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PERSPECTIVES

Getting It Right

Stop the beat to see the rhythm: excitation-contraction uncoupling in cardiac research

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Abstract

Optical mapping is an imaging technique that is extensively used in cardiovascular research, wherein parameter-sensitive fluorescent indicators are used to study the electrophysiology and excitation-contraction coupling of cardiac tissues. Despite many benefits of optical mapping, eliminating motion artifacts within the optical signals is a major challenge, as myocardial contraction interferes with the faithful acquisition of action potentials and intracellular calcium transients. As such, excitation-contraction uncoupling agents are frequently used to reduce signal distortion by suppressing contraction. When compared with other uncoupling agents, blebbistatin is the most frequently used, as it offers increased potency with minimal direct effects on cardiac electrophysiology. Nevertheless, blebbistatin may exert secondary effects on electrical activity, metabolism, and coronary flow, and the incorrect administration of blebbistatin to cardiac tissue can prove detrimental, resulting in erroneous interpretation of optical mapping results. In this "Getting It Right" perspective, we briefly review the literature regarding the use of blebbistatin in cardiac optical mapping experiments, highlight potential secondary effects of blebbistatin on cardiac electrical activity and metabolic demand, and conclude with the consensus of the authors on best practices for effectively using blebbistatin in optical mapping studies of cardiac tissue.

blebbistatin; cardiac physiology; excitation-contraction uncoupler; optical mapping

INTRODUCTION

Optical mapping is a fluorescence imaging approach used to study physiological processes of cardiac tissue and cell preparations, and it is used extensively in basic cardiac electrophysiology research. The great utility of optical mapping is that it provides unparalleled insight into the spatiotemporal dynamics of electrophysiology and excitation-contraction coupling (ECC), albeit with a few key technical hurdles. The field of cardiac optical mapping sprang from the initial work of Salama and Morad, who first recorded optical action potentials from frog hearts using voltage-sensitive dyes (1). Prior to optical approaches, absolute transmembrane potential was measured from tissue preparations and intact hearts using glass microelectrodes, although this approach is technically challenging and spatial information is limited by the number of electrodes that can be inserted into the tissue (2). Electrical activity can also be mapped using arrays of electrodes placed in contact with the tissue (3, 4). However, electrode array mapping is limited by the number and spacing of electrodes and the electrograms are often contaminated by electrical artifacts that occur during pacing and defibrillation. In contrast, the development of optical mapping offered a new mapping approach with superior spatial resolution (5) that is determined by the image sensor specifications (sensor size, number of pixels, and quantum efficiency) and the optical field of view (6). Additionally, the use of calcium-sensitive probes enabled simultaneous measurements of electrical activity and intracellular calcium cycling, as demonstrated by Choi and Salama at the turn of the century (7) and now provides unprecedented insight into ECC parameters in normal and diseased hearts (8–13).

EXCITATION-CONTRACTION UNCOUPLING

The primary challenge of imaging cardiac tissue lies in the very function of the heart itself, pressure development through contraction. Distortions of optical signals acquired from cardiac tissue that result from unconstrained myocardial

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contraction are often much larger than the physiological signal transduced by a fluorescent probe. To reduce these distortions, hearts have been mechanically constrained against a glass window or with a nylon mesh (14, 15) while optically mapping the epicardium. A limitation of mechanical constraint is that the contact pressure could reduce coronary flow, causing local ischemia. Any residual motion could still negatively impact the accuracy of measuring action potential duration (APD) or APD restitution due to persistent motion artifact (16–18), especially at the edges of the tissue. More recently, motion tracking and ratiometric imaging have enabled optical mapping of transmembrane potential in unconstrained contracting perfused hearts, including working heart preparations (16, 17, 19–22). Further developments in optics and motion tracking algorithms might one day eliminate the need for motion suppression during optical mapping.

