DMT NORMALIZATION GUIDE, VOL.2.0

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CHAPTER 1 - INTRODUCTION

The purpose of this document is to:

- Introduce the reader to the concept of length/tension relationships and vessel Normalization.
- Familiarize the reader with the terms commonly used in myography-based research
- Explain the importance and mathematical principles of vessel wall tension/transmural pressure and length/tension relationships
- Provide detailed instructions on how to determine optimal baseline experimental conditions for vessel function studies using the DMT Normalization Module
- Point out critical methodological aspects that affect data quality and reproducibility
- While it is ideal to first grasp all the concepts related to Vessel Normalization, those wishing to expedite their work are invited to view step-by-step instructions for determining Normalization parameters using AD Instruments' LabChart® DMT Normalization Module*[1] by skipping to Chapter 5, "Vessel Normalization Methods: The DMT Normalization Module"

1 **The DMT Normalization Module was developed jointly by DMT and their business partner, AD Instruments.*

CHAPTER 2 - NORMALIZATION/WIRE MYOGRAPHY NOMENCIATURE

Below are some of the basic terms commonly used in vessel function literature and methodology related to wire myography.

- **Length/Tension Relationships:** A characteristic of myosin and actin filaments whereby the amount of stretch applied to the muscle influences the extent of the actin-myosin interaction and hence active tension that can be achieved; determining the amount of applied stretch needed for maximal active force is required for proper myography studies
- **Normalization:** An aspect of length/tension analyses in which a vessel is stretched to an internal circumference that reproduces the wall force exerted on the vessel at a suitable resting transmural pressure, usually 100 mm Hg for small resistance vessels
- **Transmural pressure, ΔP:** The difference in pressure between the two sides of a vessel wall (ie, internal – external wall pressure). Units are often given as mm Hg or kPa. The standard target transmural pressure used for small resistance arteries is 100 mm Hg, or 13.3 kP.
-

$$\textbf{kPa} = kN/m^2 = mN/mm^2$$

- **Effective pressure, Pi:** An estimate of the pressure necessary to expand the vessel to the measured internal circumference

$$\textbf{Pi} = \text{Wall tension}/(\text{internal circumference}/(2 \cdot \pi))$$

- **Wall tension (developed force):** The force exhibited by a vessel wall; this value is a sum of passive and active wall tension normally may be expressed in mN OR mN/mm units
- **mN/mm:** Force normalizes the data per vessel length
- **Passive wall tension:** The force exerted by a vessel wall in the absence of smooth muscle activation
- **Active wall tension:** The force exerted by a vessel wall during smooth muscle contraction excluding passive force contribution
- **Isobar:** A line on a length/tension graph indicating points of pressure corresponding to a pre-determined value (see Figure 4 for an example)
- **IC:** Internal circumference normally expressed in μm units
- **IC_{100}:** The internal vessel circumference that corresponds to the target transmural pressure, ie, the circumference of the vessel when it reaches the optimal transmural pressure (eg, 100 mm Hg). This value is determined under passive conditions for a brief tutorial on how an IC_{100} value is determined,

please see Chapter 5, “Vessel Normalization Methods: The DMT Normalization Module”

- **IC_1:** The internal vessel circumference at which the active force production of the vessel is maximal (ie, actin-myosin interactions are optimal); this is the ideal starting circumference for the vessel being studied
- **IC_1/IC_{100} Ratio:** Also known as the Normalization Factor (Norm Factor), this ratio depicts the relationship between the internal circumference at which the active force production of the vessel is maximal and the internal circumference at which the vessel experiences optimal transmural wall pressure (here 100 mmHg) for a given vessel. This value should be determined for the specific vessel being studied! Some studies refer to the IC_1/IC_{100} ratio as “factor k”. For a brief tutorial on how the Norm Factor (IC_1/IC_{100} ratio) can be determined for a specific vessel type, please see Sections 5.2 and 5.3.

CHAPTER 3 - CONCEPTS RELATED TO WIRE MYOGRAPHY AND NORMALIZATION

3.1 BASIC CONCEPTS OF WIRE MYOGRAPHY

Myography is the study of muscle function and applies to all muscle types – skeletal, cardiac, and smooth muscle. The functional aspects being analyzed in myography are the muscle's capacity to constrict or relax in response to action potentials, electrical stimulation, changes in ion concentrations (eg, potassium), agonists (eg, norepinephrine), antagonists, and, in the case of vessels, pressure changes. At the core of these functional analyses is the muscle length/tension relationship, a concept characterizing the interaction between the myosin and actin filaments that is necessary for muscle contraction. The length/tension concept, described in Section 3.4, indicates that the actin-myosin interface is optimal when the vessel is stretched to an extent wherein the two layers have maximal contact but with enough of a baseline stretch to provide room to contract. Normalization – which is an aspect of length/tension relationship – is the act of establishing a baseline, resting tension that maximizes actin-myosin interactions for optimal contractile response.

While all muscle types can be evaluated using some form of myography, the focus of this tutorial is on the smooth muscle found in the arterial vessel wall. This muscle type serves an important function in the vasculature, especially in resistance arteries where this vascular bed is largely responsible for blood pressure control and can be a major source of vascular dysfunction. Note that larger conduit vessels (eg, aorta) are not good candidates for the Normalization approach described in Section 5.2 due in part to their thick muscular walls, whereas other large arteries (eg femoral, carotid) have been successfully subjected to this process. The majority of vessels studied with this Normalization process are resistance arteries.

Of the myography techniques established for the study of vessel function, one of the most widely used is wire myography. A basic step in wire myography is to artificially mimic a vessel's physiologic transmural pressure and thus the wall tension experienced by the vessel at that pressure. This is accomplished by feeding two wires through the vessel lumen; one wire is affixed to a sensitive force transducer to measure changes in wall tension and the other is attached to a micropositioner that allows for precise control of the distance between the two wires. Section 3.5 explains how the wall tension (force) and wire gap – which is used to calculate the internal circumference (IC) – are related. Because experiments are conducted at a set IC this is considered an isometric method.

3.2 NORMALIZATION DEFINED

As noted above, vessel Normalization is the act of pre-stretching an intact vessel segment to an IC that produces a suitable physiologic resting transmural pressure for the experimental conditions being tested (ie, it mimics the natural in vivo state of the vessel in terms of pressure and circumference). It is a specific type of muscle length/tension analysis (Section 3.5). In short, this is a means of standardizing the vessel circumference in order to yield maximal responses to vasoactive agents and provide reproducible results.

For most small resistance vessels, a baseline (ie, relaxed) transmural pressure of 100 mm Hg is sought. The internal circumference at which this pressure is achieved is referred to as the $IC_{100.}$ Determining the IC_{100} requires the incremental measurement of force generated by the vessel wall at several vessel circumferences - from each force measurement taken at a known circumference the corresponding transmural pressure is calculated using the Law of La Place, which is covered in Section 3.5.

The IC_{100} that is determined, however, may not produce the optimal pre-tension to be applied to the vessel; as noted previously, the reason for this is related to how actin and myosin interact. Instead, the baseline pre-tension force required for optimal response is achieved at the IC_1 distance. This can be determined in a few ways, including by multiplying the IC_{100} with a Normalization Factor, ie, $IC_{100} \times IC_1/IC_{100}$. This ratio must be determined experimentally for the each specific vessel type and species. [While it is common practice to use a published Normalization Factor value, it is recommended that each laboratory establish this value for their own studies (see Sections 5.2 and 5.3).] After applying this correction to the IC_{100}, the actual circumference applied to the vessel can be less (eg, as with some mesenteric vessels) or greater (eg, as with some femoral arteries) than the IC_{100} values determined for that vessel.

