pyridinium betaine (di-4-ANEPPS) because it exhibits large fractional fluorescence changes during an AP (8–15%), low toxicity, and stable signals (2–4 h) (8).

Optical mapping of activation and repolarization in the heart

The spread of activation and repolarization are important for our understanding of the mechanisms responsible for the normal cardiac rhythm and the factors underlying the initiation and perpetuation of arrhythmias. Much has been learned regarding the ionic basis of the cardiac AP using intracellular microelectrodes on isolated myocytes and papillary muscles. However, intracellular microelectrodes cannot be used to simultaneously record AP from hundreds of cells in intact hearts. Activation and repolarization patterns on the heart’s surface have been extensively studied using surface electrodes. Although much has been learned from surface electrode mapping techniques, many questions were not resolved due to technical limitations. For instance, activation sequences were difficult to interpret during rapid synchronous depolarization, as in sinus rhythm, or during slowly changing, low-level depolarization, as in fibrillation. Repolarization measured with an electrogram often does not coincide with the actual repolarization at the recording site. Surface electrodes also lack the sensitivity to detect impulse propagation through the complex three-dimensional structure of the atrioventricular node and cannot detect potential changes during defibrillation shocks or immediately postshock. The ability to simultaneously record optical APs at high spatial and temporal resolution allows us to visualize details that were previously unattainable.

Figure 2A describes our present mapping apparatus, which is based on a 16 × 16 element photodiode array, an electronic interface consisting of 256 independent amplifiers, analogue-to-digital converters, and a computer for data acquisition. Data is acquired at 12-bit accuracy at high sampling rates (up to 8,000 frames/s) and is analyzed with software developed in-house. Light from tungsten halogen lamps is passed through 520 ± 30-nm interference filters and focused on the surface of a heart muscle in a chamber. The fluorescence from the heart is collected with a camera lens, passed through a 630-nm cutoff filter, and focused on the photodiode array by a second lens. An image of a chosen region of the heart is magnified and focused on the surface of the array. The magnification can be varied from 6× to −4×. Each diode detects light from a volume of tissue, and the AP recorded by a diode is the sum of APs from cells within a volume equal to the area of tissue focused on the diode times twice the depth-of-field of the camera lens. For a lens with a high numerical aperture, the depth-of-field is narrow, such that optical APs are recorded from cells near the surface of the heart (6).

Map of APs and of activation

Figure 2B illustrates a map of APs recorded from the anterior surface of a Langendorf-perfused guinea pig heart. A symbolic map of the array is shown, with the AP recorded by each photodiode displayed in its respective location. Techniques have been developed to analyze optical APs and determine APDs while addressing concerns about movement artifacts. Activation and repolarization time points are taken at the maximum first derivative \(|(df/dt)_{max}|\) of the AP upstroke and the maximum second derivative \(|(d^2F/dt^2)_{max}|\) of the AP downstroke, respectively.
(Fig. 2C). These time points represent the times when most of the cells viewed by a diode activate and repolarize, respectively, and the APD is the difference between \( (dF/dt)_\text{max} \) and \( (dF/dt)^2 \). Theoretical and experimental analysis showed that the repolarization time point identified through \( (dF/dt)^2 \) is equivalent to 97% recovery of the AP back to baseline and is coincident with the refractory period of the AP (5). The use \( (dF/dt)^2 \) to detect repolarization offers several advantages; the detection of \( (dF/dt^2)_{\text{max}} \) can be computer automated, it detects repolarization time points reliably, even in the presence of movement artifact, and it is possible to simultaneously map repolarization and refractoriness (5). From the activation and repolarization time points recorded in one heartbeat, a computer algorithm generates isochronal maps to visualize activation and repolarization waves (3, 5, 11).