However, fully contracting perfused heart preparations may pose an additional set of challenges, especially in studies of ischemia, hypoxia, and metabolism, in that crystalloid buffers are typically used as a perfusate (23). This is important because the heart's contractile machinery is responsible for ~75% of its metabolic demand (24), and as such, crystalloid perfusates may lack adequate oxygen-carrying capacity to meet myocardial oxygen demand during elevated heart rates (20, 23, 25–27). This is likely why coronary flow rate in Langendorff-perfused hearts can be several times greater than what is measured under normal physiological conditions (28, 29). Indeed, perfused heart experiments where the crystalloid perfusate was supplemented with an oxygen carrier, such as erythrocytes or other circulating transporters like perfluorocarbons, have revealed the important impact of myocardial oxygen delivery on the electrophysiology, mitochondrial energetics, and contractile performance of perfused hearts (25, 30–32). Relevant to optical mapping, a limitation of supplementing perfusate with erythrocytes is that hemoglobin absorbs light within visible wavelength bands that overlap with the fluorescence of most voltage- and calcium-sensitive probes, necessitating a mapping approach that prevents the erythrocyte-supplemented perfusate from entering the mapped field of view (33). Furthermore, new near-infrared potentiometric probes that fluoresce beyond the absorption band of hemoglobin have recently been used to optically map in vivo and ex vivo blood-perfused hearts (14, 17, 34, 35). The success of such experiments is an exciting step forward in elevating the physiological relevance of optical mapping.

In most optical mapping studies, motion artifacts are suppressed by administering an excitation-contraction (EC) uncoupling agent to the tissue that prevents myocyte contraction by inhibiting actomyosin cross-bridge cycling. Many early cardiac optical mapping studies used 2,3-butanedione monoxime (BDM), also known as diacetyl monoxime (DAM), and cytochalasin D (CytoD) as EC uncoupling agents. Although the precise mechanism of action of these two compounds is not fully understood, these EC uncouplers generally act as noncompetitive inhibitors of muscle myosin II by inhibiting myosin ATPase, thereby disrupting actin-myosin polymerization to reduce or eliminate contraction (36–38). Since these compounds are nonspecific, relatively high concentrations are often needed to completely inhibit contraction throughout the tissue. The high concentrations and lack of specificity lead to measurable effects on ion channel kinetics, changes to intracellular calcium handling, and altered action potential morphology. As an example, BDM has been shown to decrease L-type calcium current and reduce calcium transients (39–41), flatten the restitution curve, and induce APD shortening (42). Conversely, in mouse hearts, CytoD and BDM have been reported to increase APD and refractory periods and slow conduction velocity (43). Peak systolic and diastolic calcium appears elevated in mouse hearts exposed to CytoD; however, BDM has the opposite effect. Interestingly, the side effects of these uncouplers may be species dependent (40, 41, 43).

In 2003, blebbistatin (Bleb) was introduced as a more potent and specific myosin II inhibitor (44). It was quickly adopted by many optical mapping laboratories due to its ease of use (it is less toxic than CytoD) and its efficacy in halting cardiac contraction without the apparent side effects observed with other uncoupling agents (45, 46). Named for its ability to disrupt cell motility (44, 47), blebbistatin has proven useful in a range of research areas including cancer research, cell differentiation and migration, cytokinesis, neuroscience, as well as skeletal, smooth, and cardiac muscle research (48). To assess a snapshot of the use of EC uncouplers in cardiac optical mapping research over time, we evaluated articles published in *American Journal of Physiology-Heart and Circulatory Physiology* (AJP) for the type of EC uncoupling agent used for optical mapping. A total of 164 articles published from 1993 to 2020 were identified from a search of the journal website (accessed on 14 May, 2021 by L. M.S.) using the terms: optical mapping in combination with the following keywords: "2,3-butanedione monoxime" (n = 52) "diacetyl monoxime" (n = 15), "cytochalasin D" (n = 37), and "blebbistatin" (n = 63). The three papers that appeared in both the search for "diacetyl monoxime" and the search for "2,3-butanedione monoxime" were only counted once. As shown in Fig. 1, since its first use for optical mapping in 2007, blebbistatin has all but replaced the other available EC uncouplers. In fact, in the last 3 years alone, blebbistatin was used twice as often (10–13, 49–58) as any other uncoupler for imaging applications (59–65). The use of BDM/DAM and CytoD for optical mapping has essentially ceased; yet due to its lower cost, BDM/DAM may still be a reasonable choice for larger animal (i.e., nonrodent) studies (59, 66–71), which require larger amounts of an EC uncoupler (72). Clearly, blebbistatin is currently the most popular choice in cardiac optical mapping (Fig. 1), as it can be used at much lower concentrations and is less toxic compared with the other EC uncouplers (42, 43, 45, 73).