3.3 RATIONALE FOR VESSEL NORMALIZATION

As with all experimental protocols, setting consistent baseline condition is necessary to obtain reproducible results. Normalization is the process of standardizing the baseline experimental conditions for vessel function measurements assessed via myography.

The initial, passive conditions at which the vessels are set represent a major potential source of error and therefore need to be consistent from sample to sample. There are numerous reasons for this. First, as noted above, by using the same initial resting tension for each vessel, reproducible results can be obtained. Setting the resting tension to a pre-determined value also optimizes the reactivity of the vessel in response to vasoactive agents, a concept explained, in part, by the active length/tension relationship (see Section 3.5 below). Finally, it is advisable to evaluate the viability and structural integrity of the tissue before commencing with experimentation. Each of these factors are addressed by normalizing the vessel baseline tension.

In summary, Normalization is important because:

- It standardizes baseline experimental conditions allowing for direct comparison of vessels
- It optimizes vessel response
- The sensitivity of the vessel to agonists is stretch dependent
- The vessel's active response is stretch dependent
- It allows the user to assess the viability of the tissue

3.4THE NORMALIZATION FACTOR AND THE ACTIN-MYOSIN INTERFACE

While it may seem intuitive to simply set the resting tension at the IC_{100} value, the basic physiological properties of smooth muscle may require an adjustment. The rationale for adjusting the baseline resting tension, as noted previously, is that vessels produce maximal active response in accordance with the actin-myosin interactions for the specific vessel type, and these interactions may not be optimal at the $IC_{100.}$ The actin-myosin interface is optimal when the vessel is stretched to an extent where the two layers have maximal contact but with enough of a baseline stretch to provide room to contract. However if not enough stretch is applied the opportunity for contraction is reduced, and conversely too great a stretch both damages the tissue and diminishes the actin-myosin interaction. These concepts are illustrated by the figures below.

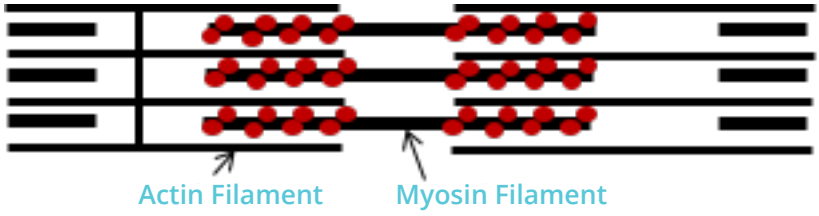


Figure 1. Ideal baseline stretch. This example shows a sarcomere pre-stretched to allow for a maximal constriction, but not so far as to compromise myosin/actin interactions. The stretch distance applied in this situation is equal to IC_1. This represents the ideal baseline condition for experimentation.

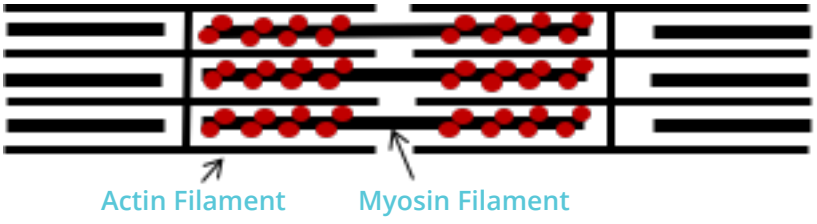


Figure 2. Suboptimal baseline stretch. In the above example there is a lot of myosin/ actin interaction, however without any baseline stretch there is no room for the muscle fibers to contract. This is a sub maximally stretched condition.

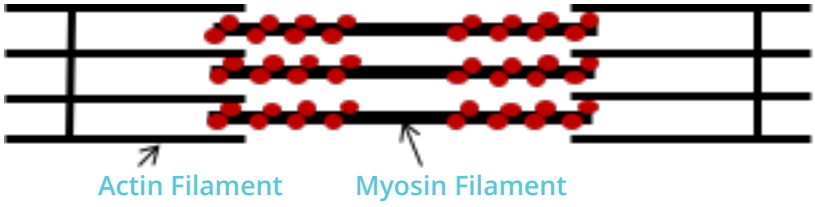


Figure 3. Excessive baseline stretch. In the above example, the muscle is stretched too far, limiting myosin/actin interactions. In this case the muscle will be unable to exert a maximal vasoconstrictive response due to this poor amount of interaction. Damage (tearing) may also occur as a result of the excess tension.

As the prior three figures illustrate, the extent of baseline stretch determines the degree of actin-myosin interaction. In order to develop a consistent, maximal constrictive response it is important to maximize these actin-myosin interactions while allowing room for further constriction.

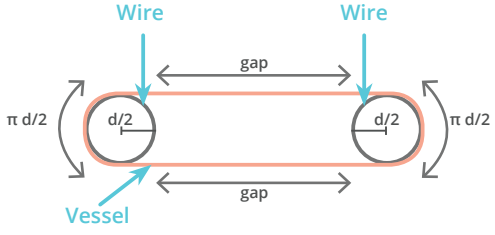
3.5 RELATIONSHIP BETWEEN INTERNAL CIRCUMFERENCE AND VESSEL WALL TENSION

The relationship between vessel wall length (ie, internal circumference, usually in mm units), vessel wall tension (usually presented as mN/mm), and the effective pressure (Pi) is described mathematically by **La Place's Law**:

Pi = Wall tension/(internal circumference/(2 × π))

Base on the above formula, it becomes apparent that as the IC increases the transmural pressure decreases. Conversely, under isometric conditions (ie, where the IC is maintained constant), as would be used while conducting myographic experiments, an increase in wall tension would result in an increase in transmural pressure, and vice versa.

When determining IC values, recall that in wire myography the vessel is stretched between two wires of known diameter positioned at a known distance from each other, represented by the "gap" in the figure below, which is manipulating by adjusting a micrometer (or "micropositioner"). This set-up, when looking through the mounted vessel lumen, looks conceptually like this:



REMINDER: As the above suggests, raw micrometer readings do NOT reflect the diameter of the vessel; CHANGES in readings, from a zero-stretch baseline, provide the gap distance.

The internal circumference (IC) may therefore be calculated with the following formula:

IC = 2 (πd/2) + 4 (d/2) + 2 x gap
= πd + 2d + 2 x gap
= d(π + 2) + 2 x gap

If d = 40µm (steel wire)

IC = 205,6µm + 2xgap

When the wires are just touching, the gap = 0 and, in the example above, IC_0 = 205.6 µm. If the micrometer is moved 50 µm, then the gap = 50 µm, and this can then be entered into the above equation to determine the IC at each stretch. For example, if the starting micrometer reading is 1550 µm, and the micrometer is then moved to 1600 µm, the difference is 50 µm, and this is your gap. If the next stretch is also 50 µm (1600 µm to 1650 µm), the new gap is 100 µm.

