How to improve the overall quality of cardiac morphometric data

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Gerdes AM. How to improve the overall quality of cardiac morphometric data. Am J Physiol Heart Circ Physiol 309: H9–H14, 2015. First published May 8, 2015; doi:10.1152/ajpheart.00232.2015.—By the mid-1990s, experts realized that drugs leading to improved ventricular remodeling were doing something remarkable in cardiac patients. The “age of cardiac remodeling” had begun. This created an experimental need for high-quality assessment of changes in cardiac tissue composition, including myocyte shape, myocardial fibrosis/collagen, and vascular remodeling. Many working in the field today have little or no training related to recognition of fixation artifacts or common errors associated with quantitative morphology. Unfortunately, such skills had become somewhat of a lost art during the ages of cardiac physiology in the mid-20th century and molecular biology, gaining prominence by the mid-1970s. Consequently, cardiac remodeling studies today are often seriously flawed to the point where data are not reproducible and subsequent researchers may be chasing the molecular basis of a nonexistent or erroneous phenotype. The current unacceptably high incidence of irreproducible data is a serious waste of time and resources as recently noted in comments by the National Institutes of Health director. The goal of this “how to” article is to share some lessons I have learned during nearly 40 years of assessing morphological changes in the heart. It is possible for any laboratory to routinely publish highly reproducible morphological data that stand the test of time and contribute to our fundamental knowledge of cardiac remodeling and the molecular mechanisms that drive it.

myocardium; morphometry; cardiac myocytes; capillaries; fixation artifacts

THERE IS GROWING CONCERN among scientists and funding agencies like the National Institutes of Health that data from many published studies are not reproducible, even those in some of the most prestigious scientific journals in the world (3). I have long felt that much of the published data on ventricular remodeling in heart disease also suffers from this problem. When reading a paper in this field, the chances are very high that I will not get past the remodeling data before dismissing the paper and moving on, particularly if the key mechanistic findings are dependent on the morphological phenotype. Rarely do I feel that these weak data are intentional. A critical problem is that important basic skills needed to properly assess and perform accurate morphology have never been learned by many scientists in the field. This is particularly obvious in some of our new trainees who have largely focused on acquiring skills in molecular biology. But, I suspect that many expert reviewers also quickly glaze over the remodeling data, accepting what is stated, and go straight to the molecular/cell signaling data. It is particularly unfortunate if scientists waste precious time and resources chasing after something that is not real. In this tutorial, I would like to provide some insight to those interested in cardiac remodeling with the hope that much of this grief can be prevented. The literature is replete with examples of questionable data. Rather than damaging some of my treasured scientific friendships, I will avoid citing specific examples. Instead, I will provide my own unpublished (thankfully) examples. Hopefully, these “lessons learned” will provide readers with some ideas to improve their morphological data.

Cardiac Tissue Preservation and Other Artifacts Affecting Measurements

Some of the “old timers” really got this. One of my favorite studies was published by Roberts and Wearn in 1941 (15). To the best of my knowledge, this study provided the most accurate data ever published regarding changes in myocardial capillaries and their relationship to cardiac myocytes in normal and hypertrophied human hearts. How did they do it? Amazingly, they obtained hearts so fresh they were able to reestablish cardiac contraction before rapidly fixing and processing tissue. They were also extremely particular about artifacts that might affect their data. In normal adult human hearts, they noted mean capillary density (CD) values of ~3,300/mm². This is virtually identical to carefully collected data from normal adult rodents (5) and large mammals (6). Roberts and Wearn also noted a significant reduction in CD in hypertrophied human hearts, a finding confirmed in many animal studies.
Many other studies published over the years show much lower values for CD, likely because of a number of potential sources of variation, including poor quality control regarding selection of good cross sections, differences in contractile state of hearts being sampled, inconsistent labeling of capillary endothelial cells, and tissue separation artifacts. When selecting fields for CD measurements, researchers will find it helpful to use only fields with circular capillaries. Some may be partially collapsed if not perfusion fixed, but if the majority of capillaries are circular, rather than oval, and data are collected from areas without tissue separation, results should be consistent. The effect of myocyte contractile state may also be a concern. In contracted areas, capillaries will be pushed further apart, leading to reduced density values. It is best to arrest all hearts in diastole, e.g., with KCl, procaine, or 2,3-butanedione monoxime (BDM), to minimize this source of variation. If this was not done and there is some contractile variability between hearts, CD in each heart can be normalized to resting unloaded sarcomere length (1.90 μm, the typical length of diastolic arrested heart) in the following manner.

