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Review

Studying dynamic events in the developing myocardium

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ABSTRACT

Differentiation and conduction properties of the cardiomyocytes are critically dependent on physical conditioning both *in vitro* and *in vivo*. Historically, various techniques were introduced to study dynamic events such as electrical currents and changes in ionic concentrations in live cells, multicellular preparations, or entire hearts. Here we review this technological progress demonstrating how each improvement in spatial or temporal resolution provided answers to old and provoked new questions. We further demonstrate how high-speed optical mapping of voltage and calcium can uncover pacemaking potential within the outflow tract myocardium, providing a developmental explanation of ectopic beats originating from this region in the clinical settings.

Contents

1. Biomechanics at cell and organ level

1.1. From whole hearts down to cellular level

To study dynamic electrical and mechanical events occurring in the heart, simplification of the complex, three-dimensional *in vivo* system is often advantageous. This can go down to the single cell level, since isolated cardiomyocytes can spontaneously beat *in vitro* and cell culture setup is useful for studying subcellular and

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molecular events or drug screening purposes. If myocytes are grown *in vitro* for a prolonged period of time, they form a sheet – a functional syncytium connected by gap junctions, simplifying thus the three-dimensional geometry of myocardium into a single plane.

Historically, cells were cultivated just on plain plastic, or surfaces coated with various extracellular matrix molecules (collagen – isotropic or aligned, fibronectin) to promote attachment and influence cellular properties (Atance et al., 2004). A strong stimulus for muscle growth is work load, or stretch; so subjecting cell cultures grown on elastic membrane to mechanical loading using a pump-powered cell stretcher (Kofidis et al., 2004; Miller et al., 2000) is a way to model differentiation normally occurring during development. The next step in bringing the cell culture more closely to *in vivo* situation is growing the cells on more sophisticated 3D scaffolding (Atance et al., 2004; Evans et al., 2003) to form tissue constructs of various complexity (Tobita et al., 2006). Each of these new technological advancements, enabled by parallel development of chemistry (new polymers), mechanical engineering (stretching apparatuses) and biology (cell isolation protocols, differentiation of cardiomyocytes from stem cells) allowed answering new sets of questions. Similar to the situation *in vivo*, cell culture conditions markedly influence the functional parameters of cells; here we will focus on their conduction properties, and extend it back to the whole organ level. This area has so far received comparatively little interest in tissue engineering aimed at construction of implantable artificial myocardium (Yildirim et al., 2007), but in addition to perfusion and mechanical properties of tissue-engineered constructs, it is of vital importance for integration with the host myocardium and electrical stability.

1.2. Tissue geometry and conduction properties

Mechanical loading has profound effects on growth, behavior, differentiation and conduction properties in isolated myocytes (cell cultures). Similarly, for *in vivo* studies, people tend to view genes at the root of everything, and sometimes forget that muscles in particular are critically dependent on mechanical stimuli, and organisms in general on epigenetic influences (Pesevski and Sedmera, 2013). What is less clear is the importance of tissue geometry, also referred to as myocardial architecture, which can be elegantly simplified *in vitro*. Patterned cardiomyocyte cultures, enabled by combination of printed circuit technology and cell culture by the Rohr lab in Bern, Switzerland (Kucera et al., 1998; Rohr et al., 1999) are just an example how availability of new technology helped to document the importance of tissue geometry for cardiac electrical conduction. Strands of myocytes, mimicking bundles of conduction fibers, carry electrical impulses at much higher speed than a large expansion of planar myocardial sheet, which acts as a sink. In such system, effects of various pharmacological agents or gap junctional uncoupling can be studied with ease. It was long believed that the role of cardiac fibroblasts is mostly structural support of the heart, and electrically they function as a mere isolator. Importance of fibroblast–myocyte interactions was revealed in well-defined co-culture experiments (Rohr, 2012) and showed that gap junctional, and possibly also electrical, communication exists between these two cell types.

