

Measuring Sarcomere Length

Overview

What does sarcomere length represent

The elementary unit of contraction in striated muscle, cardiac or skeletal, is the sarcomere. To illustrate the organization of the sarcomere, a diagram of its structure plus 2 micrographs are shown below (fig.1). The sarcomere is defined on both ends by the Z-band in which the thin filaments are anchored, with the thick filaments in between. The optical properties of the thick and thin filament are strikingly different. The region with only thin filaments is almost transparent and is labeled the Isotropic, or I-band. The thick filaments are optically dense, resulting in a dark appearance and are referred to as the Anisotropic, or A-band. Due to the repetition of sarcomeres in the muscle, the dark A-bands and light I-band alternations result in a striated appearance. Under the light microscope, one sarcomere length would thus be the distance from the middle of one light band to the middle of the next. This cannot be measured very accurately since one sarcomere is only 1.5 to 4 microns long (depending on muscle type and degree of stretch). Alternative methods are required to acquire accurate length estimates for a range of sarcomere lengths.

Measuring sarcomere frequency

The approach used by our software is to estimate the frequency of the striation pattern, i.e. how many sarcomeres can be found per μm . Although no knowledge of the frequency analysis techniques used is required to be able to use the software, it is good to know the principles behind the measurements to be able to understand which images give good signals and which will not. Below follows some background information along with some techniques to optimize the measurement.

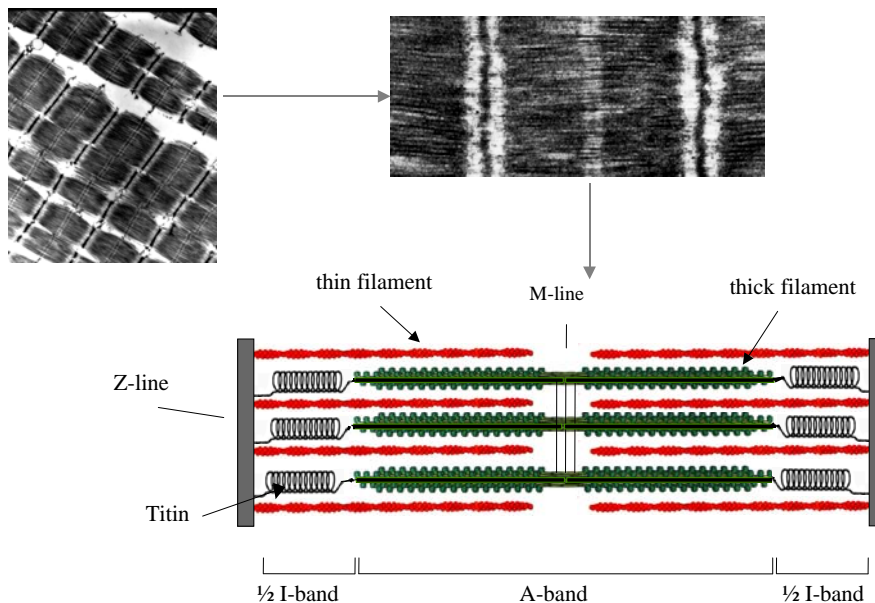


Figure 1. The top two pictures are electron-microscopy micrographs from cardiac muscle, showing the sarcomere appearance. The lower picture gives a schematic of the ultrastructure of the myofilaments in one sarcomere. This structure underlies the striated appearance of muscle (pictures courtesy of Drs. Granzier and Trombitas).

Making the measurement

Step 1 – Initiate a new experiment

Under the **File menu** select **New**. If an experiment employing the SarLen recording task is included in the experiment parameters, the following *Sarcomere Length Status Bar* will appear at the bottom of the screen (fig. 2). With this *Sarcomere Length Status Bar* the user will be able to optimize image quality for sarcomere length measurements.

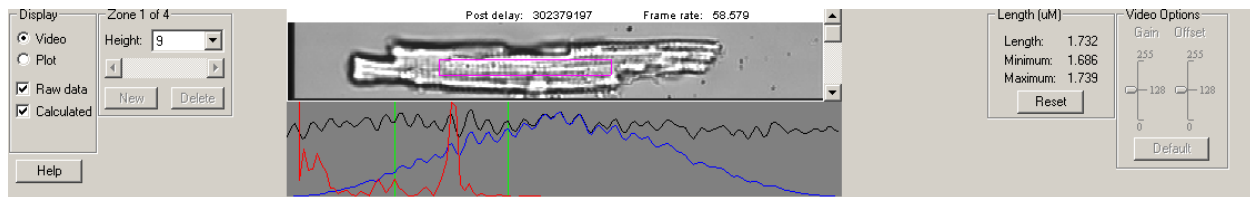


Figure 2. Sarcomere Length Status Bar

Step 2 – Position the muscle in the video display

In the above example, the video display is a quarter of a full video image from the analog MyoCam to generate sampling rates of 240Hz. When the image is captured at high sampling rates, fewer lines are recorded. With the MyoCam-S, frame rates of 250Hz will dictate a field of 91 lines (with each line being 774 pixels across for an array of 774x91 pixels). This restricts the camera's field of view to a small fraction of the field of view through the microscope's eyepieces. The viewing window will display approximately 80 to 90 lines. When more lines are present than can be displayed in the *Sarcomere Length Status Bar*, you can use the "position" slider to the right of the video display to scroll through the field of view.

