

## Introduction:

### What is Normalization?

Studies which use isolated pieces of tissues require the results to be standardized (normalized), either during the collection or when analyzing the data. Data from isolated tissue studies are expressed as relative to a known maximal response by the tissue to an agent/drug or expressed relative to a generated force. Studies using elastic tissue require that the tissue have a pre-tension prior to conducting experiments, however, factors such as the amount of elastic tissue, sensitivity of the tissue due to differing pre-tension would be difficult to determine for each piece of tissue.

The normalization calculation and procedure was devised, validated and published by Prof. Michael Mulvany using rat mesenteric arteries (for further details see Mulvany and Halpern, Circulation Research 1977, 41: 19-26). This procedure allows the user the ability to define the preexperimental conditions and thereby standardize each piece of tissue.

There are three major reasons for normalizing elastic tissue:

1. Size of elastic structures (i.e. such as vessels) can only have meaning if the conditions are clearly defined.
2. The sensitivity of muscular preparations to agonists is dependent on the degree of stretch, therefore the experimental conditions, such as pretension of the tissue, need to be clearly defined.
3. As the active response of a tissue is dependent on the extent of stretch (according to the active tension internal circumference relation), it is useful to set tissues to an internal circumference which gives the maximum response, according to the amount of muscular tissue in the sample.

Mulvany conducted the initial validation studies of the normalization procedure using rat mesenteric arteries and found that the best experimental conditions were obtained if one defines the vessel size as being the size (internal circumference) when the vessel is fully relaxed and under a transmural pressure of 100 mmHg. In determining and setting the internal circumference in relation to a set pressure means that each tissue can be standardized. This standardization takes into account the amount of elastic tissue (smooth muscle) mounted on the wire myograph. Therefore the aim of the normalization procedure is to determine, for a vessel mounted on the myograph, the internal circumference ( $IC_{100}$ ) at which the vessel would have if relaxed and under a transmural pressure of 100 mmHg. (N.B. In earlier published literature,  $IC_{100}$  was referred to as L100).

# The Normalization Process:

## What is Involved in Normalization?

Once the tissue is mounted on the myograph, normalization is performed by progressively stretching the tissue and recording the micrometer readings and force values. It also requires knowing the internal circumference, amount of tissue (wall length) mounted on the wire and the effective pressure (Pi) which is essentially an estimate of the pressure which would be necessary to stretch the tissue to the measured internal circumference.

- Internal Circumference: calculated according to the measured distance between the wires and the known diameter of the mounting wires.
- Wall length of the tissue: segment length is determined using a calibrated eyepiece and dissecting microscope (N.B. the wall length is equal to twice the tissue segment length as there is tissue above and below the wires).
- Wall tension: the measured force divided by the wall length.