1.3. Importance of physical conditioning for myocardial growth and differentiation

Effect of altered mechanical loading – an epigenetic stimulus – on developing heart and its conduction system could be studied also at the organ level, *in vivo* as well as *in vitro*. Elegant studies by Thompson and associates (Thompson et al., 2003) showed that at the early developmental stages, the fate of cardiomyocytes in the tubular heart (proliferation vs. differentiation) is plastic, and could be reversed by simply inverting the slice of the cardiac tube inside out. This gradient persists also in the trabeculated heart, and could be modeled mathematically (Damon et al., 2009). Cardiovascular development from biomechanical perspective was reviewed by Larry Taber (Taber, 2001). Growth and remodeling are two important processes occurring during both development and adaptations of the cardiovascular system to changing functional requirements. Most cardiac growth during prenatal development is based on hyperplasia (Clark et al., 1989; Sedmera and Thompson, 2011). At the tissue level, an important biomechanical parameter is the residual strain, changes in which are a sensitive indicator of active remodeling (Taber and Chabert, 2002). This regional growth can be easily measured as an opening angle obtained by cutting open a circular section of the vessel or heart. A decrease in opening angle following creation of pressure overload by conotruncal banding correlated with induced growth 12 h after the procedure. These events can be also modeled mathematically. Schroder and colleagues (Schroder et al., 2002) found that the material properties of the developing heart are regulated by mechanical loading and that microtubules play an important role in this adaptive response during cardiac morphogenesis. Specifically, there was an increased amount of both total and polymerized beta tubulin the hypoplastic left ventricle. This smaller ventricle was also stiffer (analyzed by increased hysteresis loop); both parameters were normalized by the treatment with colchicine, which induced microtubule depolymerization.

During the transition from the trabeculated to compact myocardium, spiraling of myofibers within the left ventricular compact layer is the major factor of fetal myocardial differentiation (Jouk et al., 1995; Sedmera et al., 2000). Tobita and associates analyzed the angle of myocyte inclination during normal and abnormal hemodynamic loading (Tobita et al., 2005); they found that increased pressure loading accelerated this normal morphogenetic process, while there was a delay in the settings of hemodynamically-induced left ventricular hypoplasia. Therefore, hemodynamically induced changes in myocardial architecture in these models (Sedmera et al., 1999) that are based on changes in cell proliferation (deAlmeida et al., 2007; Sedmera et al., 2002a) could be the morphological substrate of altered electrical pathways. These were investigated as well using optical mapping on isolated hearts (Hall et al., 2004; Reckova et al., 2003). We found that increased pressure loading accelerated maturation of ventricular conduction system, while there was a dysfunction of the (morphologically normal) left bundle branch in left ventricular hypoplasia. At the molecular level, these changes were paralleled by up/down regulation of conduction system differentiation marker connexin40.

The hemodynamic unloading of the developing heart could be easily taken to extreme by culturing the spontaneously beating, but not pumping heart *in vitro*. In such settings, we noticed not only an arrest of normal differentiation of the ventricular conduction system, but actually a regression towards even more immature conduction patterns (Sankova et al., 2010). To test whether these profound changes were not simply an artefact of organ culture, we performed re-loading of the ventricle by a droplet of viscous silicone oil, which stretches the ventricle and was shown previously to considerably increase myocardial oxygen and glucose consumption (Romano et al., 2001). Remarkably, this led to a complete rescue of conduction phenotype to *in vivo* values, showing that simple myocyte stretch, rather than hemodynamic shear stress transmitted through the endothelium, is the governing factor in early conduction system differentiation. It agreed well with our older data testing the importance of hemodynamically induced signaling via endothelin receptors, which was found to be important during

the later (bundle branches differentiation), but not the early stages of conduction system formation in the chick embryonic heart *in vivo* (Sedmera et al., 2008).

2. Functional imaging of the developing heart

2.1. History of electrophysiological recordings in impulse propagation studies

These functional studies, including those performed on cell cultures, would not be possible without adequate technology for recording of impulse propagation in the heart. The golden standard for action potential recordings are microelectrodes, including those arranged in arrays (sock, brush, or balloon electrodes). They work very well on large adult hearts (sheep, pig, human), but are of limited use in embryos (spatial issues, fragility). Nevertheless, a few carefully positioned electrodes poked into the isolated chick embryonic heart allowed determination of general direction of impulse propagation and enabled postulation of ventricular trabeculae as nascent network of the ventricular conduction system (Arguello et al., 1986; de Jong et al., 1992). Using just two electrodes, Chuck and colleagues (Chuck et al., 1997) discovered the transition in ventricular activation of the chick embryonic heart from primitive base-to-apex direction to mature apex-to-base pattern, and correlated this event with ventricular septation. These results were later confirmed using high-resolution optical mapping studies, discussed in more detail below. For certain question, and in particular in larger, late gestation avian hearts, microelectrodes are still useful, as was proved recently by the Leiden group (Kolditz et al., 2008, 2007). Two exploration electrodes, together with simultaneous recording of volume-conducted ECG, were enough to demonstrate the accessory atrio-ventricular pathways occurring normally during fetal avian development, and their increased frequency in a model of epicardial ablation that results in deficient formation of fibrous atrioventricular insulation.