Step 3 – Alignment of the preparation

To get proper sarcomere length measurements, it is important to align the striation pattern vertically within the field of view. Since the striation is transverse, this usually means having the muscle cell or fiber parallel with the field of view. Improper alignment will lead to an underestimation of the true sarcomere length. Don't worry too much about this: the amount of error due to improper alignment is equal to the cosine of the angle between vertical and the actual position of the sarcomere. So being off by 5 degrees results in less than a one percent error. A visual estimate is sufficient for proper alignment.

Step 4 – Identify the region of interest from which the sarcomere length will be measured.

The sarcomere length will be measured from a user-defined region-of-interest (ROI). The ROI is the square enclosed by the magenta box in the video display. Using the mouse, the box can be moved and the width and height can be adjusted. The height of the box in number of video lines is indicated in the top-left corner of the video status bar. Include at least 7 sarcomeres in the ROI to maximize the accuracy of the measurements. The estimated sarcomere length in your ROI is calculated and updated continuously and the value is displayed on the right-hand side of the status-bar.

Steps 5 through 8 describe the techniques the software uses to calculate sarcomere length from the ROI. These are included to provide the background necessary to recognize the contribution of the alignment, signal quality and intensity, ROI placement and thresholding to optimize data quality.

Step 5 - Obtaining a density trace from the image

All the calculations will be performed on a density trace derived from the ROI. Each pixel in the image is assigned a value to indicate its brightness, from 0, black, to 256, white. Plotting the pixel values from a horizontal line in the image gives the density trace. The density trace is plotted as a black line in the line graph when “video” and “raw data” are selected from the options box of the display. Due to the alternating dark and light A- and I-bands, the density trace has a sinusoidal appearance with the wavelength of the sine representing the sarcomere length. The density trace will be used to calculate the average frequency of the sine wave which can then be converted to wave length (sarcomere length).

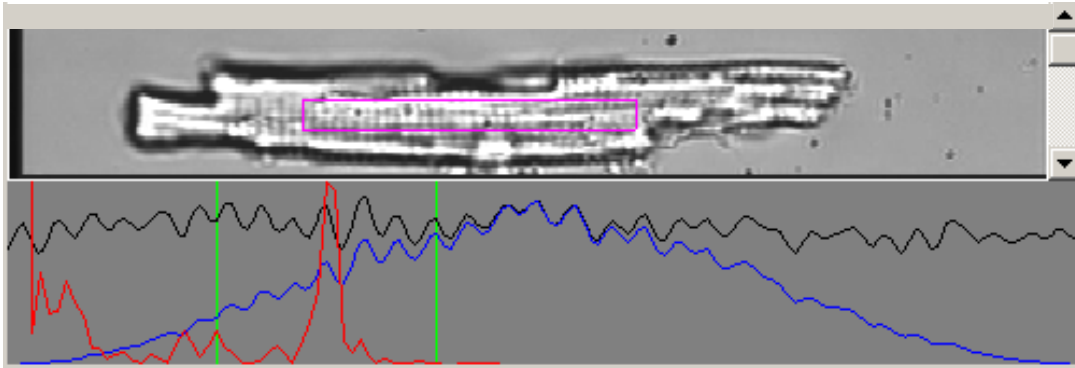


Figure 3. The SarLen video image (top) and density trace (bottom, black). The Hamming window (bottom, blue), FFT power spectrum (bottom, red) and power spectrum thresholds (bottom, green) are also displayed.

Step 6 - Applying a Hamming window to the density trace

A periodic signal (a signal that regularly repeats itself in space or time) can be dissected into a series of sine waves with differing amplitude and frequency. The frequency can be evaluated by a mathematical analysis called a *Fourier transform*. One of the conditions for a Fourier transform is that the periodic signal is infinite. This is obviously not the case with the density trace that consists of a limited number of pixel values. We can make it appear infinite from a mathematical point of view however. This is done by multiplying the density trace with a cosine function which makes both ends of the trace approach zero. The cosine function used here is called a Hamming window. It serves to both repeat the periodic signal and minimize boundary discontinuities (see [SarLen & FFTs](#) for more information).