The effective pressure, is then determined using the Laplace relation, given by

$$P_i = \text{Wall tension}/(\text{internal circumference}/[2 \cdot \pi])$$

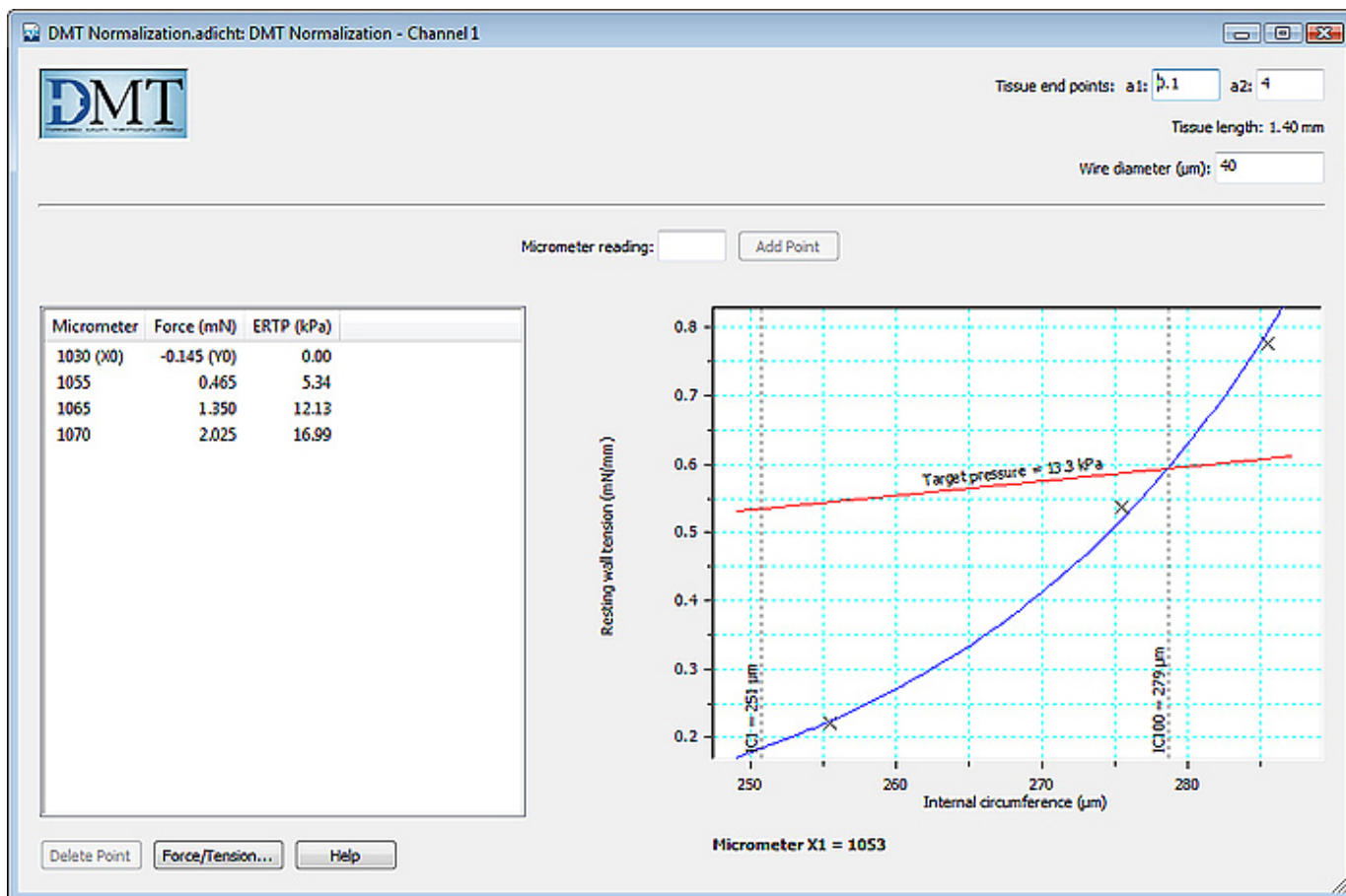


Figure 1: DMT Normalization window with calculated tissue length and Micrometer X1.

From the micrometer readings and wire diameter, the internal circumference is calculated and then

plotted against the calculated resting wall tension (effective pressure, E RTP) after each progressive stretch of the tissue (Figure 2). Once the effective pressure exceeds 100 mmHg (13.3 kPa) the stepwise stretching of the tissue can be stopped.

Micrometer	Force (mN)	ERTP (kPa)
1030 (X0)	-0.145 (Y0)	0.00
1055	0.465	5.34
1065	1.350	12.13
1070	2.025	16.99

Figure 2: The Data Points table of the Normalization window displaying the micrometer settings, force generated and calculated effective pressure (ERTP).

An exponential curve is then fitted to the internal circumference pressure data and, using Laplace's equation, the point on the curve corresponding to 100 mmHg is determined and denoted  $IC_{100}$  (Figure 3). Once  $IC_{100}$  is calculated, the internal circumference ( $IC_1$ ) is set to 90% of the  $IC_{100}$  ( $IC_1 = 0.9 \cdot IC_{100}$ ). Numerous studies have determined that for mesenteric microvessels, setting the tissue to  $IC_1$  establishes a maximal active force production of the tissue. It is important that the factor,  $IC_1/IC_{100}$ , be determined for each particular tissue type investigated.