2.2. Optical methods for visualization of electrical impulse spreading

However, alternative optical methods, recently reviewed (Boukens and Efimov, 2014), exist for studying spread of electrical activation in excitable tissue by means of supravital staining with voltage-sensitive dyes (Kamino et al., 1981). This approach depends on several technological platforms. First, of course, is the availability of suitable probes that must be stable and robust enough to give sufficient signal of either voltage or intracellular ion concentration. Then, epifluorescence microscope is commonly used for studying smaller samples (embryonic hearts, cells), but for larger hearts, incident illumination systems (typically using light emitting diodes) and barrier filter in front of a macroscope lens are widely used (Fedorov et al., 2007). Last, the amount of data recorded from numerous channels (10 × 10 in the beginning, over 100 × 100 nowadays) at high sampling rate (typically over 1 kHz at 16 bits per channel) requires an adequate computing power – typically dedicated RAM as an intermediate step, either on-board or in the computer attached to the photodiode array or high speed camera. Improvements in any of these potential bottlenecks could, and often did, result in new discoveries.

Major problems of optical probes for studying dynamic events are their instability, photobleaching with associated toxicity of breakdown products, and low response. The most popular voltage-sensitive dye, di-4-ANEPPS (Witkowski et al., 1997) is excited by a broad range of wavelengths, but the voltage-dependent response in emitted fluorescence is in the range >590 nm. Once incorporated to the membrane, it is fairly rapidly internalized, so modification of the lipophilic part by a longer aliphatic chain (di-8-ANEPPS) is advantageous in some preparations, such as isolated cells (Kucera et al., 1998). Novel, longer wavelength dyes use excitation wavelengths better penetrating to the tissues and less toxic for the cells for improved survival (Sakai et al., 1998).

The first detectors employed were light-sensitive photodiodes, which are individually tunable and have an excellent signal to noise ratio. They were arranged into a customizable photodiode array, positioned over an image projected by the imaging setup. This approach was pioneered by the Kamino group in Japan (Hirota et al., 1985; Kamino, 1991; Kamino et al., 1981). The system was used to study the earliest sites of electrical activity in the chick and rat heart, as well as the effect of atrial myocardial architecture (pectinate muscles) on impulse propagation in frog atria (Komuro et al., 1986). Photodiode arrays are today commercially available, and still in use to study events from cell level (Kucera et al., 1998) to early rabbit embryonic heart (Chuck et al., 2004).

An interesting approach used another new technological breakthrough, notably laser scanning system, to study changes in voltage (Dillon and Morad, 1981). These authors showed in the frog heart that myocardial architecture is an important determinant of preferential conduction pathway. While this system did not become widely accepted, laser scanning confocal microscope in a line scan mode, which can achieve speeds up to 2 kHz, is rather popular to study calcium transients and sparks in isolated cells (Toischer et al., 2010), and is useable also for voltage sensitive dyes in isolated hearts (Fig. 1), where it has the advantage of possibility to select precisely the depth from which the signal is acquired, and makes thus data interpretation less complex.

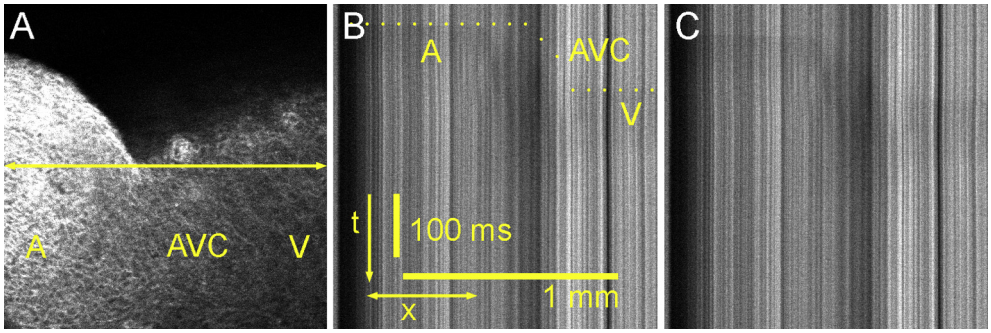

Fig. 1. Proof of principle demonstration of high speed line scan imaging of voltage in the embryonic heart. A: XY scan of a Stage 21 chick embryonic heart in a plane containing atrium (A), atrioventricular canal (AV) and ventricle (V). B: Xt recording at 1 kHz along the line indicated in A showing the slowing of the propagation velocity in the AV canal. Beginning of the action potential is indicated by the yellow dots. C: raw data from B.

