# USER GUIDE, VOL.2.1

## TISSUE BATH SYSTEM - 720MO

DMT

# TISSUE BATH - 720MO

## USER GUIDE

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# CHAPTER 1 - TISSUE BATH OVERVIEW

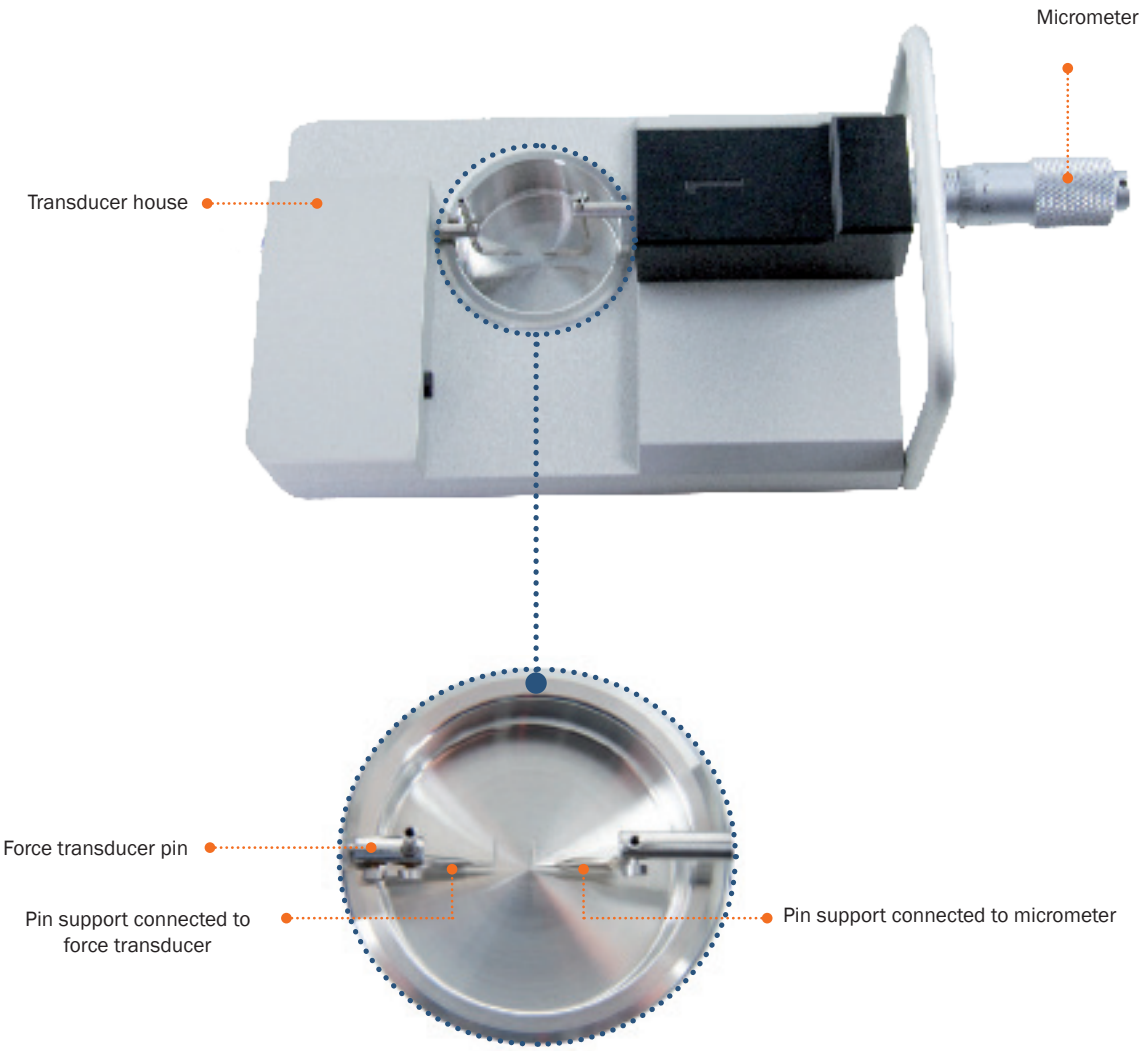



Figure 1.1 *Tissue Bath with close-up of chamber*

# CHAPTER 2 - SETTING UP THE TISSUE BATH

## 2.1 Changing and adjusting the mounting supports

Each chamber can accommodate mounting supports for larger segments (>500 µm). Because the mounting supports can be changed easily, experiments can be performed with different vessels of varying internal diameter from 500 µm up to 10 mm. Continuous use and repeated greasing of the transducer arm holes will cause some misalignment of the mounting supports. The mounting supports will need occasional adjustments. Changing and adjustment of the mounting supports is performed using the following step-by-step procedure.