Blebbistatin's mechanism of action as an EC uncoupler is that it inhibits the ATPases associated primarily with class II myosin isoforms in an actin-detached position, yet it displays little affinity for the rest of the myosin superfamily (47, 74, 75). Importantly, when applied at a concentration that eliminates cardiac tissue contraction, it was reported to have little measurable effect on intracellular calcium handling, action potential morphology, ECG parameters, sinoatrial node activity, and activation patterns (45). Unlike CytoD and BDM/DAM, which must be used at higher concentrations to halt contraction (80 μM to 10 mM), blebbistatin is effective at much lower circulating concentrations (5–15 μM). Notably, even at higher concentrations (25 μM), sinoatrial node cells

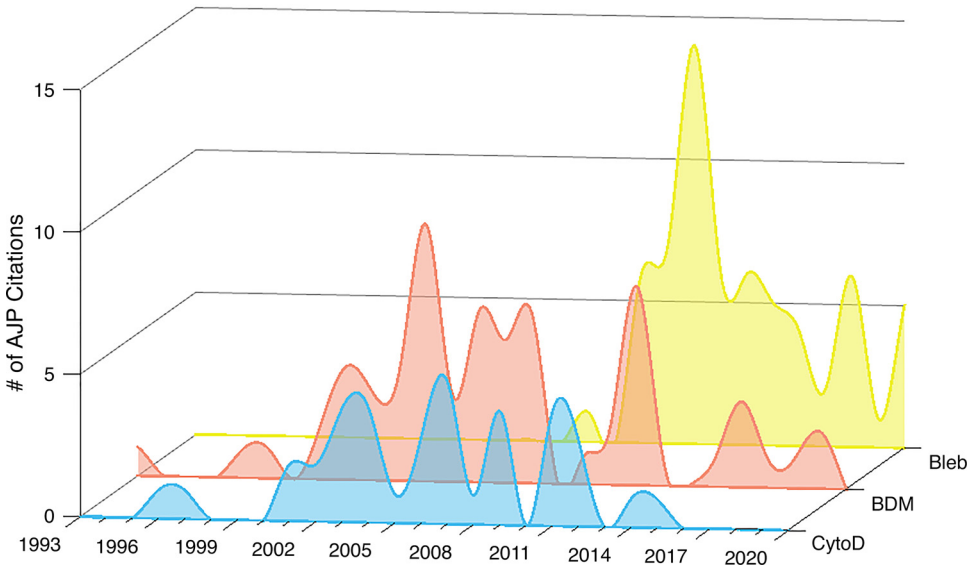

Figure 1. Annual number of *American Journal of Physiology-Heart* publications from 1993 to 2020 that used excitation-contraction uncoupling agents [CytoD, BDM (DAM), Bleb] in cardiac optical mapping studies. BDM, 2,3-butanedione monoxime; Bleb, blebbistatin; CytoD, cytochalasin D; DAM, diacetyl monoxime.

retain their intrinsic firing rate with no measurable changes in calcium handling, unlike BDM (73). Although blebbistatin has proven to be an incredibly useful compound for cardiac optical mapping studies, it too comes with caveats, including some degree of photoinstability, cytotoxicity, and intrinsic fluorescence (76, 77). There have also been reports of secondary effects on electrical activity, metabolism, and vessel occlusion in excised perfused heart experiments (16, 78).

ELECTROPHYSIOLOGICAL AND METABOLIC CONSIDERATIONS

In the foundational 2007 study by Fedorov et al., the effects of blebbistatin on isolated rat cardiomyocytes and excised perfused rabbit hearts were first reported. Blebbistatin was found to inhibit cardiac contraction without measurable effects on cardiac electrophysiology end points, including sinoatrial node activity, epicardial conduction velocity, repolarization, and intracellular calcium cycling (45). Several years later, using monophasic action potential recordings from perfused rabbit hearts, Lou et al. corroborated that BDM flattened the APD restitution curve, but blebbistatin did not; nor did it change monophasic APD (42). Brack et al. later confirmed that atrioventricular and ventricular conduction remained unchanged after administering blebbistatin, but they reported effects on APD restitution, ventricular effective refractory period, and the threshold for ventricular fibrillation in perfused rabbit hearts (78). Recently, Kappadan et al. also reported that blebbistatin prolonged APD in rabbit hearts by 25% (16), and recent in vivo studies found that blebbistatin prolonged APD and slowed conduction velocity in swine hearts (17). It is still unclear whether these observed effects are directly attributed to blebbistatin nonspecifically interacting with ion channels or transporters, or rather, a secondary consequence of the altered metabolic state of the heart during EC uncoupling, as oxygen demand and ATP consumption are reduced (Fig. 2) (26).