2.3. High-speed cameras for optical mapping

However, significant new information about cardiac electrophysiology could be gathered from simultaneously increasing the temporal resolution to the domain of milliseconds, and spatial to micrometers. The Morley group relied on a Dalsa camera with frame rate just below 1000 fps and spatial resolution 64 × 64 pixels (Gutstein et al., 2001; Hall et al., 2000; Jalife et al., 1998, 1999; Morley and Jalife, 2000; Morley and Vaidya, 2001; Morley et al., 1999; Rentschler et al., 2001, 2002; Tamaddon et al., 2000; Vaidya et al., 2001). For dedicated systems, such resolution was deemed completely satisfactory, as any increase in spatial resolution would be tied with decreased pixel size and therefore number of photons hitting it per unit of time, and there would be a problem with data streaming (for example, one second recording of 100 × 100 pixels at 16 bits and 1 kHz takes about 25 Mb – a significant problem for the personal computers in the 1990s and even 2000s). Another plague of optical recordings of beating hearts are motion artifacts. In principle, such mechanical events could be, and were, used for analysis of the heart rhythm (Buechling et al., 2009; Raddatz, 1997), but for voltage studies they pose a considerable problem, especially for analysis of repolarization. Therefore, motion inhibitors (excitation-contraction uncoupling agents) such as BDM (Efimov et al., 1997), cytochalasin D (Biermann et al., 1998; Jalife et al., 1998), blebbistatin (Efimov et al., 1997; Fedorov et al., 2010, 2007; Jou et al., 2011; Sankova et al., 2012) were introduced. These agents enabled considerable improvement in signal quality and are considered indispensable by many investigators. However, they have caveats in their own respect due to their inherent toxicity, effects on action potential duration or pacemaking potentials. Alternative methods were therefore developed for improvement of signal to noise ration, such as signal averaging (Rentschler et al., 2001, 2002) or pixel tracking using dual wavelength imaging (Leaf et al., 2008). They seem to work well as long as the heart beat is regular and the conduction pathway does not differ from one cycle to another, which is most of the time true (Sankova et al., 2012). However, pixel tracking requires either two camera system or rapid wavelength switching, compromising somewhat the spatial and/or temporal resolution.

Unlike sock or balloon electrodes, which truly allow mapping of the heart chamber in three dimensions, the optical approach is typically restricted to a single view, reducing the three dimensional organ in a single plane. This is accentuated by some groups that further flatten the imaged surface to reduce the ambiguity of the curved surface (Larsen et al., 2012), but such artificial stretching could alter conduction properties of the myocardium due to physical uncoupling or action on stretch-sensitive ion channels. The problem of a limited area of view, particularly troublesome when studying complex arrhythmias such as ventricular fibrillation (Boukens and Efimov, 2014), could be solved by using dual/multiple camera systems (easily achieved in a hanging Langendorff preparation), flipping the heart (Ammirabile et al., 2012; Sedmera et al., 2004), or using angled mirror (Gurjarpadhye et al., 2007) for simultaneous viewing of ±75% of the heart surface. This last approach sacrifices some of the spatial resolution of the camera, but saves considerable expenses of the setup.

2.4. New developments in high-speed imaging: getting the high resolution, too

Increase in hardware performance and availability of ready-to-use systems enabled recordings of multiple parameters at once, such as simultaneous voltage and calcium imaging (Chen et al., 2010). The first such dual recording was reported by Efimov and associates (Efimov et al., 1994). The main advantage of such approach is to study electromechanical dissociation during ischemia. In such settings, long calcium transients with no ATP can occur without any voltage changes, but also alternans is possible either at the level of voltage or calcium changes (Choi and Salama, 2000). Dual simultaneous imaging can provide exact correlation, impossible with any other method.

Newer generation of cameras is marked by "competition" between two different designs – CCD vs. CMOS or sCMOS (for continuous