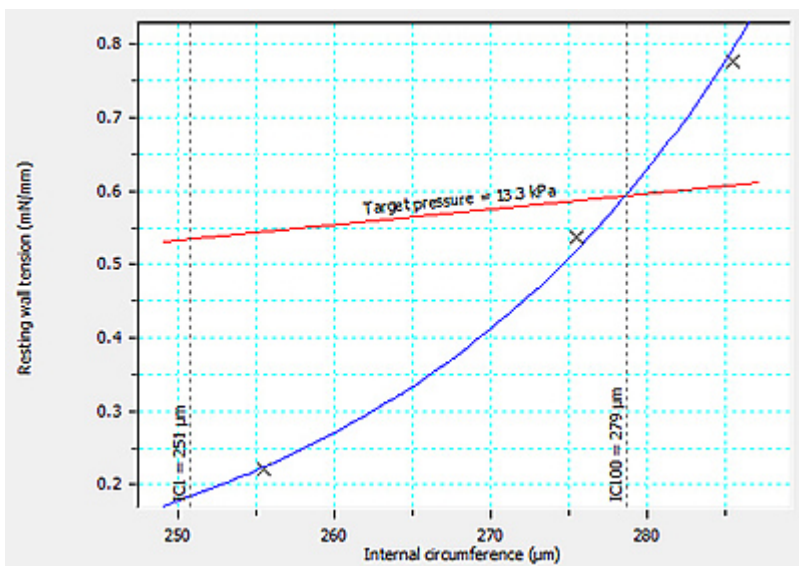


Figure 3: The Normalization Window graph of internal circumference versus resting wall tension.

The module uses these values to display the Micrometer pretension setting (Micrometer XI) for that particular tissue. The above process is conducted on each individual tissue setup.

## Module Features:

The DMT Normalization Module adds a DMT item to the LabChart Menu Bar. Researchers can conduct experiments on multiple tissues preparations and channels and therefore normalization will need to be determined for each individual preparation in each channel. For this purpose a Normalization Window

is provided for each visible LabChart channel. The module also provides a Normalization Settings dialog window in which parameters such as the microscope eyepiece calibration, target pressure,  $IC_1$  / $IC_{100}$ , online averaging time, delay time and play sound are set by the user (Figure 4). These settings are universal and each parameter is applied to calculations in each Normalization Window.

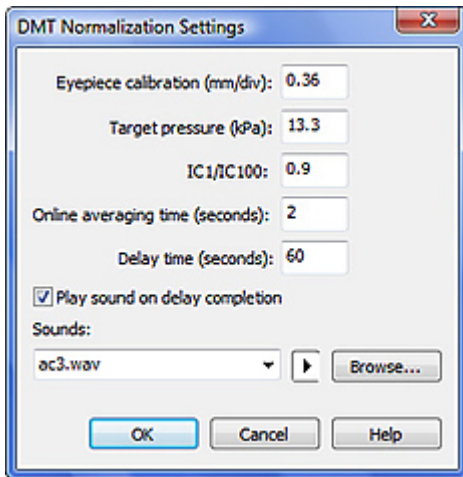


Figure 4: Normalization Settings dialog

#### **The module calculates:**

- tissue length,
- internal circumference ( $IC_{100}$ ), calculated from the force signal recorded in LabChart, and
- provides the final micrometer setting required to stretch the tissue to  $IC_1$  (Micrometer X1).

## **Module Settings:**

### **Normalization Settings**

For further details on each of these features and a detailed explanation of the procedure for conducting tissue normalization see the DMT Normalization Module User's Guide.

#### **Eyepiece calibration**

Experimentally, tissue length is measured with a calibrated eyepiece (mm per division) on the dissecting microscope. The eyepiece graticule readings at the two ends of the tissue segment (a1 and a2) are entered into the Normalization Window and the tissue length calculated.