\[
\text{Corrected CD} = \frac{\text{measured CD} \times 1.90/\text{observed sarcomere length}}{}
\]

For example, in a heart with sarcomere lengths averaging 1.50 μm in the sampling area, multiplying measured CD values by 1.27 (1.90/1.50) will correct for the reduction in CD resulting from fixation in a contracted state. Since capillaries are anisotropic (directionally dependent), CD is a good measure to assess capillary changes from cross sections.

Length density (LD) is another helpful method for quantitation of vascular density changes. Unlike CD, LD measurements are not affected by variation in sectioning angle. This measurement is particularly helpful to assess myocardial arteriolar changes where structures tend to be more isotropic (e.g., similar properties in all directions) (16).

\[
\text{LD (mm/mm}^3\) = \frac{\Sigma (a/b)l}{M}
\]

LD is the sum of all ratios of maximum (a) and minimum (b) external arteriolar diameters in the tissue sampling area (M).

Readers are encouraged to explore the large body of literature published by Karel Rakusan and Robert Tomanek (13, 14, 18, 19) for additional information regarding quantitation of microvascular changes in myocardium. A simple formula provided by Rakusan et al. (14) is also helpful in determining sample numbers needed. To reduce sampling error to <1%:

\[
N = \frac{(100 \text{ SD/mean})^2}{1%}\]

To reduce sampling error to <5% (more reasonable):

\[
N = \frac{(20 \text{ SD/mean})^2}{5%}\]

So, in a pilot study, one needs to collect morphometric data from a representative sample (large sample is better in this case) and determine sample number needed (N) for the given level of accuracy using SD and mean from that sample into the above formulas. The same formula can also be used to determine if animal numbers are sufficient. In the case of measuring isolated myocyte length, for instance, we typically observe that sampling 40 myocytes per animal generally falls between N for 1 and 5% sampling error.

A common error is to report data “per microscopic field” without respect for variations that may occur from tissue separation or shrinkage. Some fields may contain larger areas of tissue separation than others. While the goal is to collect all data from beautiful, well-preserved tissue sections with minimal artificial tissue separation, this may not always be feasible. For relative group comparisons, one could collect data based on solid tissue. For instance, fibrosis can be determined from tissue samples by determining the ratio of collagen area to the area of all solid tissues (collagen + myocytes + other cells). This approach will lead to an overestimation of actual volume percentages since all clear spaces are being ignored, but relative comparisons between groups should be valid.

Tissue shrinkage artifact is another potential source of error (8). Before determining isolated myocyte volume by Coulter Channelizer, we fix isolated myocytes in 1.5% glutaraldehyde in 0.07 M phosphate buffer since this fixative preparation does not alter cell volume (8). While some minimal changes in tissue volume can occur with different fixatives and this may vary by tissue type, tissue processing is generally a much larger source of error. For instance, we have noted an ~30% shrinkage of isolated myocyte volume (as assessed by Coulter Channelizer) after hematoxylin-and-eosin processing (unpublished data). This is similar to reports by others (1). If corrections are desired, one can measure tissue section area before and after processing to determine shrinkage. But, if everything is processed in the same manner, group differences are unlikely.

Sections also become compressed by the knife during sectioning. While this may lead to a 20% reduction of individual profile areas in a thin section being used for transmission electron microscopy (TEM), section compression, which occurs in the sectioning direction only, is reduced as section thickness increases. For instance, in one micron plastic sections typically collected before thin sections for TEM, compression is only ~1%. The extent of tissue compression can be determined by comparing measurements from the block face to tissue sections. Section compression artifact is minimal with large thick sections and can essentially be ignored.

One of the most important things to avoid is cutting fresh heart tissue and dropping into fixative at room temperature. There is a pronounced response of fresh heart to cutting injury. Myocytes near the cut surface hypercontract, leading to dense contraction bands and loss of normal sarcomeric structure. Figure 1 shows freshly cut left ventricle from a normal pig, excised and placed into formalin at room temperature. The dense areas at the edge represent contraction bands, although such changes can also be observed in deeper areas of normal heart fixed in this manner. Wavy fibers, a change often reported in early myocardial infarction (MI), can also be seen in normal hearts fixed in this manner.