As a shortcoming of applying the Hamming window, sarcomeres in the middle of the ROI will weigh more heavily into the estimated sarcomere length than the ones at the edge of the ROI, since the pixel values in the middle of the ROI are multiplied by a much higher number. To give a sense of the relative contribution of the different parts of the ROI, the Hamming window as applied to the density trace is plotted in blue in the line graph (fig. 3).

Step 7 - Zero padding

The fast Fourier transform analysis requires that the number of points upon which it is performed is a power of two. Since the ROI and the related density trace have an arbitrary number of points, the computer adds zero values until the next power of two (e.g. a density trace with 82 points will be zero-padded to 128 points).

Step 8 - Calculating the power spectrum

The FFT transforms the density trace from the spatial domain to the frequency domain. Its output is in the form of the power spectrum. The power spectrum is usually represented as a graph in which relative

contribution of each frequency to the density trace is plotted (see figure 3). Peaks at low frequencies correspond to the slow change from dark to light in the image. The sharp peak at intermediate frequency represents the most dominant pattern in the density trace, which are the dark-light transitions of the sarcomeres. Having a sharp peak in the power spectrum provides direct visual feedback and allows the user to determine if a good measurement will be made.

Step 9 - Finding the peak in the power spectrum

The average sarcomere length is derived from the peak of the power spectrum. The peak detection is a multi-step, interactive process. First the user has to limit the values the peak can assume. If there is for example a dark/light transition in the image, this will result in a strong low frequency component of the power spectrum, with a peak that is possibly higher than that of the sarcomere length. The program allows the user to exclude such values by putting in a lower and upper limit for sarcomere length. The upper and lower boundaries are set manually by moving the two vertical green lines in the line graph. The values of the upper and lower limit are displayed in the length box of the status bar (fig. 3).

The software will look for the maximum value of the power spectrum between the upper and lower limit. Once the maximum is found, it uses the surrounding point to fit the peak. The peak of the fitted curve is stored as the “sarcomere frequency”. The advantage of this method that it gives the program sub-pixel resolution.

Step 10 - Converting the frequency to sarcomere length

The sarcomere length found from the power spectrum will be in “sarcomeres per pixel”. The software automatically converts this to pixels per sarcomere. In order to get the appropriate value in μm a conversion factor has to be provided by the user and stored in the software. There are many techniques to perform the spatial calibration. SarLen in versions of IonWizard 6.1 and newer include a video calibration dialogue, older versions can be calibrated using the SoftEdge cell length or [SarLen](#) recording tasks.

See [SarLen Algorithm](#) for further more information regarding the fast Fourier transform.

Optimizing Sarcomere Length Signal Quality

Overview

The sarcomere length estimates by the software can only be as good as the material it is provided with. Sarcomere length signal quality is dependent upon the video image quality and the region of interest. Techniques available within the IonOptix system for optimizing the video image quality and tips for choosing the proper region of interest are discussed below.

Brief definitions of nomenclature

Throughout the procedure of optimizing the image quality, the user should become familiar and comfortable with the following nomenclature:

Gain: the amplification of video signal reported by the camera.

Offset or Black level: the DC offset of video signal reported by the camera.

ROI: region-of-interest, the user-defined part of the image upon which the analysis will be performed.

Adjusting the video gain and offset to optimize image contrast

A number of steps are involved to get from your specimen to a sarcomere length measurement. Each step will add noise to the signal. The number one priority is thus to start out as clean as possible and that is by setting up the microscope properly. Several illumination modes are available to image your specimen. For muscle specimens, cardiac or skeletal, phase-contrast or contrast modulation optics will give the best possible contrast. When your objective doesn't support such contrast enhancing techniques, and most of the commonly used oil-immersion fluorescence objectives do not, properly set up Kohler or brightfield illumination will provide sufficient resolution and contrast to observe a striation pattern with the camera.

Once the microscope is set up properly, the best place for contrast enhancement is the camera control. The IonOptix MyoCam and MyoCam-S provide gain and black level control. Increasing the gain will provide greater signal amplitude, while the offset will simply move the density trace up or down. All things being equal, the best results will come from a sine wave with a large (but not saturating) amplitude. If you're using the analog MyoCam camera, both the video power supply and the frame grabber card in the computer have gain and offset controls. The video power supply has controls on its front panel, the frame grabber's can be operated digitally from within the SarcLen status bar (fig. 4). If you're using the digital MyoCam-S, the gain and offset can be operated from the SarcLen status bar as well.

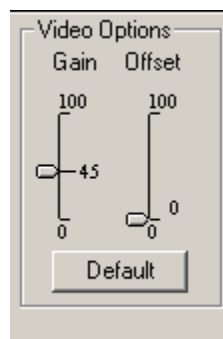


Figure 4. Video Controls found in the SarcLen status bar (the gain scale is dictated by either the frame grabber or camera present).