**FIGURE 2.** Maps of APs, activation, and repolarization. A: schematics of a mapping apparatus. A Langendorf-perfused guinea pig heart was stained with 1-(3-sulphonatopropyl)-4-(2-di-n-butylamino)-6-naphthylvinylpyridinium betaine (di-4-ANEPPS), placed in a fluid-filled chamber, and illuminated with 520 ± 30-nm excitation from a halogen lamp. Image of heart was focused on array with a camera lens, and photocurrent from each diode was amplified, digitized, and stored in computer memory. B: symbolic map of APs. APs were recorded from different sites and shown in their respective locations. Anterior surface of heart was imaged on array, and this orientation applies to subsequent figures. C: analysis of optical APs. Each AP recorded by a diode was analyzed by a computer algorithm to determine first \( (dF/dt) \) and second \( (dF/dt^2) \) time derivatives, which were used to detect activation and repolarization time points, respectively.
Optical mapping revealed for the first time that the repolarization sequence is a propagating wave and, like activation, is guided by the ventricular fiber structure. The ventricular wall is composed of multiple layers of elongated cells, with the longitudinal axes of the cells parallel to each other within each layer. The longitudinal axes of the layers rotate going from the endocardium to the epicardium, resulting in a three-dimensional anisotropy of the ventricular wall. Thus activation patterns recorded from the surface are a manifestation of AP propagation and the anisotropy of the surface and deeper layers of cells. The effects of fiber structure in deeper layers on activation patterns at the surface were determined by recording activation on the epicardium while pacing the heart from different depths and correlating these patterns with histological analysis of the underlying fiber structure (6). As shown in Fig. 3A, stimulation on the anterior surface of the heart initiates an activation pattern that is determined by the fiber structure on the surface of the heart. Local velocity vectors (calculated from the activation time delay between a diode and its nearest neighbors) depict the orientation of the local wave front (Fig. 3B) and align with the longitudinal axis of the surface fibers (6). Pacing the center of the left ventricle initiates an elliptical activation pattern, with a major and a minor axis oriented parallel to the longitudinal and transverse axes of the fibers. The tissue anisotropy produces fast ($\Theta_l = 0.85 \pm 0.05$ m/s) and slow ($\Theta_t = 0.44 \pm 0.04$ m/s) conduction velocities aligned with the longitudinal and transverse axes of epicardial fibers, respectively (6). Conduction velocities $\Theta_l$ and $\Theta_t$ apply to all layers, and the transmural velocity (i.e., between layers) was shown to be equal to $\Theta_l$, the transverse conduction velocity (6). Conduction velocities depend on intercellular coupling through gap junctions and vary along different directions because the wave front travels along a more tortuous path in the transverse compared with the longitudinal direction. In contrast to anisotropic activation initiated by a local bipolar depolarization, a rapid synchronous activation is produced in sinus rhythm driven by pacemaker cells. The AP from the sinoatrial (SA) node spreads across the atrial chamber, is delayed at the atrioventricular node (AVN), spreads to the conductile system (His bundle and Purkinje fibers), the septum, and transmurally across the ventricular walls. Activation on the epicardium appears as a large region of synchronous depolarization that breaks through from endocardium to epicardium and is completed in 3–5 ms (Fig. 3C) (6, 11). The same rapid activation patterns were measured in sinus rhythm and when pacing the heart on the SA node or on Purkinje fibers lining the septum or the endocardium. Hence, Purkinje fibers depolarize the ventricles in a manner akin to shocks applied by large-plate electrodes, resulting in synchronous activation of the heart with apparent longitudinal and transverse conduction velocities of $2.66 \pm 0.11$ m/s and $1.65 \pm 0.09$ m/s, respectively (6, 11). Ventricular tachycardia (VT) induced by burst stimulation (25–50 Hz for 3 s) produces complex activation patterns with different morphologies from beat to beat. A hallmark of activation patterns during VT is that the conduction velocities are slow, as if initiated by a local depolarization rather than Purkinje-driven depolarization of the ventricular myocardium. Optical mapping showed that subthreshold stimulation of Purkinje fibers interrupted VT by restoring electrical coupling between Purkinje cells and the myocardium (11). Thus arrhythmias are characterized by slow propagation (at high frequencies), which requires that the Purkinje fibers be damaged and/or uncoupled from the myocardium.

Maps of repolarization

Until recently, it was difficult to obtain a detailed picture of repolarization and refactoriness using conventional surface electrograms and intracellular electrode techniques. The technical difficulties of mapping repolarization prompted speculations on whether repolarization is a real propagating wave (i.e., repolarization of a cell drives the repolarization of an adjacent cell) or a “phase” wave resulting from the intrinsic repolarization of individual cells. Experimental studies showed that repolarization can be modified by the direction of propagation, suggesting that the manner in which cells are depolarized can influence repolarization (14). Another study reported a dependence of APD on fiber orientation, but there were no measurements of spatial distribution of APDs (9). Simulations of the myocardium in one and two dimensions demonstrated that electrotonic coupling between cells must mask at least partially the intrinsic heterogeneities of APDs between cells (7).