In particular, several important aspects of using perfused hearts for optical mapping arise from the fact that the heart's metabolic machinery is intertwined with electrical excitation

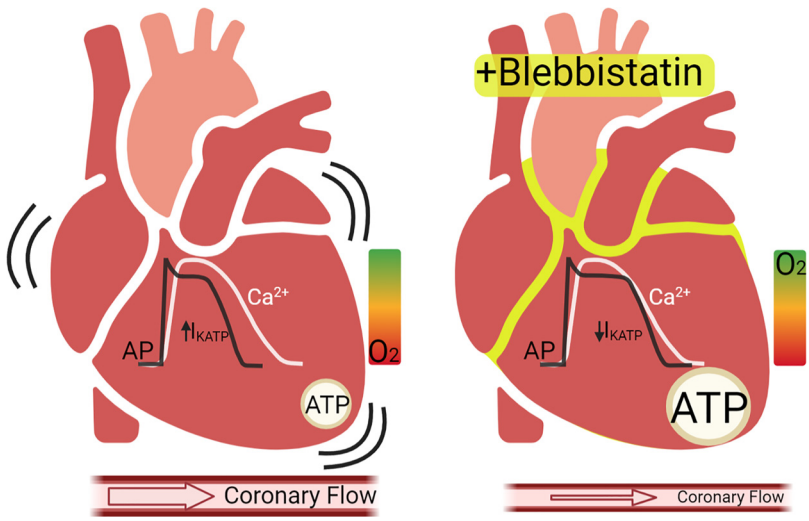

Figure 2. Major metabolic effects of blebbistatin on perfused hearts. *Left:* beating hearts perfused with crystalloid buffers may have significant I_{KATP} due to low ATP/ADP ratio, which can shorten the action potential duration. *Right:* blebbistatin treatment reduces metabolic demand, increases ATP/ADP ratio, and may decrease I_{KATP}, resulting in a longer action potential duration. Coronary flow is also typically reduced under these conditions. Notably, intracellular calcium transients are minimally affected by blebbistatin. AP, action potential; Ca^{2+}, intracellular calcium transient; I_{KATP}, ATP-sensitive potassium channel current. Created with BioRender.com, and published with permission.

and mechanical output and feedback mechanisms are dependent on oxygen supply and ATP availability (79). After excitation-contraction is uncoupled, perfused hearts have a fourfold drop in oxygen demand, which dramatically slows cellular metabolic processes throughout the tissue (24, 26). This reduction in myocardial metabolic demand could dramatically impact the sarcolemmal ATP-sensitive potassium channel current (I_{KATP}), essentially reducing the repolarizing current to zero when cellular ATP/ADP ratio is high, such as when there is no cross-bridge cycling during EC uncoupling. Indeed, previous studies in rabbit hearts perfused with fully oxygenated crystalloid perfusate found that I_{KATP} was active during sinus rhythm and this activity increased when heart rate increased (20). Therefore, administering blebbistatin to a perfused heart would then result in a loss of contraction, a large reduction in oxygen demand, an increase in cellular ATP/ADP ratio, a reduction in sarcolemmal I_{KATP}, and a longer APD.

Consideration of this reduced metabolic rate is especially important when using EC uncouplers for research inquiries that have an ischemic component (27, 80). This aspect was highlighted in previous experiments of globally ischemic rabbit hearts, wherein blebbistatin administered before ischemia prolonged (by ~100%) the time required for mitochondrial membrane potential to collapse as well as the time to asystole (80). This underscores the fact that EC uncoupling dramatically slows the effect of ischemia, so the downstream physiological changes must be considered within this context.