Choosing the region of interest

There are two important factors to consider when choosing the ROI. The most obvious one is of course that the region is encompassing the sarcomeres you are interested in. Second, that the ROI results in a good density trace for the FFT. Following are a few guidelines about how to take care of the second part:

Length of the ROI

The software can come up with a reasonable sarcomere length estimate for as little as 4 sarcomeres. For an unbiased estimate however, try to include at least 7 sarcomeres. Be aware that, as explained above, the sarcomeres in the middle of your ROI carry more weight in the sarcomere length calculation than the peripheral ones. The algorithm will perform best with more sarcomeres, assuming that the sarcomeres within the region are uniform. The number of sarcomeres chosen must be balanced against their regularity and uniformity.

Height of the ROI

The height of the ROI is also important: the more lines that are averaged, the better the signal to noise ratio in the density trace. Conversely, when the cell is not well aligned, the striations will extinguish themselves. The result can be a loss in signal as shown by the disappearance of a clear peak in the power spectrum, or big spikes in your length data.

For further illustration, figures 5 through 7 (recorded using an earlier version of SarLen) show a few specific cases:

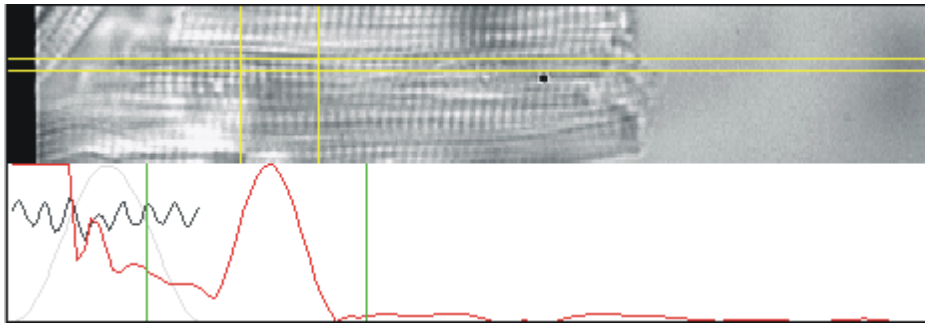


Figure 5. The SarLen algorithm makes accurate and unbiased length measurements down to as little as 7 sarcomeres.

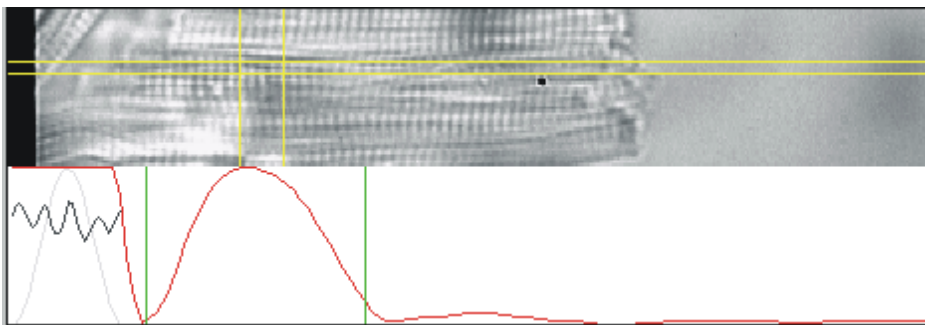


Figure 6. Fewer sarcomeres can be measured but the method loses accuracy, as evidenced by a broad power spectrum.

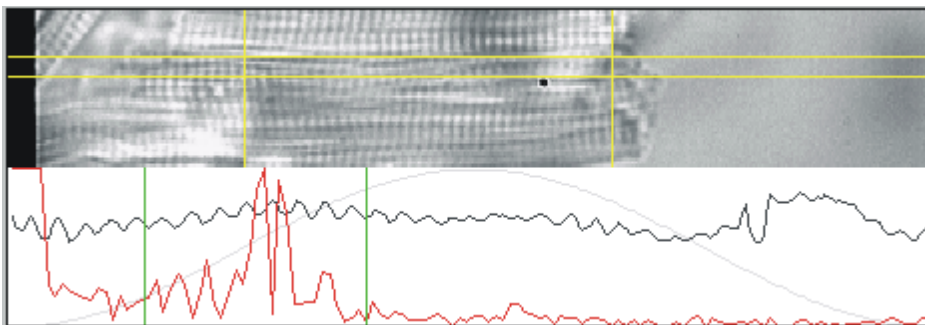


Figure 7. Two peaks usually indicate that the ROI contains 2 populations of sarcomeres. In this case the ROI is on the border of two different cells

Acknowledgements

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