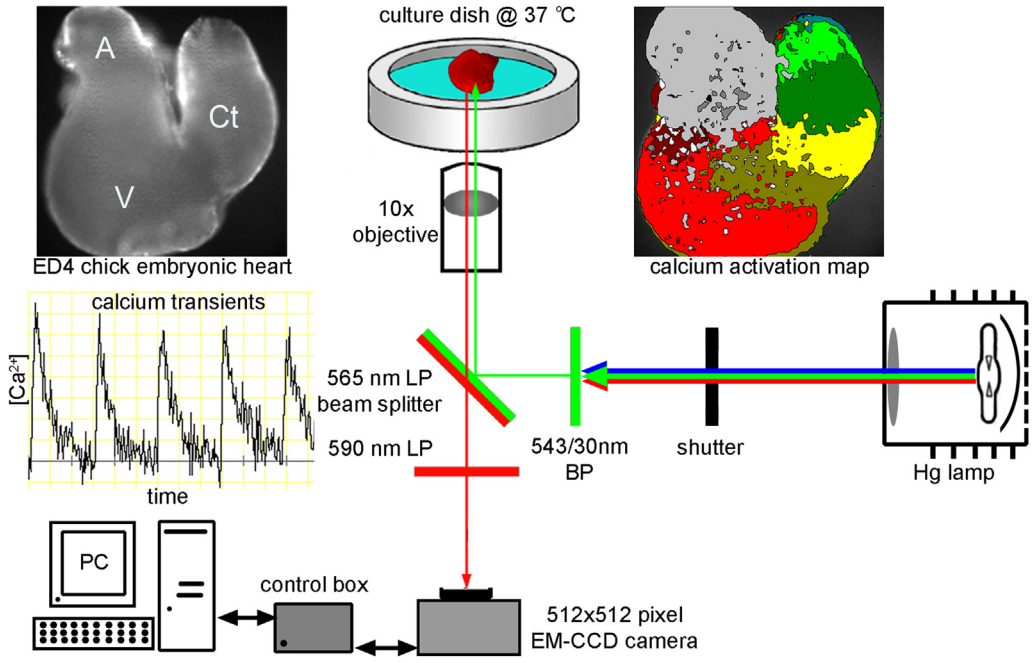

Fig. 2. Setup for voltage (di-4-ANEPPS) or calcium (rhod-2) imaging. The system is centered around an inverted microscope (Nikon Eclipse) with objectives ranging from 4× (whole heart imaging) to 63× water immersion (isolated cells). Current high-speed camera has a maximum resolution of 512 × 512 pixels and can achieve rate of 1300 fps, although not simultaneously.

updates and discussion of this topic, see http://www.microscopy-analysis.com/). While the use of add-on image intensifiers (Reckova et al., 2003; Sedmera et al., 2003) was gradually abandoned, EM-CCD cameras still present very sensitive and reproducible detectors, and due to their speed at full frame rate are useful also in other low-light application (e.g. spinning disc confocal, light sheet microscopy). Newer CMOS sensors possess excellent sensitivity and readout speeds up to 10,000 fps in dedicated systems (Ultima L) are available in practice. Recent developments widen the spectrum of cameras usable for optical mapping – the newest cameras (e.g. Andor, Hamamatsu) allow full frame (over 1 megapixel) high resolution imaging at over hundred frames per second; with sub-array and binning, rates over 1,000 fps could be easily achieved at still reasonable spatial resolution. Such versatile systems might open the door to the area of high-speed imaging even to labs that do not primarily intend to build such setups.

2.5. Calcium imaging in the developing heart

Measuring calcium concentrations in time at relatively low temporal resolution was successfully performed in the past in events such as egg fertilization or cell signaling (Brooker et al., 1990; Sun et al., 1992). High-speed imaging of calcium in embryonic mouse heart (Valderrabano et al., 2006) resulted in increased sensitivity and allowed signal detection also in the atrioventricular canal, enabling detection of various arrhythmias. As important events in the cardiac myocytes occur rather rapidly (e.g. calcium sparks) and at a small spatial scale (parts of cells), both high speed and high resolution are desirable. Thus, our experimental approach (Fig. 2) of imaging normal and stressed embryonic hearts from 100 × 100 to 512 × 512 pixel, 0.5–20 ms resolution presents a significant technological improvement that brings important new pieces of information, such as precise localization of sites of conduction block and uncovering of ectopic pacemakers (Ammirabile et al., 2012; Benes et al., 2014; Hoogaars et al., 2007; Leaf et al., 2008) in the isolated embryonic heart model.

Our current setup for high speed/high resolution calcium and voltage imaging is depicted in Fig. 2. It allows for speed from about 50 fps (full frame mode) up to 1300 fps (ROH mode – with 128 × 128 pixel subarray and 8 × 8 binning, 3/4 of the chip therefore being used for readout). Data acquisition and processing is performed as described previously (Nanka et al., 2008; Sankova et al., 2010), with the exception of different staining protocol with the rhod-2 calcium indicator. Calcium signal has the advantage over voltage in being positive (i.e., increase, rather than decrease of fluorescence), and higher signal-to-noise ratio is especially advantageous in regions with lower signal amplitude, such as the atrioventricular canal and the outflow tract (Reckova et al., 2003).