Optical maps of APs in guinea pig hearts showed that, in the left and right ventricular walls, repolarization patterns were closely associated with fiber orientation and were largely independent of the activation pathway (6, 12). APDs were shorter on the endocardium compared with epicardium at physiological cycle lengths (200–400 ms) (6). Figure 3D illustrates that repolarization does not begin at the first site to depolarize but begins near the apex, then spreads anisotropically towards the base in 18–24 ms. Irrespective of the method used to activate the heart (e.g., sinus node, pacing at various sites on the endocardium or epicardium), APDs were consistently shorter at the apex and became progressively longer towards the base (6, 12). Hence, repolarization is not a phase wave but is spread anisotropically, with repolarization velocities of $0.53 \pm 0.11$ m/s parallel to the longitudinal fiber axis and $0.31 \pm 0.10$ m/s transverse to fiber axis (6). Once repolarization begins at cells with the shortest APDs, it spreads like activation but at slower velocities. The findings indicate that gradients of APDs are caused by intrinsic differences in APDs at the cellular level, which are due to spatial heterogeneities in the expression of ionic channels involved in the repolarization of the AP.

Gradients of APDs in ventricular muscle are species dependent, since the spatial distributions of K+ ionic channels involved in the repolarization of the AP are species dependent, resulting in different repolarization sequences. For instance, dog ventricular muscle exhibits “spike and dome” APs on the epicardium but not on the endocardium because of an additional transient outward current, $I_{to}$ (not found in the guinea pig). The heterogeneous distribution of $I_{to}$ results in longer APDs on the epicardium.
compared with the endocardium, and the heterogeneity of APDs is rate dependent because \( \delta \) is inactivated at short cycle lengths (1). In rat hearts, there are spatial heterogeneities in the levels of mRNA expressing K+ channel Kv4.2, resulting in spatial variations of APDs (4). Thus intrinsic APD gradients are species dependent and are important for understanding normal cardiac repolarization and function. Knowledge of the normal spatial and temporal heterogeneities of repolarization and refractoriness is
essential for our understanding of the initiation and perpetuation of arrhythmias. For example, a single premature impulse applied on the left ventricle of the guinea pig heart propagates faster near the apex than near the base because of the spatial heterogeneity of refractory periods intrinsic to epicardium (Fig. 3). The slowing of conduction and functional lines of conduction block occur even in a “healthy” ventricle due to heterogeneities of APDs. Although molecular biology and biophysics have identified the ionic channels and currents that are heterogeneously distributed in the heart, optical probes offer new insights on how spatiotemporal heterogeneities of APs govern propagation and repolarization and help us visualize arrhythmias with previously unattainable details.

Optical mapping of AP propagation across the AVN

Another contribution of optical techniques has been the mapping of impulse propagation across the AVN and the elucidation of the mechanisms responsible for AVN delay. The AVN is the only electrical connection between the atria and the ventricles and is located between the interatrial septum (IAS) and the interventricular septum (IVS) in a region called the triangle of Koch. The node is composed of a spindle-shaped compact network of small cells whose main function is to delay activation between the atria and ventricles and coordinate their sequential contractions. The AVN region consists of five morphologically distinct cells: 1) transitional cells that comingle with 2) atrial cells, 3) tightly packed midnodal (N) cells which form the compact node, 4) elongated lower nodal (NH) cells, and 5) cells of the penetrating AV bundle embedded in the central fibrous body. AVN cells are divided into three zones based on their electrophysiology: 1) atrionodal (AN) cells form a transitional zone between the fast conducting atrial tissue and the N zone; 2) The N zone is an area of slow conduction and AP upstrokes; and 3) the NH zone is a transitional zone between the N zone and His fibers of the conductile system.