OBS
THE TRANSDUCERS ARE FRAGILE AND SENSITIVE TO MECHANICAL STRAIN. BE VERY CAREFUL WHEN CHANGING OR ADJUSTING THE MOUNTING SUPPORTS!

### 2.1.1 Changing the mounting supports (figure 2.1):

1. Use the micrometer to separate the supports as far apart as possible.
2. Use the small screwdriver provided to gently loosen screw “D” on the support attached on the transducer side. Screw “D” is the screw on the support closest to the transducer.
3. Gently pull the support away from the transducer pin.
4. Loosen screw “B” on the micrometer side with the appropriate Allen key.
5. Pull the pin support away.

NOTE
NUMBER THE MOUNTING SUPPORTS WITH THE NUMBER OF THE CHAMBER THEY WERE REMOVED FROM USING A PERMANENT MARKER. STORE THE MOUNTING SUPPORTS IN THE PROVIDED PLASTIC CASE. NUMBERING THE MOUNTING SUPPORTS WILL SAVE TIME WHEN THE MOUNTING SUPPORTS ARE CHANGED AGAIN, MINIMIZING THE AMOUNT OF ADJUSTMENTS NEEDED AFTER EACH CHANGE.

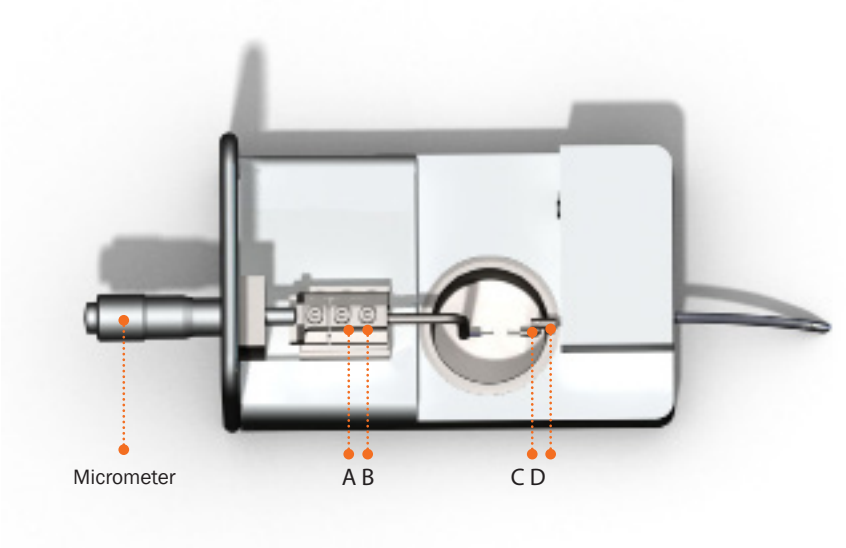



Figure 2.1 *Illustration of the screws for changing supports and adjustment of the pins*

### 2.1.2 Fine adjusting the pins for larger vessels (figure 2.2 and 2.3)

1. Loosen screw “A” to move the micrometer-side arm holder sideways
2. Loosen screw “B” to move the micrometer-side pin toward or away from the transducer.
3. Loosen screw “C” to align the transducer-side tissue holding pin horizontally.
4. Loosen screws “D” and “E” to align the heights of the tissue holding pins vertically.