Chick embryonic hearts subjected to hypoxia fall into atrioventricular conduction block (Sedmera et al., 2002b; Tran et al., 1996). This could unmask a potential backup pacemaker. We observed occasionally temporary conduction blocks during stabilization of the preparation prior to imaging. Our analysis showed that they were of different kinds, ranging from sino-atrial, atrioventricular, to ventriculo-conotruncal, similar to *in vivo* block induced by digoxin in ED4 embryo (Paff et al., 1964). The embryonic outflow tract is myocardial at this stage, and a distinct wave on ECG for corresponds to its activation (Sabourin et al., 2011; Sarre et al., 2006). Pacemaking activity of low frequency is known to be present in the isolated outflow tract (Raddatz, 1997; Sarre et al., 2009), but the exact location of this pacemaker was not known. In the settings of conduction block, we observed activity of the conotruncal pacemaker in two of 59 hearts; the activity originated at the base of the outflow tract with a frequency of 30 bpm at 37 °C in the example depicted in Fig. 3. The atrial rate was in this case 142 bpm, typical for this stage *in vitro* (Sedmera et al., 2002b).

3. New frontiers in dynamic imaging

3.1. Ectopic or backup pacemaker in the outflow tract myocardium

We then set to investigate whether similar properties are present also in the mammalian heart. In the mouse, experimental creation of complete AV block by cutting the atrioventricular junction with fine scissors did not result in manifestation of ectopic activity (24 fresh hearts cut at ED11.5 or 12.5), analyzed by voltage sensitive dye method described in detail previously (Sankova et al., 2012). However, when ED11.5 mouse embryonic hearts were cultured (originally for different purposes – mechanical unloading and pharmacology, to build upon previous studies of Sankova et al. (2010) and Rentschler et al. (2002)) for 24 h in M16 media, we observed frequently (in 36%, 9 of 25 spontaneously beating hearts) a complete atrioventricular block with atrial rate of 88 ± 28 bpm, while the ventricles were activated retrogradely by a low-frequency (37 ± 9 bpm) ectopic backup pacemaker in the base of the outflow tract (Fig. 4). This phenomenon was also observed in hearts cultured from ED10.5 to ED11.5, although at lower frequency (2 of 9 hearts). The retrograde propagation was different from the activation of the freshly isolated chick heart in block (compare with

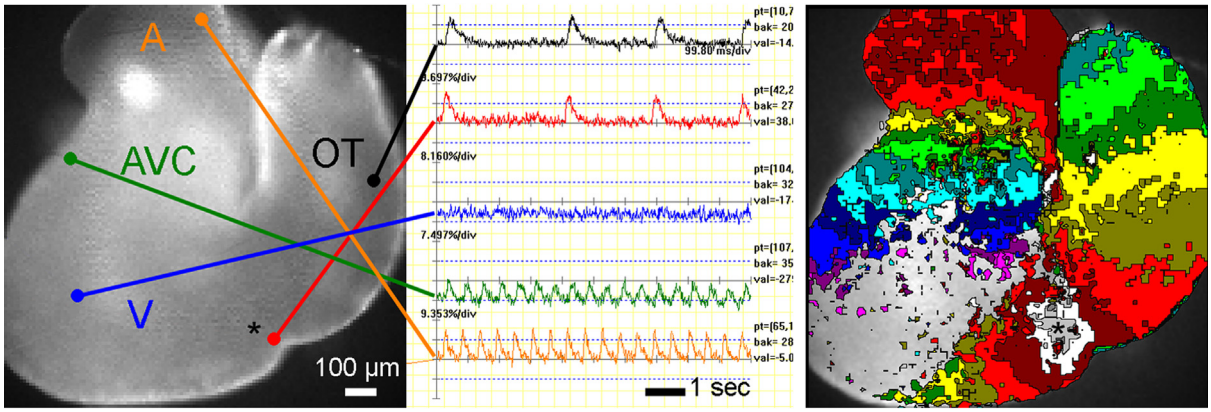

Fig. 3. Ectopic pacemaker in the outflow tract of ED4 chick embryonic heart. The heart is in complete atrioventricular block, located at the transition of the atrioventricular canal and ventricle; by co-incidence, the timing of the ectopic beat correlates with the normal cardiac cycle initiation. A, atrium; AV, atrioventricular canal; OT, outflow tract; V, ventricle. Color isochrones are in 7.68 ms intervals. Scale bar 1 mm.