3.6 CONTRIBUTIONS OF PASSIVE AND ACTIVE STRETCH TO MUSCLE LENGTH/ TENSION RELATIONSHIPS

As noted, the circumference of a vessel is related to wall tension through the **Law of La Place:**

Pi = Wall tension/(internal circumference/(2 × π))

with wall tension receiving contributions from the structural aspects of the vessel wall (passive stretch) and the muscle contraction contributions of the smooth muscle (active stretch). In the absence of agonist, only passive tension contributes to the relationship; this is the vessels' state when normalizing the vessel with a known IC1/IC100. In the presence of an agonist (eg, potassium ions, noradrenaline, phenylephrine, etc.), the smooth muscle contributes a tension force the magnitude of which is influenced by the baseline stretch, as described in the prior section. The individual contributions of passive and active stretch are shown on the length/tension graph below: This set-up, when looking through the mounted vessel lumen, looks conceptually like this:

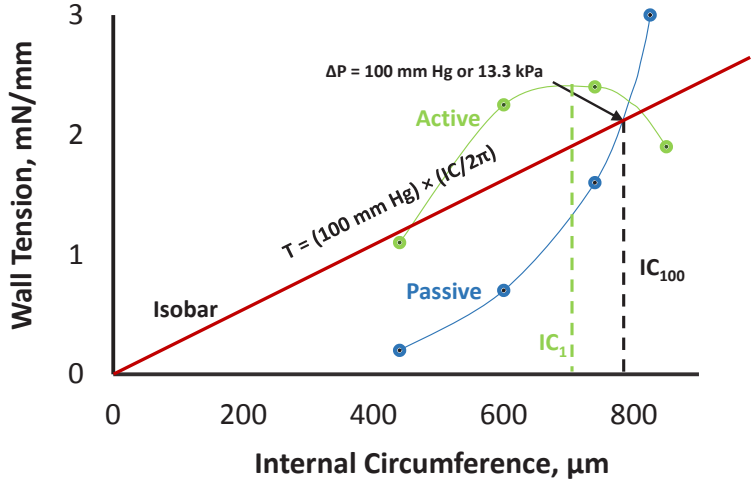


Figure 4. Length/tension graph. The above plot represents a typical length/tension graph with the individual contributions to the total length/tension plot broken out separately. (The components of this graph will be described in detail in Chapter 4.) The passive stretch line (blue) reflects the tension exhibited by a vessel when increasing stretch is applied using the myograph micrometer (ie, in the absence of agonist). This plotted line is representative of the data obtained during the Normalization procedure which, as noted previously, is performed to find the IC_{100} value (ie, the circumference at which the vessel achieves a wall tension consistent with a transmural pressure of 100 mm Hg). The active stretch line (green) represents the increase in tension beyond the passive stretch (ie, only force contributions from muscle contraction). This force is generated in vessels administered a smooth muscle agonist (vasoconstrictor). Not shown is the plot with both active and passive stretch; this plot is what would be obtained when exposing the vessel to agonist. The goal is to pre-stretch the vessel to the circumference that produces the maximum agonist response; this value is represented by IC_1 (dashed green line). From the above data, one can derive the IC_1/IC_{100} (Normalization Factor) which is consistent among vessels of the same type and species (see Sections 5.2 and 5.3).

CHAPTER 4 - LENGTH/TENSION RELATIONSHIP GRAPHS DECONSTRUCTED

In Chapter 3 we summarized the basic physiological mechanisms behind the length/tension relationships exhibited by muscle with the main focus being on actin-myosin interactions and how these play a role in physiologic and pharmacological aspects of muscle reactivity. Here we will provide a succinct overview of how these mechanisms are assessed by describing the contributions of passive and active tension to the primary graphical representation of these phenomena, the muscle length/tension plot.

4.1 PASSIVE TENSION GRAPHICAL CONCEPTS

Following the basic, agonist-free steps described in Chapter 5 of this tutorial produces a passive length/tension graph like the example below. The LabChart Normalization Module will read "Resting Wall Tension" on the Y-axis label.

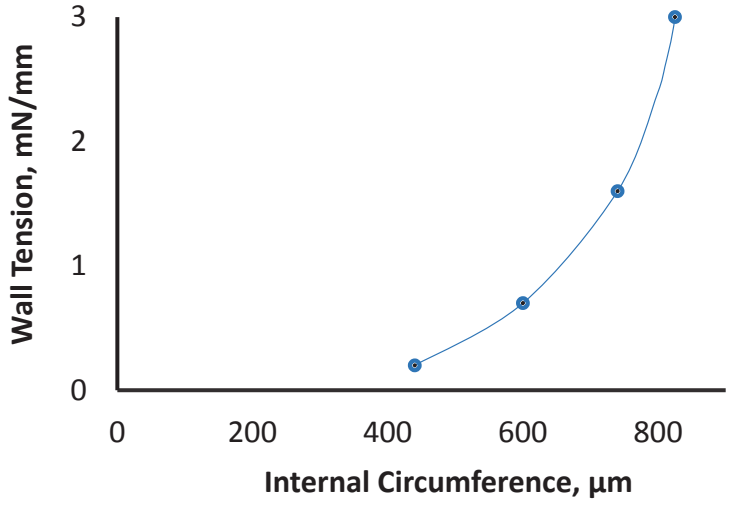


Figure 5. In the above plot, symbols represent data points obtained by the user and subsequently used by the software to produce the length/tension relationship trace. Note: the values in the above graph are arbitrary and are for conceptual illustration purposes only.

The DMT Normalization Module software adds to this plot a red isobar line labeled "Target Pressure"; in the representation below (Figure 6) the label also displays the formula for this line. Also shown in Figure 6 below is the pre-selected "IC_{100}"value (dashed black line).

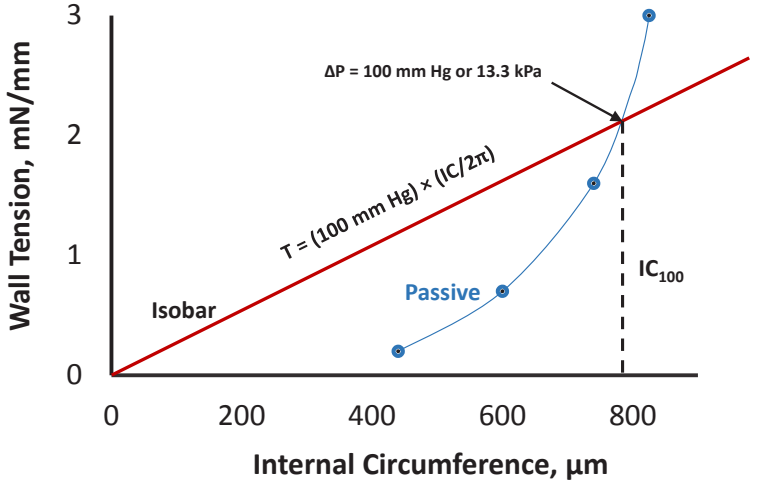


Figure 6. Example of a passive tension curve generated in the DMT Normalization Module with the IC_{100} value indicated on the graph. Assuming that the transmural pressure (Δp) selected is 100 mm Hg (13.3 kPa), the software will use the data to calculate the isobar formula (ie, Target Pressure, red in the above figure) corresponding to the line T = (100 mmHg) x (IC/2π). In LabChart the preselected value is displayed in kPa units. The dashed black line represents the calculated circumference for a transmural pressure of 100 mm Hg, ie, the IC_{100} value.

As described in Chapter 5, an IC_1/IC_{100} value is input at the start of the Normalization procedure. Once enough points have been added to the plot the module will use this information to extract an IC_1 value; graphically, the program indicates this by addition of a dashed vertical line labeled "IC_1", as shown in Figure 7.