Reducing warm time is critical after removal of the heart. As with preserving viability of explanted hearts for transplant, quickly cooling myocardial tissue for subsequent structural assessment is critical. Ideally, hearts should be arrested in diastole and coronary vessels quickly perfused with a cold isotonic physiological solution, followed by rapid perfusion with the fixative of choice (e.g., glutaraldehyde for TEM; formalin or paraformaldehyde for immunolabeling). The prewash is important, since fixative coming in contact with blood will quickly form clots and prevent good perfusion fixation. Since fresh frozen tissues are often needed for biochemical assessment, it is often undesirable to perfusion fix the whole...
heart. In this case, injection of KCl (0.2 M KCl in PBS) into the right or left ventricles to arrest in diastole (5), followed by quick extraction and immersion in ice-cold isotonic solution, would be a good approach. After the hearts are adequately cooled, cutting injury should be prevented and tissue pieces can be quickly collected and placed in cold fixative or flash frozen. Figure 2 provides an example of well-preserved tissue labeled for arteriolar LD and CD morphometric measurements from hearts that also provided fresh frozen tissues for mRNA and protein assessment (20).

Many older studies examined structural changes in diseased hearts by TEM using cardiac biopsies. Typically, “pathological” changes in myocyte structure were reported from areas showing hypercontracted myocytes with sarcomere lengths well below the normal physiological range. One needs to realize that myofibrils are tethered at the Z lines by cytoskeletal components running from the sarcolemma (costameres) to the nuclear envelope. This cytoskeletal tethering system also maintains mitochondria in nice orderly rows between myofibrils during contraction. It is easy to visualize how reported “pathological” changes in myocyte cytoplasm observed in hypercontracted myocytes are more likely due to dramatic disruption of cytoskeletal components and rearrangement of organelles (e.g., myofibrillar separation, loss of myofibrils, large spaces filled with untethered mitochondrial or nonspecific cytoplasm). One should be skeptical of changes attributed to pathology when observed in tissue samples fixed under such nonphysiological conditions. More recently, pathologists discovered that biopsies can be placed in BDM to promote relaxation before fixation. As a result, TEMs from BDM-treated human biopsies usually show improved structural preservation.

Many published TEMs of “perfusion”-fixed hearts also show autolytic features, suggesting poor fixation (e.g., myofibrillar disarray, mitochondrial cristae damage and reduced density). Often the authors publish a high-power TEM showing such changes without any verification that the selected site was properly perfused with fixative. Manuscript reviewers should...
raise this issue when purported changes are suspicious. Figure 3 shows myocyte structural changes in a TEM from a patient with dilated cardiomyopathy that was “perfusion” fixed. But, one needs to note the red blood cells clogging the adjacent capillaries (also noted in thick section of this area). In this case, the “pathological” changes are likely due to poor fixation of this area. Figure 4 shows a TEM from a patient with dilated cardiomyopathy that was adequately perfusion fixed. Myocyte structural integrity appears normal. Unless there is clear evidence the tissue was rapidly perfused with fixative or quickly cooled to allow adequate penetration of cold fixative in sufficient time, one needs to be suspicious of changes that may be artifacts.

In my early career, TEM changes in myocardium from hyperthyroid rats were investigated. Previous reports suggested structural changes in hyperthyroidism similar to those shown in Fig. 3. In my experiments, such changes were noted in hyperthyroid rats, but never in properly perfusion-fixed areas. Penetration of fixative also takes time, several millimeters per hour in the case of formalin. Tissue temperature during this diffusion process and more rapid deterioration in stressed pathological samples compared with controls are important variables. Also, one needs to realize that control and pathological samples can be treated exactly the same way with different results. For instance, fresh tissue from hyperthyroid and control rats can be placed into fixative (same protocol) with “pathological” alterations noted in the center of tissue blocks from hyperthyroid animals but not controls. One would expect fixative diffusion time to be more critical in preserving the hypermetabolic tissue from hyperthyroid rats than controls. In summary, one needs to be suspicious of structural changes, particularly at the TEM level, that could be due to fixation artifacts rather than true pathology. Cooling tissues is also critical if there is any delay from diffusion of fixative.