Appropriate oxygenation of perfused cardiac tissue is therefore a critical factor for mimicking in vivo conditions and must be carefully considered during experiments using crystalloid perfusate. Contracting hearts, either retrograde (Langendorff) or anterograde perfused (working heart) with crystalloid perfusate, have an increased risk of myocardial hypoxia compared with EC uncoupled hearts (25–27). This is revealed by action potential triangulation at faster pacing frequencies, which is associated with hypoxia (oxygen demand exceeds oxygen supply) and is linked to K_{ATP} channel activation, as explained above. Furthermore, APD restitution curves in contracting rabbit hearts can be achieved down to 100 ms; however, in blebbistatin-treated hearts, electrical alternans are observed at slower rates (16). Altogether, changes in APD, APD restitution, and conduction velocity after administering blebbistatin may therefore be, at least in part, attributed to the dramatic change in metabolic rate. In this way, if a vigorously contracting crystalloid-perfused heart, which may be partially hypoxic with significant I_{KATP}, is uncoupled to a state with increased ATP availability, the reduction in I_{KATP} and subsequent APD prolongation could be substantial.

A related observation is the pronounced effect of blebbistatin on coronary flow rate. In perfused rodent hearts, the administration of blebbistatin results in increased perfusion pressure and decreased flow rate over time. In perfused rabbit hearts, blebbistatin initiates a brief period of increased coronary flow, but as contractions wane and metabolic demand diminishes, vasoconstriction initiates a reduction in the coronary flow rate (78, 81). In summary, it is important to consider that experimental end points measured from EC uncoupled tissue are measured during the condition of a very low rate of cellular metabolism and should therefore be interpreted accordingly.

INTRACELLULAR CALCIUM CYCLING CONSIDERATIONS

In contrast to reports that CytoD is associated with higher diastolic intracellular [Ca^{2+}] and BDM prolongs intracellular Ca^{2+} transients (43), these parameters appear to be minimally affected by blebbistatin. In the original study by Fedorov et al., blebbistatin was associated with an increase in the fluorescence of an intracellular Ca^{2+}-sensitive probe (Fluo-5F, AM), which the authors attributed to Ca^{2+} release into the cytoplasm during relaxation and/or the intrinsic fluorescent properties of blebbistatin (45). Using isolated rat cardiac myocytes, Farman et al. reported an elevation in diastolic Ca^{2+} in the presence of 0.5 μM blebbistatin when Fluo-4, but not Indo-1, was used as the calcium indicator dye (82). This observation may also explain APD prolongation after administering blebbistatin, as increased intracellular [Ca^{2+}] can alter both the APD and intracellular calcium transient amplitude. This phenomenon mirrors the action of CytoD, albeit at higher concentrations (43). In contrast, Jian et al. reported a reduction in the calcium transient amplitude when blebbistatin was applied to cardiomyocytes under strong mechanical load (stiff gel), but the resulting calcium transient amplitude was not different from cells with either no or smaller (soft gel) mechanical load (83). These findings highlight the important role of mechanotransduction in mediating cellular signaling but suggest nominal direct pharmacological effects of blebbistatin per se on intracellular Ca^{2+} cycling in both isolated cell and perfused hearts.

BEST PRACTICES FOR PREPARING AND USING BLEBBISTATIN

In addition to the physiological effects of EC uncoupling, there are additional technical considerations that could influence optical mapping outcomes and data interpretation when using blebbistatin. First, blebbistatin is reported to undergo photoinactivation that is associated with toxicity in cell culture models (84). Although, to date, this result has not been validated in experiments of perfused cardiac tissue, as constant epicardial illumination for >1 h with UV light did not affect optical action potentials (76). Second, it is important to use an adequate concentration of blebbistatin to halt contraction throughout the tissue to most effectively minimize motion artifacts. The literature commonly reports the use of a circulating blebbistatin concentration of 5–10 μM, although we have found that blebbistatin works best when a higher concentration is administered as a bolus dose to the heart (up to 50 μM), which dilutes in the perfusion system to a 10 μM circulating concentration. If tissue motion returns, an additional bolus dose can be applied. If a very low level of motion persists then the resulting motion artifact can be removed from the mapping data using a nonrigid motion registration algorithm (85), as recently described (58). Depending on desired experimental outcomes, some level of residual motion artifact (or foregoing the use of an uncoupler at all) may be acceptable. For example, activation