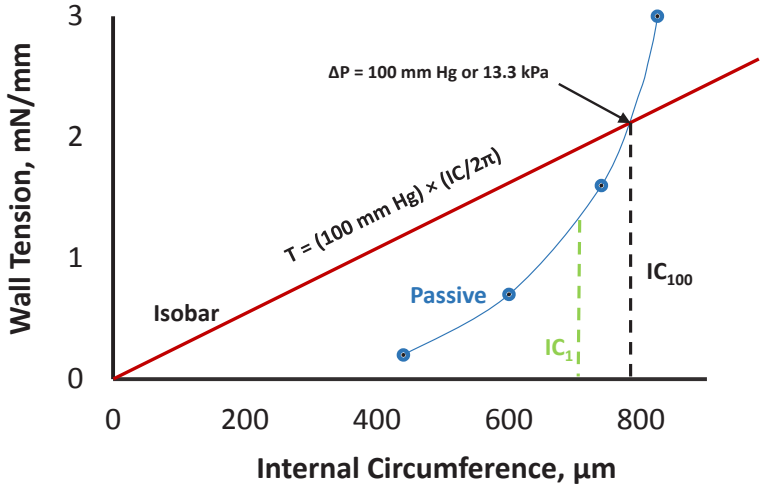


Figure 7. The red isobar line, as noted previously, is represented by the line equation used by the software to determine the IC_1 – or normalized lumen internal circumference (green dashed line) – for the given Normalization Factor (IC1/IC100).

4.2 ACTIVE TENSION GRAPHICAL CONCEPTS

The IC_1 value is the internal circumference of the stretched vessel at which the vessel produces the optimal active response. This is illustrated in Figure 8 (in contrast to the passive stretch, as shown in Figure 7). An active tension graph is not generated by raw data; the data obtained from consecutive agonist-induced contractions at different IC´s (stretches) reflects the sum of the contributions from passive and active tension. An active tension graph is produced by subtracting the passive tension data from the data obtained when agonist is present at each stretch. Below is an example of how such an active force length/tension graph might appear. The red circle indicates the range over which maximal actin-myosin interactions – and hence active response – occurs. The IC_1 should be selected from this range.

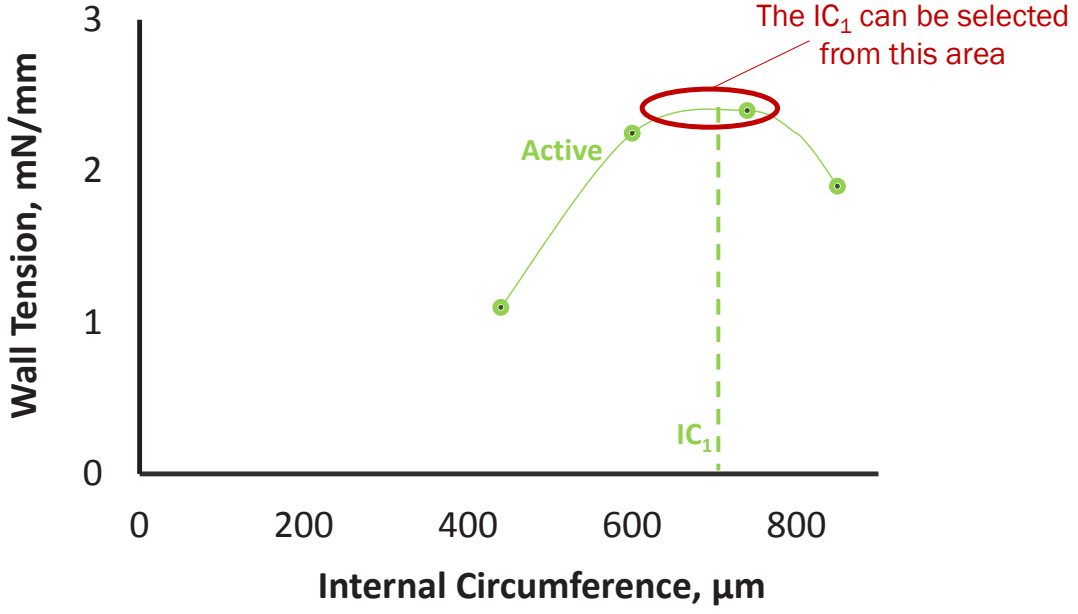


Figure 8. Example of an active tension curve generated with the IC_1 value indicated on the graph. The IC_1 value is the internal circumference that correlates with optimal stimulated contractions. The red circle indicates the range over which an acceptable IC_1 value can be selected. (Note these are arbitrary values and not values you would expect during an actual experiment. These values are only to illustrate the concept of how the IC_1 value selected).

When both passive and active plots are overlapped the result is Figure 9.

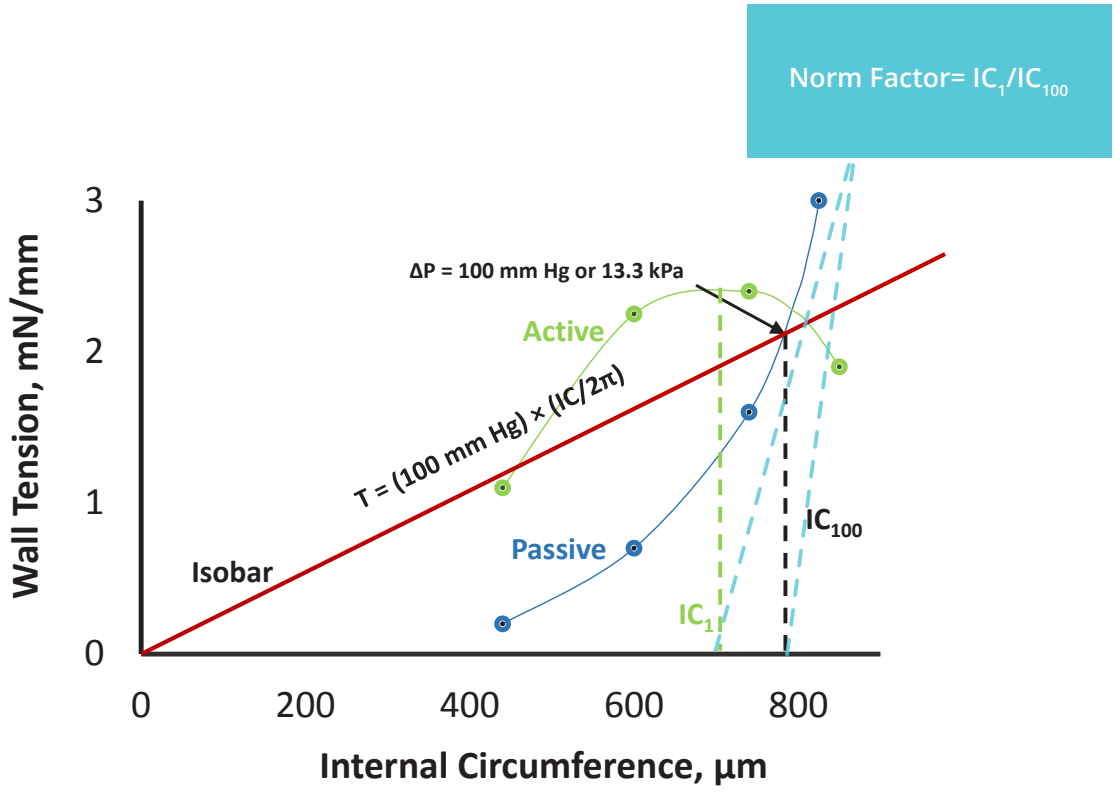


Figure 9. Example of passive and active tension plots with the IC_1 and IC_{100} values indicated. The IC1/IC100 ratio is the calculated ratio of the internal circumference derived from the active response curve divided by the internal circumference derived from the passive tension curve. Note that the values above are arbitrary (they are for conceptual illustration purposes only).