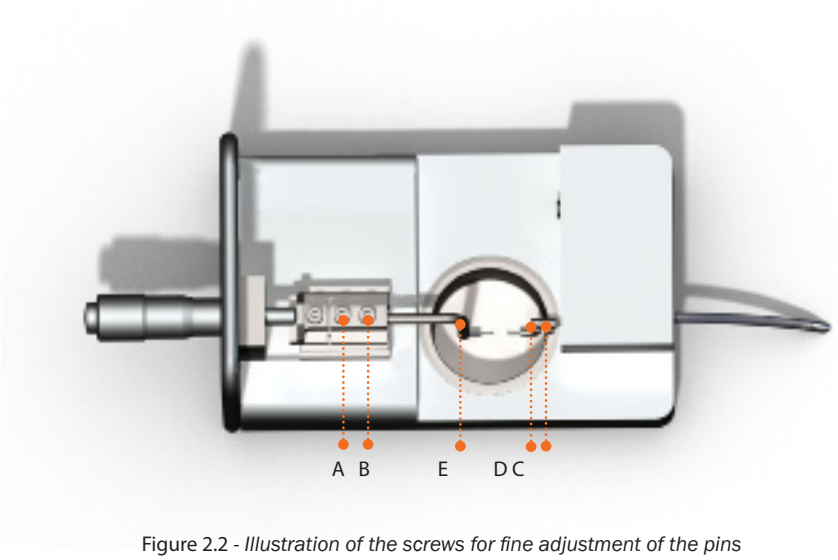



Figure 2.2 - *Illustration of the screws for fine adjustment of the pins*

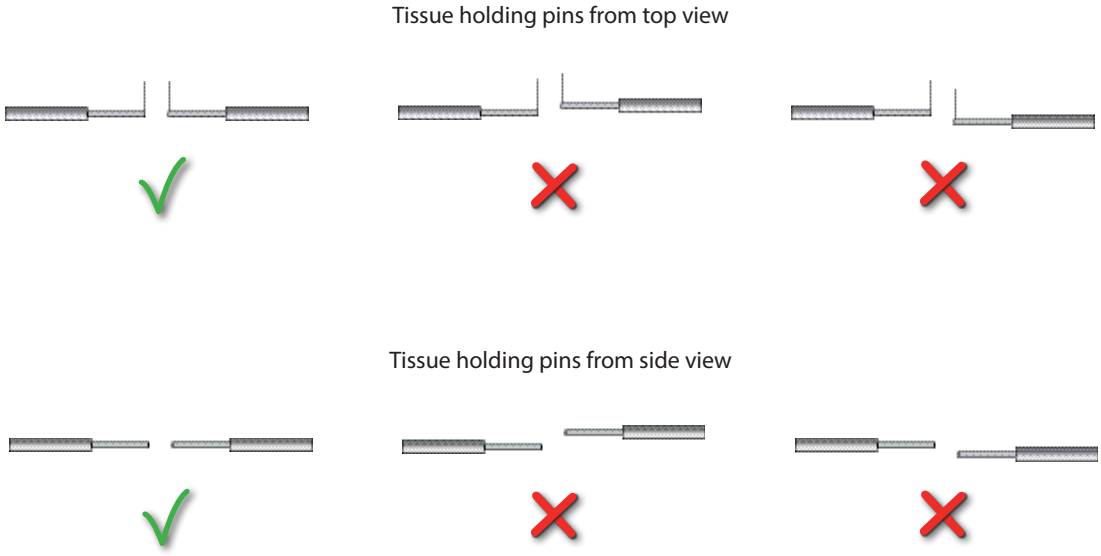



Figure 2.3 - *Illustrations of properly aligned tissue holding pins (depicted on the far left) and incorrectly aligned pins (depicted in the middle and far right).*

## 2.2 Calibration of the force transducer

As a part of the general maintenance of the Tissue Bath, DMT recommends that the Tissue Bath is force calibrated at least once a month. The Tissue Bath should also be force calibrated every time the Multi Interface has been moved. Although lab benches are all supposedly perfectly horizontal, small differences in lab bench pitch can affect the calibration of the system. The Tissue Bath should also be calibrated if the system has been idle for longer than a month. A step-by-step procedure is explained in chapter 3.5.1.2 in Multi Myograph System - User Manual.

# CHAPTER 3 - EXPERIMENTAL SET-UP

This chapter contains experimental set-up for the Tissue Bath. For dissection of a vessel, please see Procedures for investigations of small vessels using a small vessel Myograph by M.J. Mulvany.

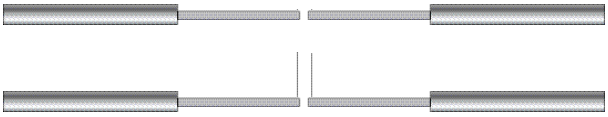
## 3.1 Mounting protocol

Below is a short description of how to mount a tissue ring preparation on the mounting pins of the 720MO system. DMT deliver the 720MO system with 200µm pins allowing the user to mount ring preparation with an internal diameter from 450µm and up. For really large tissue ring preparation with the ability to make high force contractions the 200µm pins may be too thin. DMT can delivery mounting pins with the following thickness:

L-shaped mounting pins (Diameter): 200µm, 250µm, 300µm and 400µm.