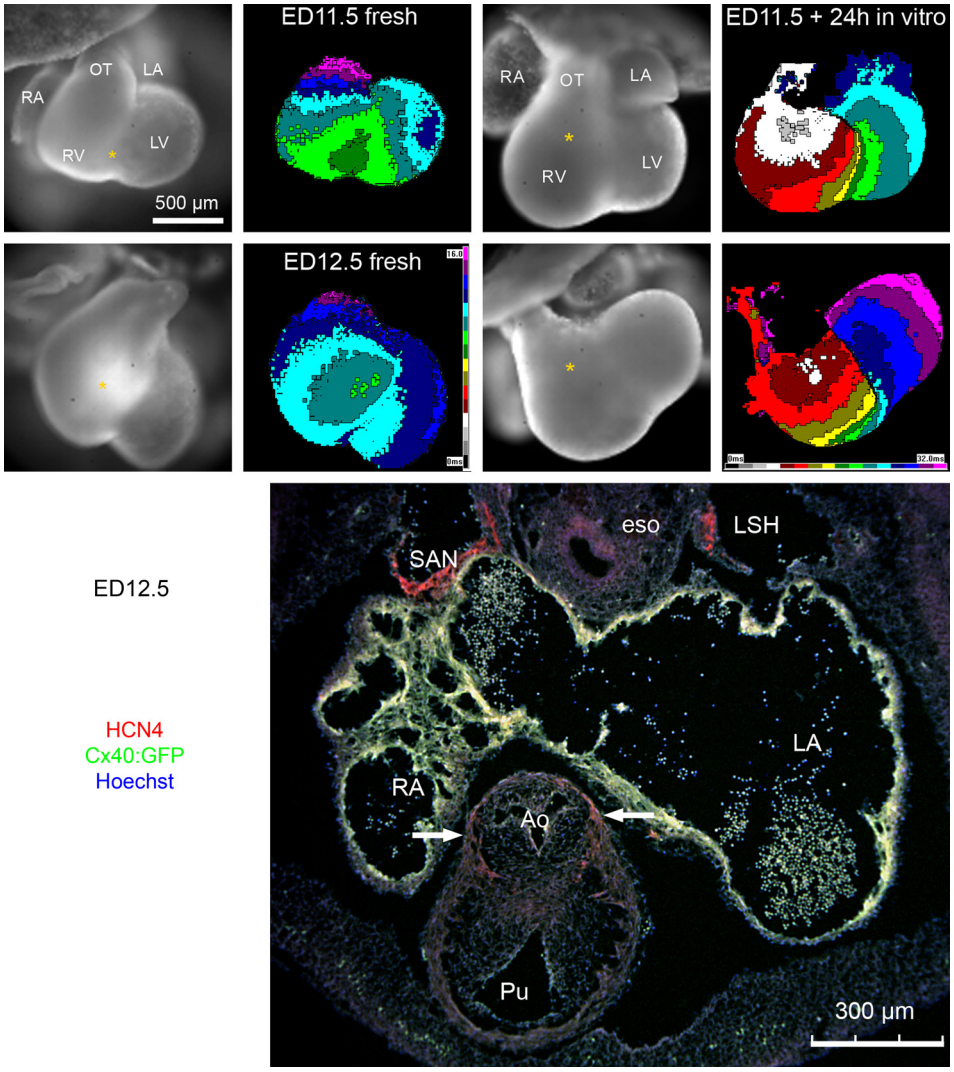

Fig. 4. Ectopic activation of cultured mouse embryonic hearts. Typical ventricular activation of freshly isolated hearts proceeds either via the primary interventricular ring (ED11.5), or from the right apical breakthrough (ED12.5). The site of first activation is marked with an asterisk. Colors represent 1 ms isochronal intervals. Two examples showing the ventricular activation from the outflow tract region illustrate the position of the ectopic pacemaker at the base of the outflow tract (OT; orange asterisks). Note that the conduction is considerably slower, color isochronal intervals being 2 ms. Transverse histological section through this region shows higher intensity of staining for HCN_4, responsible for the pacemaking activity, in this region; normally, the highest levels are found in the area of the sinoatrial node (SAN), with some remnants in the left sinus horn (LSH) myocardium. Staining performed in conjunction of study by Benes et al. (2014). Ao, aorta; eso, esophagus; LA, left atrium; LV, left ventricle; Pu, pulmonary artery; RA, right atrium; RV, right ventricle.

Fig. 3), where the wavefront did not propagate back to the ventricular myocardium.