CHAPTER 5 - VESSEL NORMALIZATION METHODS: THE DMT NORMALIZATION MODULE

Before beginning this protocol, please note the following:

The "IC_1/IC_{100}", or "Normalization Factor", is the ratio of the internal circumference at which the maximum response to a vasoconstrictor (eg, 40µM norepinephrine plus KCl) is observed divided by the internal circumference at which a transmural wall pressure of 100 mm Hg (eg, IC_{100}) is observed. Therefore, multiplying the IC100 by this ratio gives the starting internal circumference at which an optimal response (eg, IC_1) may be anticipated.

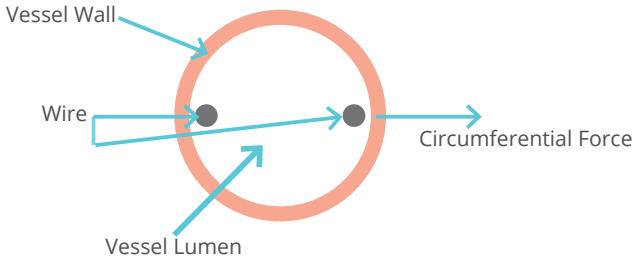
The 0.9 Normalization Factor has been determined to be appropriate when studying rat mesenteric arteries, though investigators are always encouraged to determine the Normalization Factor for their vessel samples. A protocol for determining the optimal Normalization Factor for a given tissue type is provided in this chapter.

It is possible for the Normalization Factor to exceed 1.0 (eg, rat femoral arteries produce maximal responses to vasoconstrictors at 1.1 times the IC_{100}) (Slezak, Waczulikova et al. 2010).

WARNING: The Norm Factor IC_1/IC_{100} value should always be determined for the specific vessel type being used! The default setting, 0.9, is just that, a DEFAULT, and not necessarily the correct value for use with your specific vessel/species!

5.1 IC_1 AND NORMALIZATION FACTOR DETERMINATION

As noted previously, in order to set a consistent baseline condition for studying vessel smooth muscle function one must pre-stretch the vessel, in the circumferential direction (see below), to an extent that achieves ideal actin-myosin interactions. The amount of pre-stretch must be determined experimentally for each vessel.



There are two common ways to identify this optimal pre-stretch value. The first requires the user to determine the length/tension relationship for each individual vessel; this can be done by sequentially stretching the vessel while measuring force (wall tension) both under passive and activated (ie, with the addition of a vasoconstrictor) conditions at each IC. The difference of these data points (tension in the presence of agonist minus passive tension) provides a measure of the active force. As shown in Figure 8 of the previous chapter, the optimal stretch – the IC_1 – can be derived from this active response curve. With this approach the vessel stretch is returned to an IC that corresponds to the peak active response. All subsequent experiments are performed at these isometric conditions. Note that this approach will not be described in detail here but can be extrapolated from the following sections.

The second approach is to utilize a pre-determined correction factor – the IC_1/IC_{100} ratio, or Normalization Factor – to simplify the process, allowing the user to perform only passive stretches to identify the optimal pre-stretch. This is effective because the IC1/IC100 ratio is generally accepted as being consistent between vessels from the same vascular bed derived from the same animal model.

When using the DMT Normalization Module in LabChart, the default value for the IC_1/IC_{100} ratio is 0.9. This ratio is the appropriate IC_1/IC_{100} ratio for mesenteric resistance arteries for typical rat strains. If a different resistance artery and different species of animal is used, a new IC_1/IC_{100} ratio should be determined for the specific artery of interest. This chapter provides step by step details on how to determine the proper IC_1/IC_{100} ratio (Normalization Factor) for the animal model/vessel type being studied.

5.2 NORMALIZATION PROCEDURE WITH IC1 DETERMINATION:

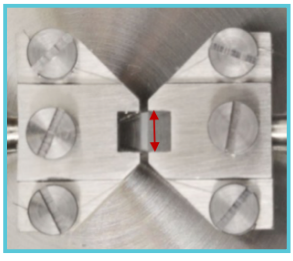
The purpose of this section is two-fold: first, to provide detailed step-by-step instruction on how to use the DMT Normalization Module (in AD Instruments' LabChart 8 software suite) to obtain the IC_1 (and the micrometer setting at which the IC_1 is achieved), and second, to go through the procedure for determining the Normalization Factor (Norm Factor) for any specific vessel type in a specific animal model. (Mulvany and Halpern 1977, Warshaw, Mulvany et al. 1979). A second approach to determining the IC_1/IC_{100}, which makes some assumptions regarding the range of IC_1/IC_{100} values applicable to the vessel, has been published elsewhere (Slezak, Waczulikova et al. 2010, Xiao, Ping et al. 2015) and represents a simpler, albeit more restrictive, method. This second protocol will be presented separately at the end of the section. What follows below is a step-by-step, detailed, "cook book" approach for successfully utilizing the DMT Normalization Module interspersed with notes and suggestions to help optimize the process.

To proceed with the Normalization Module:

1. Begin by isolating the vessel of interest and keeping the intact vessel in cold/room temperature physiological saline solution (PSS; suggested recipe in Appendix A) until ready for mounting

NOTE: For a video of how to isolate and mount a rat mesenteric small vessel artery to a DMT wire myograph, can be requested from your DMT sales representative.

2. Mount the vessel on the wires in cold/room temperature PSS
3. Record the vessel length (red arrow in figure at right) using one of two approaches:
   - Direct measurement through the use of a ruler (mm units)
   - Measurement through use of microscope eyepiece reticles; if using this approach, be sure to record the number of mm per division (see 8a, 9a)

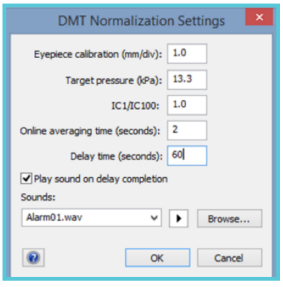


4. Place the chamber on the interface, plug into back, start bubbling O_2, and allow to heat (37°C) for 20 min
5. Set the micrometer such that the wires are nearly touching

- A force reading may be present, but no changes in force should be observed when increasing wire separation by a few microns

6. Zero the force reading on the user interface when reading stabilizes

7. Record the first diameter reading from the micrometer (needed for step 12)
   - This is the position where the gap is Zero.
8. Open the LabChart "Normalization Settings" under the "DMT" drop-down menu; a new screen will open with the following settings and default values (underlined; screen shot at right):
   a. Eyepiece calibration (mm/div): 0.36
   b. Target pressure (kPa): 13.3
   c. IC_1/IC_{100}: 0.9
   d. Online averaging time (seconds): 2
   e. Delay time (seconds): 60
   f. Play sound on delay completion (check box)
   g. Sounds (with drop-down selection menu or Browse function)

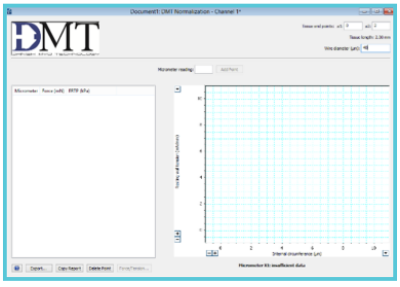


9. What follows are explanations and notes corresponding to the above:
   a. Eyepiece calibration: This represents the reticle conversion factor used when determining vessel length; if the length was determined directly (in mm), you can use "1" here then input the actual length in the DMT Normalization Screen (see section 12 below)
   b. Target pressure (kPa): The default value, 13.3, corresponds to 100 mm Hg; this is the generally accepted pressure for this procedure and represents the mean physiologic pressure of a resistance vessel. This value is usually not changed for resistance vessels. An exception to this rule would be pulmonary arteries, which see significantly lower mean arterial pressures, around 12 to 15 mm Hg (1.6 to 2.0 kPa)
   c. IC1/IC100: This is the NORMALIZATION FACTOR. As noted above, 0.9 is a DEFAULT value and is generally appropriate for use with rat mesenteric vessels.