Cardiac Remodeling: Is It Real? Does It Make Sense?

In my opinion, many of the reports of myocyte pathological changes in heart diseases should be viewed with some reservations based on the above-mentioned fixation issues. I worked with Ken Margulies’ heart transplant group several years ago in an effort to optimally perfusion fix failing human hearts. The goal was to determine if myocyte structural changes in heart failure (HF) as reported by others were observed in better preserved samples. Efforts to fix these hearts quickly enough proved to be rather challenging, and a comprehensive paper was never published. But, with more rapid fixation, myocytes from failing hearts were clearly more normal in appearance (Fig. 4). In earlier work with the heart transplant program at the University of South Florida, I noted that high-quality isolated myocytes (e.g., no membrane damage, structurally intact with resting, unloaded sarcomere lengths) from failing hearts were always fully packed with normally distributed myofibrils and rows of intervening mitochondria (7). Myocyte structural changes observed in TEMs by others were never seen in undamaged, high-quality, isolated myocyte preparations. While this issue is not definitively settled, I have never observed severe myocyte intracellular alterations in samples from animals or humans with heart disease that were optimally perfusion fixed. It goes without saying that every effort should be made to optimally fix tissues before quantitation and provide a summary of findings that accurately represents overall observations without misrepresenting the extent of those changes. Finally, one should ask the following question. If drastic and likely lethal myocytes changes are occurring diffusely throughout the heart during this snapshot in time, how does this individual stay alive? Observed changes should be reasonable, and one should make every effort to exclude fixation artifacts as a possible cause. Please note that assessment of fibrosis is generally not a problem since autolytic changes occur much slower in this tissue compartment.

The vast majority of published data on changes in cardiac myocyte shape in heart diseases are incomplete, conducted poorly using crude techniques, and often do not make sense. Typically, myocyte cross-sectional areas (CSAs) are reported. Sometimes the numbers make sense, but in other cases they can vary from known reasonable values to being off by severalfold. In a given study, when control values often differ by 200%, 300%, or more from known norms and there is little evidence of quality control, I find it difficult to believe reported experimental changes, particularly if they are of a much smaller magnitude (e.g., sensitivity of method should be in question). Mean cardiac myocyte CSAs in normal adults ranges from ~180 to 250 μm², with higher values in males (4).
Most reports are from whole tissue sections with little concern for sources of variation. Measurements are affected by differences in contractile phase (remember CSA increases during contraction), variation in sectioning angle, shrinkage or separation artifacts, and poor resolution of myocyte boundaries (8, 9). While oblique sections showing uniformly elongated myocytes are obvious and easily excluded, myocyte transverse shape is highly variable and serves as a poor criterion for judging cross sections. Selecting myocytes only from fields with circular capillaries improves measurement reliability considerably. When optimally preserved, myocytes are tightly packed with boundaries often difficult to discern even in 1-μm plastic sections. Using a cell membrane marker, such as wheat germ agglutinin or anti-laminin antibody, often improves myocyte boundary recognition. While many collect data from only the myocyte nuclear area, this results in overestimation of mean myocyte CSAs. Collection of CSA data from all discernable transversely sectioned myocytes in a sample area can result in mean CSAs that more frequently approach known values. Since the vast amount of myocyte branching occurs between adjacent myocytes at intercalated discs, rather than within a given myocyte, the chance of double sampling from an individual branching myocyte is extremely low. If preparations vary in contractile state, CSA values should be normalized to resting unloading sarcomere length, as indicated earlier with capillary quantitation. In this case, CSA values can be multiplied by observed sarcomere length/1.90 for normalization. For example an observed CSA of 300 μm² in contracted tissue (e.g., 1.50 μm) would result in a normalized value of 237 μm² (1.50/1.90 × 300). In my experience, tissue sections can be obtained relatively accurate and reproducible values for myocyte CSAs if done properly, but this is clearly not the best technique.