Several hypotheses have been proposed to explain propagation delays at the AVN. The intracellular resistance of N cells is markedly higher than that in atrial and ventricular tissues, which could account for the delay across the AVN if conduction is decremental such that the overall delay is distributed across the AVN cell network (15). Alternatively, the conduction delay following a premature impulse might not be decremental in space but more localized in N cells, in which conduction stagnates (2). Such studies have led to the realization that slow conduction in the AVN cannot be solely explained by active properties of N cells such as the maximum rate of AP depolarization \( (dV/dt)_{max} \) and that both passive and active...
properties are responsible for the inhomogeneous conduction velocity in the AVN (2). An early hypothesis of AV delay by Scherf (1929) was based on the existence of a “step-delay” across an inexcitable gap as a major contributor to the total AV delay (15). The mechanisms responsible for the AVN delay remained unclear, in part due to the inability to map impulse-propagation pathways using conventional electrode techniques. We mapped impulse propagation across the AVN and used optical sectioning to resolve electrical activity in three dimensions to elucidate the mechanisms responsible for the AVN delay. Several features of the AVN pose difficulties in the application of optical techniques. The AVN is a three-dimensional structure sandwiched between atrial and transitional cells above and ventricular cells below, such that APs from these three cell types are superimposed in the optical signals recorded from the AVN. The small dimension of AVN cells limits the amount of dye bound to the cells, resulting in low-amplitude optical APs. The staining, exposure of the node for optical access, and the immobilization of the preparation to control movement artifacts required the design of a new chamber. Optical signals from the AVN region are shown in Fig. 4A. Signals recorded from a narrow zone near the central fibrous body exhibited a set of three sequential upstrokes per cardiac beat. The first upstroke coincided with atrial depolarization and the firing of AN cells. The third upstroke was coincident with the depolarization of ventricular cells lying below the AVN in an out-of-focus plane. The second upstroke fired at intermediate time points and was only observed in a narrow (1 × 2 mm) region of the preparation. To elucidate the origins of the second upstroke, microelectrode and optical APs were simultaneously recorded from the AVN. The firing of the second upstroke was coincident with microelectrode recordings characteristic of N and NH cell APs (3). The spatiotemporal characteristics of the second upstroke confirmed that it originated from N or NH cells. Activation spread across the atrial septum in 15 ms (Fig. 4B) and, after a delay of 43 ms, the first site on the AVN fired an AP (Fig. 4C). AVN APs then spread across the node in 8 ms. A second delay occurred between the last AVN AP and the first ventricular AP. In the latter delay, \( \tau_2 \), accounted for the time to propagate from the His bundle to the apex of the ventricles and back along the IVS to the base of the ventricle. The ventricular septum fired APs at 93 ms and spread in the field of view in 4 ms. Maps of impulse propagation across the AVN were highly reproducible both in their pattern and temporal relationship. In the step-delay, \( \tau_1 \), between AN and N cells was 49.5 ± 6.59 ms (at 300-ms cycle length) and the activation time across the node was 10.33 ± 3.21 ms, indicating an apparent conduction velocity of 0.162 ± 0.02 m/s (\( n = 9 \) hearts) (3). The conduction velocity across the AVN is faster when measured with optical techniques than that inferred from microelectrode recordings because the latter were not based on maps of APs and included the step-delay in the calculation of velocity. The step-delay accounts for a major component of total AVN delay, its rate dependence, and modulation by neurotransmitters (e.g., acetylcholine). Hence, the theory of decremental propagation proposed that the AVN delay resided in the time needed for AP propagation across the node (Fig. 4B, trace 1), whereas optical maps demonstrate the existence and location of a step-delay preceding AVN conduction (trace 2).

**Future directions**

Optical mapping is a relatively new technique, but it is coming into its own as a powerful tool to address fundamental questions in cardiac electrophysiology. The method offers important advantages for the investigation of questions that can not be satisfactorily addressed by conventional methods. For instance, the dye response is impervious to shock artifacts and can be used to examine in detail the depolarization of the myocardium close and far from the shock electrodes. The high spatial and temporal resolution allows us for the first time to visualize AP wavefronts in patterned cultured cells and in hearts under sinus rhythm or fibrillation. The method is being used to map the hearts of genetically engineered mice with specific ion channel modifications to determine the mechanisms underlying arrhythmias (e.g., long QT syndrome). The development of depth-resolved images for three-dimensional mapping should extend the power of this approach.

References

8. Loew, L. L., B. Cohen, J. Dix, E. N. Flühler, V. Montana, G. Salama, and W. Jian-Young. A naphthyl analog of the aminostyryl pyridinium class of...