NOTE: IF IDENTIFYING THE IC_1 EXPERIMENTALLY, OR DETERMINING THE IC_1/IC_{100} VALUE, LEAVE THIS AT 0.9

   d. Online averaging time (seconds): Time period over which measured force is used for averaging delay time (seconds): Represents the countdown to occur once the "Add Point" button is pressed; allows the vessel adequate time to equilibrate from the prior stretch
   e. Play sound on delay completion (check box): Alerts user when data is collected, signaling that it is OK to move on to the next step
   f. Sounds (with drop-down selection menu/Browse function): Allows the user to select the audio signal for "f"

NOTE: The module will not work in Labchart monitoring mode



10. With the appropriate number of channels available, start the chart recording
11. Select the channel of interest from the drop-down DMT menu; the DMT Normalization screen will appear (screen shot at right)
12. Populate the following with the appropriate data:
    - Tissue end points: "a1" should be 0 and "a2" is the length of the vessel (in mm or reticule units)
    - Wire diameter: Input the diameter of the wire used to mount the vessels (eg, 10 µm, 25 µm, 40 µm, or 200 µm for pin-mounted vessels) the steel wire typically sent with systems is 40 µm
    - Micrometer reading: Input the value from the analog micrometer scale (users of DMT myograph model series 510A and 520A, please see the applicable DMT user manual)
13. When ready, click the "Add Point" button to record first point
14. The countdown clock will begin under the "Force" column in the left window
15. When the countdown reaches zero and the audible signal is heard (if selected), the first data point is collected and will appear in the left-hand window
    - Note that unlike the regular Normalization procedure, values from the actual force tracing will be needed to calculate IC_1
    - Continue to input the micrometer settings and passive tension data as this will allow the Normalization Module to calculate the IC_{100} for you

NOTE: It is expected that the wires will not have been separated far enough to generate any appreciable tension or transmural pressure, therefore this first point will yield a pressure value of "0" and a force near "0"

AT THIS POINT THE USER CAN EITHER PROCEED TO THE REGULAR NORMALIZATION PROCEDURE (CONTINUED ON STEP 25), OR IF THEY ARE DETERMINING THE IC1 VALUE, AND NORM FACTOR FOLLOW STEPS 16 THROUGH 24. THE IC1/IC100 VALUE IN THE "NORMALIZATION SETTINGS" SHOULD BE SET TO "0.9"; THIS WILL ALLOW YOU TO SEE THE IC100 VALUE IN THE NORMALIZATION MODULE

16. Instead of applying the next PASSIVE stretch (step 25), stimulate a vessel contraction
    - It is suggested to elicit a potassium-mediated contraction, as this will give a quick response that can easily and quickly be washed out
17. Wait for the contraction to reach a plateau before washing out the potassium-containing buffer solution
    - Three washes over 5 minutes is usually sufficient
18. Once the contraction has been washed out, the next passive stretch is applied and the subsequent micrometer reading is entered in the DMT Normalization Module; click "Add Point"
19. Repeat the contraction protocol for the next active response
20. After the contraction has reached the plateau, wash out the agonist and repeat the passive stretch, active stretch, wash process until the last passive stretch point has just gone over the 13.3 kPa (100 mm Hg) isobar line (or the isobar line for the selected transmural pressure, if different) in the DMT Normalization Module
21. For an example of what a protocol might look like, and to help visualize how the data output for a typical experiment would appear, please see Figures 10 and 11 below:

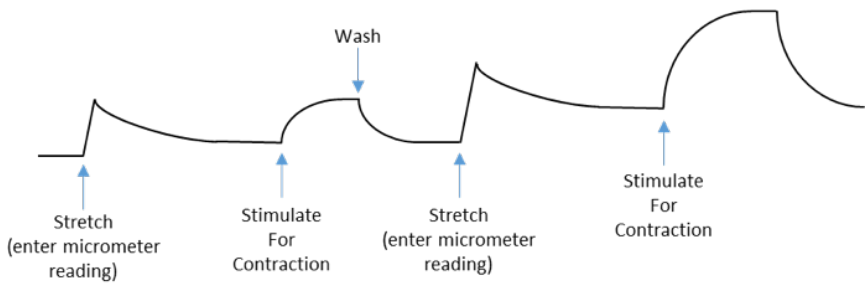


Figure 10. Representative trace illustrating an experiment to determine the active response length/tension relationship in order to determine IC1, while simultaneously obtaining the passive stretches to determine the IC100 from the DMT Normalization Module.

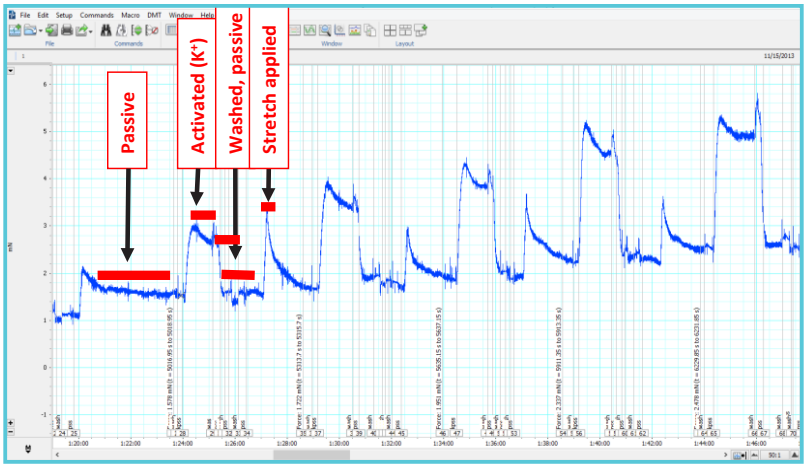
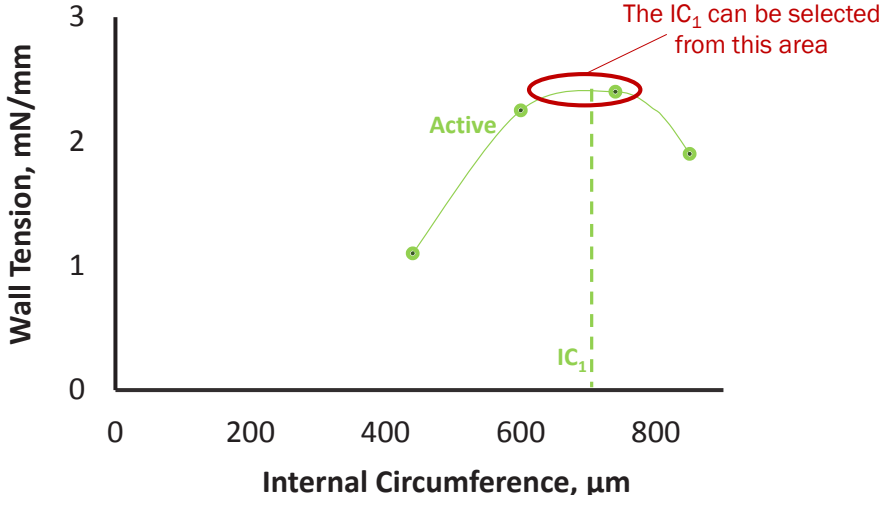
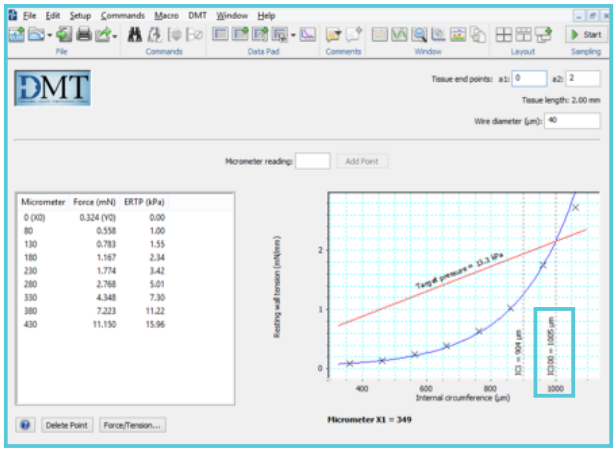