During development, the outflow tract (as well as inflow) myocardium is added to the cardiac tube from the pharyngeal mesenchyme (van den Berg et al., 2009). This cell source is referred to as the second heart field and it is distinguished by Isl-1 expression (Kelly et al., 2001; Kelly and Buckingham, 2002). Right ventricular outflow tract is a frequent site of ectopic activity in humans (Braunwald et al., 2001); our results showing the location of the ectopic pacemaker site coinciding with stronger HCN_4 expression might be responsible to a large extent for the spontaneous depolarization (Fig. 4), and thus could provide a developmental explanation for this phenomenon. In this context, a paper by Boukens and colleagues analyzed the origin of ectopic pacemaking in the outflow tract in Brugada patients (Boukens et al., 2013). Study in a mouse model revealed expression of Tbx2 in the outflow tract myocardium, responsible for maintaining the primitive phenotype (i.e., slow conduction and automaticity potential). This features disappeared in the adulthood, but could be unmasked by either genetic of pharmacological sodium channel blockade. Persistence (Reaume et al., 1995) of very slowly conducting (our unpublished data) outflow tract myocardium was also noted in the mouse fetuses with Cx43 deletion, which have generally severe conduction problems and predisposition for ventricular arrhythmias (Vaidya et al., 2001).

3.2. *Imaging the dispensable heart: lessons from the zebrafish*

Zebrafish heart is of considerably simpler design than its mammalian counterpart, possessing only a single atrial and single ventricular chamber (Hu et al., 2001). In addition, its transparency and self-contained early development make it ideal for longitudinal imaging studies. Its small size enables use of high-power optics, allowing excellent 3D rendering and cell counting; furthermore, coupled with its low metabolic requirements due to poikilothermy, heart function is not essential for embryonic survival during the

first week of development. All these features allow functional studies of genes crucial for cardiovascular development that are not feasible in the mouse model due to early embryonic lethality. It is thus not surprising that new technical advances in developmental imaging come from this field.

Particularities of this model organism allowed calcium imaging after dye injection at a single cell stage (Milan et al., 2006). Motion control, critical for optical mapping, was elegantly achieved by using the *silent heart* mutant with normal cardiac morphology and electrical behavior but no contraction. Combination of this approach allowed imaging of previously inaccessible early stages of conduction system differentiation, and showed critical requirement of neuregulin and notch signaling for atrioventricular delay development.

Optogenetics approach was used to localize the pacemaker region at various stages of zebrafish heart development (Arrenberg et al., 2010). Stable transfection with halorhodopsin and channelrhodopsin-2 allowed precise temporal and spatial control of cardiac function. In this way, patterned light illumination enabled disabling and pacing studies that allowed mapping of pacemaker shift during heart development. Light-sheet microscopy setup also bypassed the photobleaching and phototoxicity problems, reported earlier (Sedmera et al., 2003).

Confocal and 3D particles imaging using zebrafish embryos expressing fluorescent proteins under myocardial and endocardial promoters (Hove and Craig, 2012) allowed detailed, 3D monitoring of cardiac function from the earliest time points. This setup permitted fairly precise biomechanical studies and resolution of early cardiac mechanics at much higher resolution than other imaging modalities, such as ultrasound biomicroscopy (McQuinn et al., 2007).

Advantages and drawbacks of genetically encoded vs. injected calcium indicators in zebrafish studies were reviewed recently (Kettunen, 2012). It is important to mention fruitful transfer of techniques from the mouse model, where genetically encoded calcium indicators were originally reported (Tallini et al., 2007) and used to study calcium dynamics specifically in the Purkinje fibers. Transmembrane voltage indicator acting via FRET ('mermaid') was recently transfected into zebrafish embryos (Tsutsui et al., 2010), and allows screening for potentially arrhythmogenic drugs or functional evaluation of different ion channel mutations in a whole heart model.

In conclusion, we have structured this paper around new technologies, pinpointing what unexpected piece of information they uncovered in the field. Technological progress for its own sake is of little value if it does not bring any novel findings or opens new questions. In our opinion, fruitful avenues of future research in this arena will involve three-dimensional imaging studies of cardiac excitation, enabled by simultaneous improvement in light sources, optics, and computing power. This will allow precise definition of conduction pathways, hitherto inferred only from 2D recordings. Genetically encoded fluorescent probes are also of high value, both for large-scale screening as well as for spatially precise electrophysiological studies.

Editors' note

Please see also related communications in this issue by Fedorchak et al. (2014) and Ambrosi et al. (2014).

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