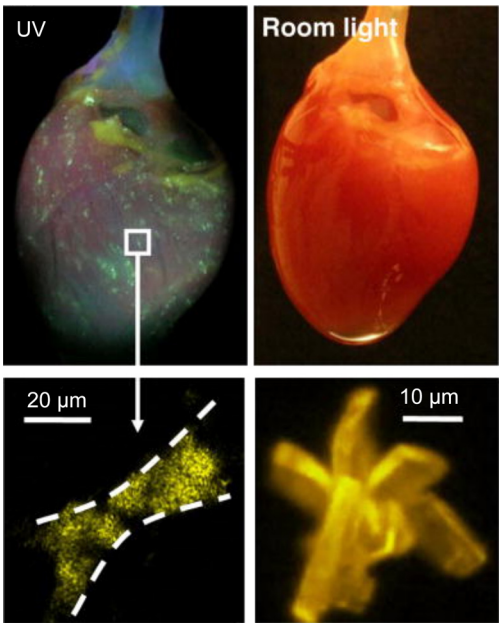

Figure 3. Blebbistatin crystallization within a perfused heart—the "disco heart" effect. *Top*: blebbistatin crystals have accumulated within the vasculature but are not visible unless the tissue is illuminated with UV light (365 ± 5 nm). *Bottom*: the size of the blebbistatin crystals, both in the vasculature (*left*) and in solution (*right*). Reprinted with permission. [Swift 2012, Pflugers Arch (76)].

times, activation maps, conduction velocity, and sites of leading pacemakers or ectopic activity can typically be accurately reconstructed even without EC uncoupling (7, 86), as contraction always occurs after the action potential upstroke. We have found that the amount of acceptable residual motion depends on the experimental design and imaging approach. For instance, motion artifact can be more pronounced at the edges of the tissue and is typically worse at higher magnifications. For each experimental design and hypothesis being tested, pilot experiments are recommended to determine the optimal blebbistatin dose.

Third, the chemical data sheets for blebbistatin report that it is soluble in DMSO in a range from 12.5 mg/mL (Caymen) up to 58 mg/mL (Selleckchem), but it has limited solubility in aqueous solutions. There is little to no information on the best approach for diluting a concentrated stock solution of blebbistatin dissolved in DMSO. We have found that this is a particularly important procedure, especially when adding a small volume of concentrated DMSO + blebbistatin solution to perfusate media, because the blebbistatin could unexpectedly precipitate out of solution due to spontaneous blebbistatin crystallization (Fig. 3) (76). To prevent this, we have found that vigorous agitation and heating of the perfusate solution (37°C–42°C) are necessary when diluting blebbistatin from DMSO stock. Furthermore, an inline filter (5 μm) is recommended to trap crystals that could occlude the coronary microvessels and capillaries (16, 76). After delivering to the tissue, the bolus dose of DMSO + blebbistatin + perfusate will be diluted by the total volume of the recirculated perfusate, ultimately resulting in a final circulating concentration of ~10 μM blebbistatin. Perfusate temperature should be maintained at 37°C for the duration of the experiment to reduce the likelihood of blebbistatin crystallization. If the blebbistatin solubility range is exceeded, or the procedure for diluting blebbistatin stock in perfusate is improper, or perfusate temperature is not maintained, then blebbistatin crystals over 20