Figure 11. Actual trace from an experiment to evaluate the active response length/tension relationship of mouse trachea in order to determine the tissue's IC_1, while simultaneously obtaining the passive stretches to determine the IC_{100} from the DMT Normalization Module. Note that the indicated steps are a sequence repeated throughout the experiment.

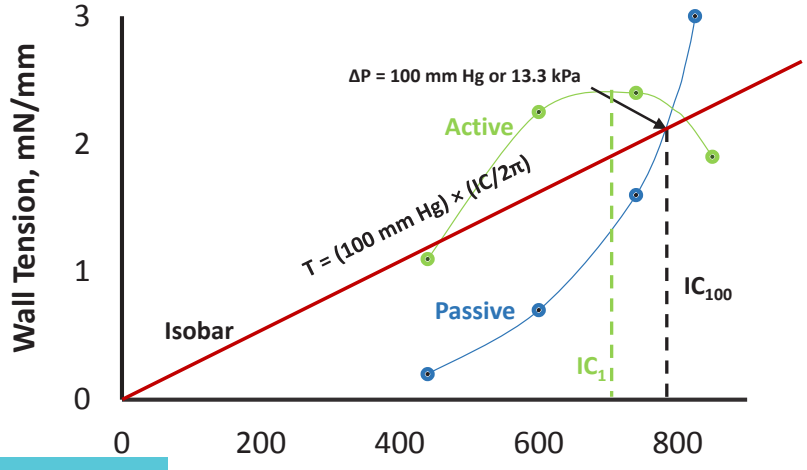
22. Extract active force data from the trace by subtracting the passive force at each stretch from the K+-activated force (ie, passive + active force) data
    - It may be useful to average passive force values from before AND after the K^+-induced activation period
23. From your data create a graph with the IC values (calculated from micrometer data) on the X-axis and active force on the Y-axis
    - For this vessel, an IC value lying within the peak activity plateau (as shown in Figure below) should be selected; **THIS IS YOUR IC_1**



- The DMT Normalization Module will provide the IC_{100} (see figure below)



- See Chapter 6 for a breakdown of the formulas used to produce these graphs



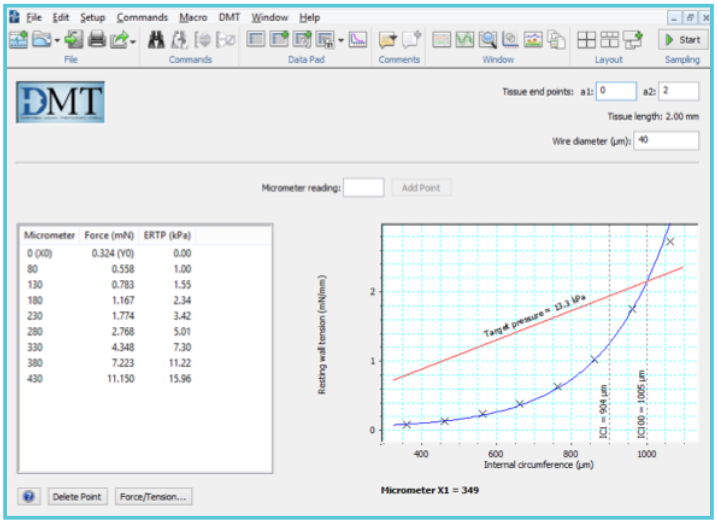
NORM. FACTOR = IC_1/IC_{100}

24. If a number of vessels are evaluated in this manner, the mean of the individually determined IC_1/IC_{100} ratios can be applied to other samples of the same vessel type without having to re-determine the IC_1 value each time for the species and vessel in question
    - Alternatively, a series of graphs generated from different vessels can be overlaid to provide a visual reference from which an IC_1/IC_{100} ratio can be selected

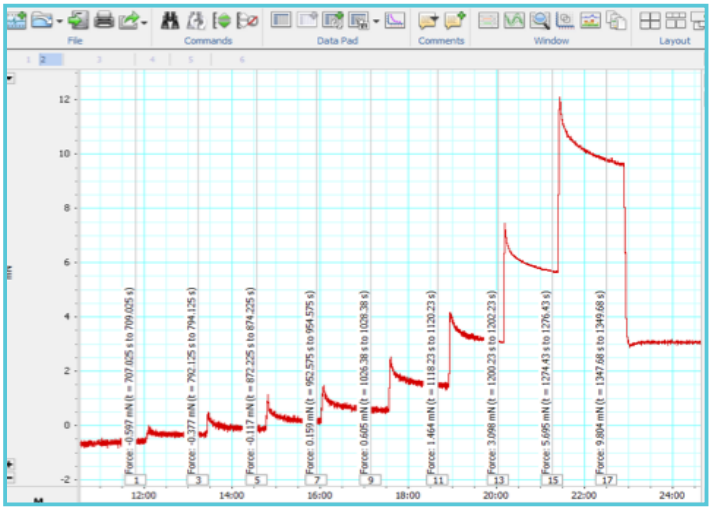
This completes the section describing how to calculate the Normalization Factor (NORM. FACTOR).

IMPORTANT:

- It may be helpful to have the main chart window open while adjusting the micrometer settings; if a large spike exceeding the isobar is observed on the trace, reduce the tension
- The micrometer "steps" used during the passive stretch do NOT need to be the same amounts for each passive stretch, eg, the first few stretches could be 50 µm each, but as the stretches get closer to the isobar line in the module, the steps can be reduced to 30 µm, 20 µm or even 10 µm. Basically, the distance used do not have to be the same from stretch to stretch
  - » For some larger vessels it may be appropriate to be more aggressive with the first few steps, eg, start with an increase of ~50 – 100 µm or even more
- A 60 second delay between readings (see 7e, 8e above) is generally sufficient to allow the vessel to acclimate to any given stretch
- Greater accuracy may be expected with a larger number of data points to generate the curve, but for best results in the normalization module try to have a minimum of 4 points for the graph, having 3 points below the isobar line and 1 point just above the isobar line
- Points may be deleted if necessary by clicking on the line and pressing "Delete"; the graph/algorithm will be recalculated
- A point too far above the isobar line may physically damage the mounted vessel, causing unreliable results during the experiment; the closer the last point is to the isobar line but just above it will result in the best Normalization possible without damaging the vessel
- Change the isobar from 13.3 kPa in the settings if the artery has a lower mean pressure, in vivo (eg., for pulmonary arteries)



25. While the prior figures provide insight into what the Normalization window output looks like, the force trace found in the main LabChart window should look something like this:



26. From the DMT Normalization window, the optimal IC for baseline – denoted IC_1 – is shown. The micropositioner setting at which this IC is obtained appears under the graph as "Micrometer X1 = (value)"; this is the value at which the user should set the micropositioner. The 510A, 520A and 630MA system will automatically adjust the micropositioner to the IC position.
27. Allow vessel to rest per user protocol requirements and commence with study

CHAPTER 6 - FORMULAS AND DATA PRESENTATION

While the math associated with length/tension relationships was outlined briefly in Section 3.3, how these data are presented can be inconsistent and sometimes confusing. Here we provide additional details related to formulas important to vessel length/tension studies with a focus on utilizing the data generated by the Normalization Module. This will be followed by a brief discussion on some of the common ways in which such data are presented in the literature.