I spent considerable time in my early career trying to determine the most accurate method to comprehensively determine myocyte dimensions. I was driven to this goal because such information was critical to fully understand ventricular remodeling and accurate calculation of myocyte number alterations in heart diseases. It was clear that isolated myocytes showed the most promise, primarily because maximum mean cell length could be measured more accurately than in whole tissue sections where maximum boundaries may fall outside the plane of section. Indeed, most histology textbooks still list normal cardiac myocyte length in adults as ~80–100 μm (whole tissue data), whereas it has been clear for several decades that mean myocyte length in adult mammalian hearts normally ranges from ~120 to 140 μm in females and ~130–150 μm in males as assessed by isolated myocytes. Surprisingly, little variation is observed in mean myocyte length from multiple sampling of the same preparations when considering that normal myocytes may range from as low as 40 μm to over 200 μm in a given preparation.

In 1986, we demonstrated a precise correlation between three independent measurements of cardiac myocyte size (9). Importantly, in the two morphometric methods assessing CSAs using TEMs of whole tissues and sections of isolated myocytes, all known sources of error were corrected for the first time (8, 9). The described isolated myocyte approach measuring changes in myocyte volume (Coulter Channelizer), length (microscopically), and CSA (volume/length) was a major technological advance for many reasons. For additional details of this method, see Gerdes and Pingitore (10). This remarkably reproducible approach was many times faster than traditional whole tissue-sectioning methods, provided comprehensive measures (not just CSA), and allowed direct comparisons of all published data regardless of age, species, sex, disease state, etc. With the use of this method, a comprehensive understanding of myocyte shape differences related to physiological growth, pathological hypertrophy, HF, sex, and species was established. Importantly, William Grossman’s theory (11) that pressure overload leads to an increase in CSAs and compensated volume overload leads to a proportional increase in myocyte length and width (translates to ~2/3 CSA and 1/3 width) was finally proven after examining many animal models of cardiac hypertrophy (4). Our theory that myocyte length alone is largely responsible for the increase in chamber diameter-to-wall thickness ratio observed in systolic HF was also proven in animals and humans (4). It was convincingly shown that increased myocyte lengthening alone accounted for the increased chamber circumference over the life span of female spontaneously hypertensive HF rats progressing to dilated HF (17). It should be noted here that we correctly identified, without exception, prior history of hypertension in humans with ischemic cardiomyopathy by noting a larger myocyte CSA in addition to cell lengthening. Nonetheless, when significant myocyte necrosis and replacement fibrosis occur, chamber dilatation can be a mixture of myocyte lengthening and fibrotic segment expansion (12). The important thing to remember is that anatomical chamber remodeling is largely reflected by parallel changes in myocyte shape. Myocyte lengthening is associated with dilatation, and wall thickening is associated with increased myocyte CSA. Unfortunately, the clear understanding of myocyte remodeling patterns described above has been muddied by a large amount of published, low-quality data.

One of the most suspicious and commonly reported remodeling changes is an increase in myocyte CSA after MI in rodents. A typical study often shows a large increase in myocyte CSA in the noninfarct zone of wild-type mice after MI, with prevention or attenuation of this change in gene-targeted or drug-treated mice. It is clear from human and animal data that little, if any, change in wall thickness occurs in the spared myocardium (e.g., posterior wall with left anterior descending coronary artery ligation) after an MI. But, over time the spared segment lengthens. Lengthening with minimal change in wall thickness of the spared segment is clearly due to an increase in myocyte length only with no change in CSA (2, 7, 21). Remodeling of myocyte shape is consistent with anatomical findings. So, if a given mouse study shows that targeting gene “x” prevents the “doubling” of myocyte CSA post-MI, one should be highly suspicious of this finding, particularly if posterior wall thickness is unchanged (typically the case). Of course, if cell shape and echocardiographic data are conflicting, suspicions about data quality are reasonable.

Summary

Careful consideration of the issues discussed should lead to improved quality of cardiac remodeling data. To summarize basic points:

1) Rapid fixation, preferably in diastole, is critical to preserve cardiac morphology.
2) Cooling heart tissue is important to limit autolytic damage before fixation occurs.
3) Avoid cutting injury to fresh myocardium if structural data are to be collected.
4) Is the morphologic/morphometric approach adequate? Have potential technical sources of error been considered in data collection?
5) Do results make sense? Do anatomical/echocardiographic data correlate with myocyte remodeling data?

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AUTHOR CONTRIBUTIONS
A.M.G. conception and design of research; A.M.G. performed experiments; A.M.G. analyzed data; A.M.G. interpreted results of experiments; A.M.G. drafted manuscript; A.M.G. revised manuscript; A.M.G. approved final version of manuscript.

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