## Target pressure

This is the transmural pressure which the DMT Normalization Module is used to calculate a corresponding tissue internal circumference.

- The default value of 13.3 kPa (100 mmHg) can be changed according to the tissue type being used.

## $IC_1/IC_{100}$

$IC_{100}$  is the internal circumference corresponding to the target transmural pressure. Normalized internal circumference  $IC_1$  is calculated from  $IC_{100}$  by multiplying it by a number less than 1 (usually 0.9). Either the default 0.9 or any value in the range 0.2 to 1.0 determined by the researcher for a particular tissue type can be entered.

## Online Normalization Procedure

This allows the user to have LabChart and the DMT Normalization Module operating online to log the force in the table, calculate the effective pressure and generate the exponential curve automatically after each progressive stretch of the tissue.

### Online average time

- The force reading corresponding to a micrometer setting is determined by averaging the force data, when using the online mode (see Delay time below). The average time is taken as the period immediately prior to the point at which the delay time ends (see below).

### Delay time

- If a time greater than zero is set in the Normalization Settings dialog, the module will operate in the online mode (for offline mode set delay time to zero).
- In the online mode, after clicking Add Point, there is a delay (a countdown of the set delay time in the Data Points table of the Normalization Window) before the force data is collected from the LabChart recording and automatically added to the table and graph.
- The delay time is dependent on the length of time required for the force reading to stabilize after each progressive stretch.

### Play sound on delay completion

- If the Play sound checkbox is selected, the module will play a sound when the delay time expires.

## Normalization Window

### Calculating tissue length (Figure 1)

- Tissue end point a1 is when the hairline of the microscope eyepiece is over the far end of the tissue segment.
- Tissue end point a2 is when the hairline is over the near end of the tissue segment.
- The tissue length is calculated and displayed in the top right corner of the window in mm.

### Wire diameter (Figure 1)

- To determine the internal circumference of a tissue, the diameter of the wire used (either 40  $\mu\text{m}$  or 25  $\mu\text{m}$ ) must be entered.

## Editing

- Edit and delete data points in the DMT Normalization window, either online or offline.
- Edit micrometer readings of points already added to the table of points.
- Delete data points from the table.

## Experimental procedure

Once mounted in the wire myograph, the tissue is progressively stretched (using the myograph micrometer) and the contractile force response recorded. Initially the force increases sharply in response to each stretch and then decreases. The force reading should be stable before the tissue is stretched again. For mesenteric vessels the recommendation is to have a set delay time of two minutes after stretching the tissue (Figure 5). In the offline mode it is important that the force data selected from the LabChart recording is collected at the same time from the beginning of each stretch.

Micrometer readings and corresponding force data can be collected while recording (online mode) as described above or offline (after the sequence of stepwise stretches have been recorded).