6.1 KEY PARAMETERS GENERATED BY, AND DERIVED FROM, THE NORMALIZATION MODULE

The data parameters of interest, and their commonly-used units, include:

- Transmural Pressure (Pi, kPa = mN/mm^2)
- Wall Tension (T, mN)
- Internal Circumference (IC, µm and mm)
- Wall Diameter (µm and mm)
- IC_1
- IC_1/IC_{100}

For IC100, the formula is simply:

$$IC_{100} = 100 \text{ mm Hg} = 13.3 \text{ kPa} = 13.3 \text{ mN/mm}^2$$

For the purpose of this discussion we will assume that the IC_1/IC_{100} ratio is already known and has been entered into the Normalization Settings window. The procedure for calculating this ration is detailed in Section 5.2.

When performing the Normalization procedure, the values to be noted are the starting micrometer setting (ie, only the wires contribute to the IC, diameter) and the final micrometer setting to achieve IC_1. Also helpful are the tension values surrounding the IC_1.

Determine the "gap" between the baseline micrometer setting and the IC_1 micrometer setting and use it in the following formula to determine the vessel IC_1;

1. IC = d (π+2) + 2 x gap

In the example below, 40 µm diameter wire are used

2. IC = 205.6 µm* + 2 × “gap”
3. Diameter = IC_1/π

When you have completed the Normalization Procedure and set the micrometer to the proper setting to achieve IC1, the tension can then be determined. Starting with the Law of Laplace:

4. Pi = T / (IC / (2 × π)) or Pi = 2 × π × T / IC

One can rearrange the above equation to solve for wall tension, T. Note the units, which are included below:

5. T (mN/mm units) = Pi (mN/mm2 units) × IC (mm units) / 2 π

The Law of Laplace, as it is commonly written, does not take into account the length of the vessel. Wall tension (T) can be further broken down into the parameters of the force exerted on the wall (F) per unit vessel length:

6. T (mN/mm units) = F (mN units) / by 2 × vessel length (mm units) or F = T × 2 × vessel length

Therefore, rearranging formula “5” above, we obtain:

7. F (mN units) = Pi (mN/mm2 units) × (IC (mm units) / 2 π) × 2 × vessel length (mm units)

The reason why this extra step is necessary is that the data output in LabChart is force (F) in mN units, not wall tension (T), which takes into account the length of the vessel. The tension that registers in the force tracing – before zeroing and proceeding with your experiments – should be close to the one calculated above, and the value should lie within the tensions of the Normalization curve data points that lie on either side of the IC_1.

Example:
In this example, a mouse mesenteric artery (2nd order) is mounted on 40 µm wire. A target pressure of 100 mm Hg (IC_{100}) is selected, and an IC_1/IC_{100} ratio of 1.0 is chosen. The vessel length is 2 mm. During Normalization, the first micrometer reading (where the wires are nearly touching) is 6940 µm. After

completing the procedure, the module indicates that the IC1 can be reached by setting the micrometer to 7207 µm. The “gap” in this scenario is the difference between these values:

IC = 205.6 µm + 2 × “gap” = 205.6 µm + 2 × (7207 µm – 6940 µm) = 740 µm

At this point equation “5”, above, is applied, noting the conversion of Pi to units of kPa (mN/mm2) and the IC to mm units:

(9) $T = Pi \times IC / 2\pi = 13.3\ mN/mm^2 \times 0.74\ mm / 2\pi = 1.57\ mN/mm$

Therefore the wall tension (T), which is standardized to vessel length, is 1.57 mN/mm. For the final force calculation, taking length into account:

(10) $F = T \times 2 \times \text{vessel length} = 1.57\ mN/mm \times 2 \times 2\ mm = 6.28\ mN$

When the user looks at the force after the micrometer if set to the proper IC_1 value, the resulting force reading should be similar to this calculated value.

6.2 COMMON DATA PRESENTATION APPROACHES IN THE LITERATURE

One potential source of confusion when evaluating literature related to myography and length/tension relationships is how data are presented. There is no standard approach, and the way data are presented can vary from laboratory to laboratory. We will point out some of the more common approaches here, but please note that we are not suggesting that any one approach is more acceptable than the other; we leave this to the reader to decide for themselves the most appropriate way to present their data.

There are essentially 2 main areas where multiple variations in data presentation can be expected: when defining the means of setting baseline tension and when tabulating length/tension data.

Baseline Tension and Length/Tension Data

Specific tension information regarding the baseline conditions used in muscle function studies is omitted – or referenced – in nearly half of the literature. Those studies that do provide baseline resting-tension data are split among those using:

- The Mulvany-Halpern method for Normalization (ie, the module's approach)
- Pre-determined tensions, provided in units of mm Hg, mN, or grams
- Length/tension analyses for the individual tissues

In many cases the authors clearly indicate that pre-tension values were determined in the laboratory for the specific vessel in question, but often little or no explanation is given as to the source of the tension values used. The literature is littered with a wide range of values for a specific vessel type in a specific animal model, suggesting that either vessels can produce reproducible tension data over a range of baseline stretches, different laboratories use widely varying approaches to identifying their baseline values, Values determined in one vessel/species are often used indiscriminately for other vessels/species, or a combination of each is at play. Regardless of the source of this variability, it is advisable that each laboratory not borrow a value from another group but instead perform their own length/tension studies to find the optimal baseline conditions for their protocols, vessels, species, equipment, environment, and personnel.

Data for vessel dimensions and reactivity also vary from laboratory to laboratory. In addition to providing a mix of diameter versus internal circumference measures, some authors elect to list wall tension while others select force. Often it is difficult to convert between tension and force measures due to insufficient vessel length data, making side-by-side comparison of data from different studies challenging.

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Warshaw, D. M., M. J. Mulvany and W. Halpern (1979). "Mechanical and morphological properties of arterial resistance vessels in young and old spontaneously hypertensive rats." Circ Res 45(2): 250-259.
Xiao, X., N. N. Ping, S. Li, L. Cao and Y. X. Cao (2015). "An optimal initial tension for rat basilar artery in wire myography." Microvasc Res 97: 156-158.

Danish Myo Technology A/S

E-mail: sales@dmt.dk
Tel.: +45 87 41 11 00
Fax: +45 87 41 11 01

DMT-USA,Inc.

E-mail: sales@dmt-usa.com
Tel.: +1 734 707 0250
Fax: +1 678 302 7013

DMT-China

Tel.: 86-21-6486 9685
Fax: 86-21-5877 0063

DMT-Asia Pacific

Tel.: +61 2 8814 1597