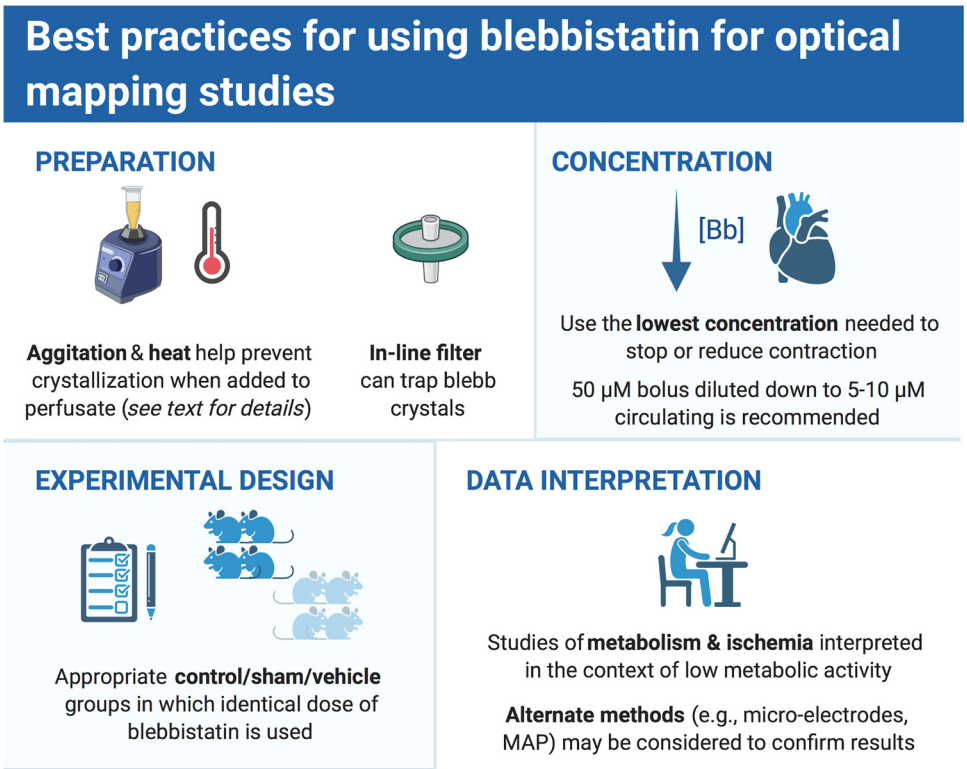

Figure 4. Authors' recommendations for use of blebbistatin for optical mapping experiments. MAP, monophasic action potentials. Created with BioRender.com, and published with permission.

μm could form in the perfusate and occlude the capillary beds (Fig. 3), leading to local areas of ischemia and increasing incidence of arrhythmias (76).

Fourth, experiments requiring the use of blebbistatin (or any EC uncoupler) should be carefully designed to ensure that appropriate control (or sham/vehicle) groups are studied in which equal doses of blebbistatin and identical methods of administering blebbistatin are used across all experimental groups. In this way, any metabolic effects of EC uncoupling should be similar between groups and any electrophysiological differences observed could be attributed to the hypothesis being tested, rather than secondary effects of EC uncoupling. Finally, for studies related to metabolism, oxygenation, and ATP availability (e.g., ischemia, hypoxia), alternative approaches that do not require EC uncoupling could be considered to validate results, including floating glass microelectrodes (87) or monophasic action potential recordings (22, 88), or isolated cardiomyocyte studies (89).

CONCLUSIONS

Forty-five years after the first optical action potentials were recorded from a frog heart, optical mapping of cardiac tissues continues to inform our understanding of basic cardiac physiology, pathophysiology, and provide insight into the mechanisms of clinical therapies for cardiac disease and arrhythmias. Since 2007, the use of blebbistatin as the EC uncoupler of choice for most experimentalists has no doubt improved the translational relevance of cardiac optical mapping studies due to minimal effects on cardiac electrophysiology and calcium handling, especially when compared with earlier pharmacological agents. Considering the electrophysiological, metabolic, and technical considerations outlined here, the authors have recommended a set of best practices for using blebbistatin as an EC uncoupler (see Fig. 4). Although optical mapping of in vivo, contracting, blood-perfused hearts may be considered the "gold standard," various hypotheses, experimental designs, animal models, and mapping of human hearts may continue to necessitate in vitro optical mapping studies, and because of its ease of use, blebbistatin is likely to remain a cornerstone of such experiments. To study the orchestrated events that underlie cardiac function, sometimes you need to stop the beat to see the rhythm.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.M.S., M.W.K., C.M.R., and N.G.P. conceived and designed research; L.M.S., M.W.K., C.M.R., and N.G.P. interpreted results of experiments; L.M.S. and C.M.R., and N.G.P. prepared figures; L.M.S., M.W.K., C.M.R., and N.G.P. drafted manuscript; L.M.S., M.W.K., C.M.R., and N.G.P. edited and revised manuscript; L.M.S., M.W.K., C.M.R., and N.G.P. approved final version of manuscript.

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