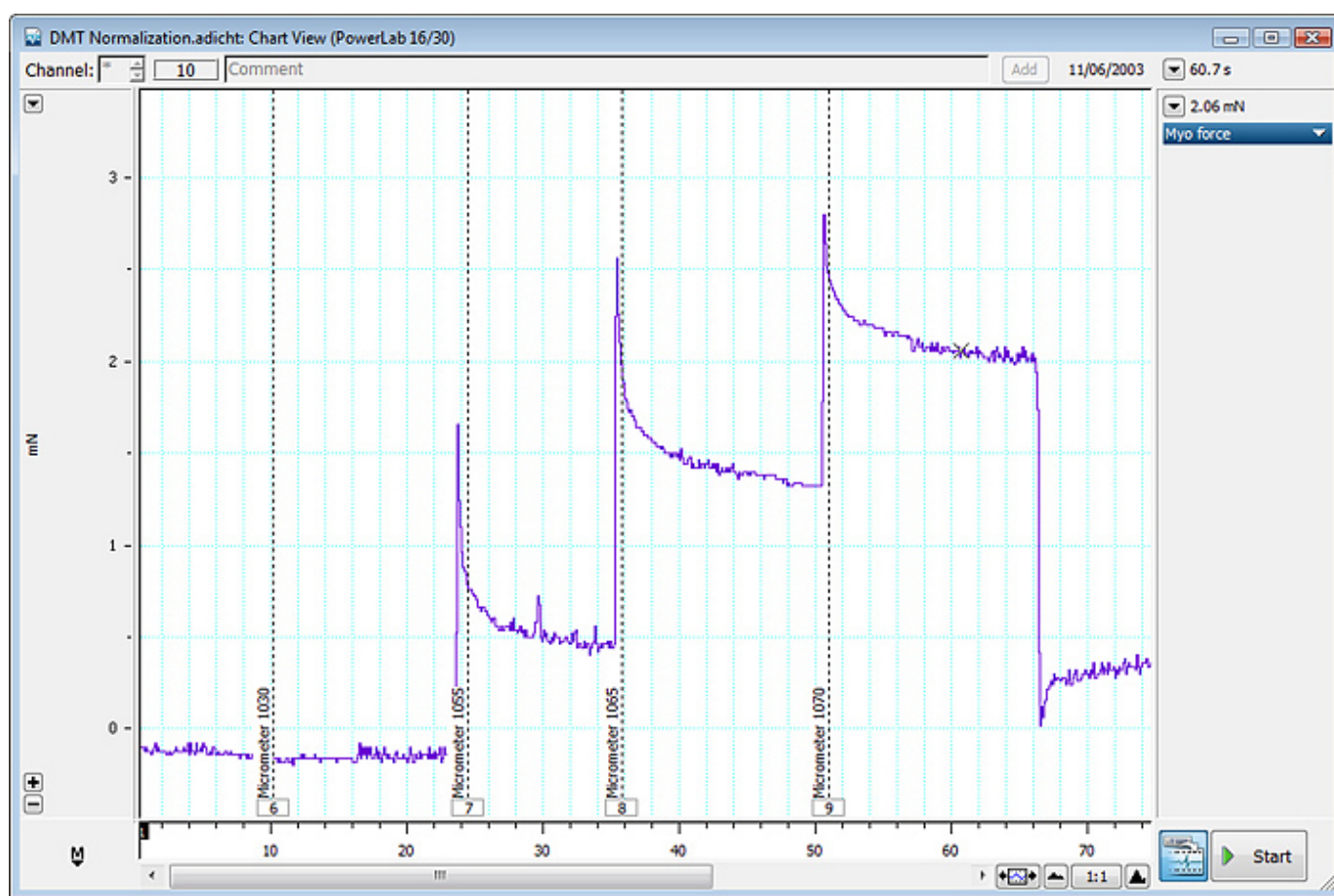


Figure 5: A LabChart recording of the developed force of a mesenteric artery, with a progressive stretching and resultant force development. Automatic comments are placed on the LabChart recording indicating the area and point from which the force measurement was made.

## Online or Offline: Which method to choose?

Choose offline if:

- You are recording normalization data on two or more channels at once.

Choose online if:

- Recording normalization data and inexperienced with the technique
- If only recording a single channel.
- For those experienced with the myograph techniques and normalization procedures, multiple channels may be normalized using the online method.
- The time taken for the force signal to stabilize after each stretch is predictable.

### **Units for experimental data**

When you are ready to record experimental data after normalization is complete, the user has the choice of using either force or tension units for the new data. The DMT Normalization Module uses the normalization data to display subsequent experimental data in units of either force (mN) or tension (mN/mm); however the normalization procedure must be performed using units of force (mN).

The Force/Tension... button is only enabled and therefore units for new data can only be changed, when the following conditions apply:

- A normalization result, which could be an extrapolated result, has been achieved.
- The normalization has been done with units of nM set up in Units Conversion.
- The LabChart channel containing the normalization data is turned on and a PowerLab is currently connected to the computer and is recognized by LabChart.