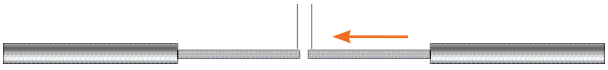
1. Fill the chamber with a given buffer (5-7ml) (see appendix 1 for Buffer recipes). DO NOT FILL THE CHAMBER TO THE EDGE OF THE CHAMBER.

2. Make sure that the tissue holding pins are properly aligned.

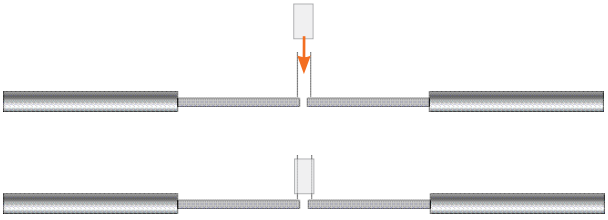


*Properly aligned pins, seen from side and above*

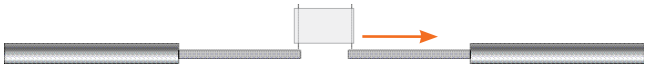
3. Use the micrometer to get the pins close together as possible without touching each other.



4. Slide your tissue ring preparation onto the two mounting pins.



5. Slowly move the pins apart using the micrometer until the force reading is increasing.



6. The tissue is now mounted and ready for a normalization protocol (see chapter 3.2) finding the optimal pre-load tension for the mounted tissue

7. Place the micrometer at the position giving the optimal pre-load tension on the mounted tissue.

8. Continue with a standard start (see chapter 3.3).

9. The mounted tissue is now ready for experiments.

## 3.2 Normalization

The importance of normalizing the preparation is three-fold:

1. Experiments with elastic preparations like vessels can only have meaning if they are performed under conditions where the size is clearly defined.

2. Clearly defined conditions are required in pharmacological experiments as the sensitivity of preparations to agonists and antagonists is dependent on the amount of stretch.

3. The active response of a preparation is dependent on the extent of stretch, which makes it important to set the preparation to an internal circumference giving maximal response.

The aim of the normalization procedure is to stretch the segment to a so-called normalized internal circumference ($IC_1$): defined as a set fraction of the internal circumference ($IC_{100}$) that a fully relaxed segment would have at a specified transmural pressure. For small rat arteries the target transmural pressure is typically 100 mmHg = 13.3 kPa.

### 3.2.1 Principles of the normalization procedure

In practice the normalization is performed by distending the segment stepwise and measuring sets of micrometer and force readings (figure 3.8). These data are converted into values of internal circumference (µm) and wall tension T (mN/mm) respectively.

Plotting wall tension against internal circumference reveals an exponential curve and by applying the isobar curve corresponding to 100 mmHg, $IC_{100}$ is calculated from the point of intersection using the Laplace relation (figure 3.9). $IC_1$ is calculated from $IC_{100}$ by multiplying a factor giving an internal circumference at which the active force production as well as the sensitivity to agonists of the segment is maximal. For rat mesenteric arteries the factor is 0.9 but both this factor as well as the transmural pressure has to be optimized for each particular segment. The normalized internal diameter is calculated by dividing $IC_1$ with π.

Appendix 2 contains a complete description of the mathematical rationale and calculations of the normalization procedure.

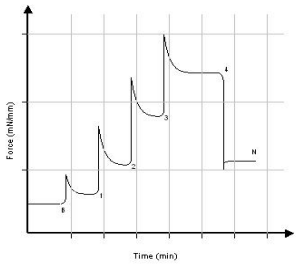



Figure 3.8 Illustration of the stepwise normalization procedure

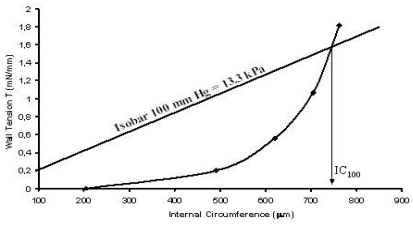



Figure 3.9 Illustration of the exponential curve fitting and determination of $IC_{100}$

## 3.3 Standard start

The purpose of performing a standard start is to:

1. Re-activate the mechanical and functional properties of the vessel segment.

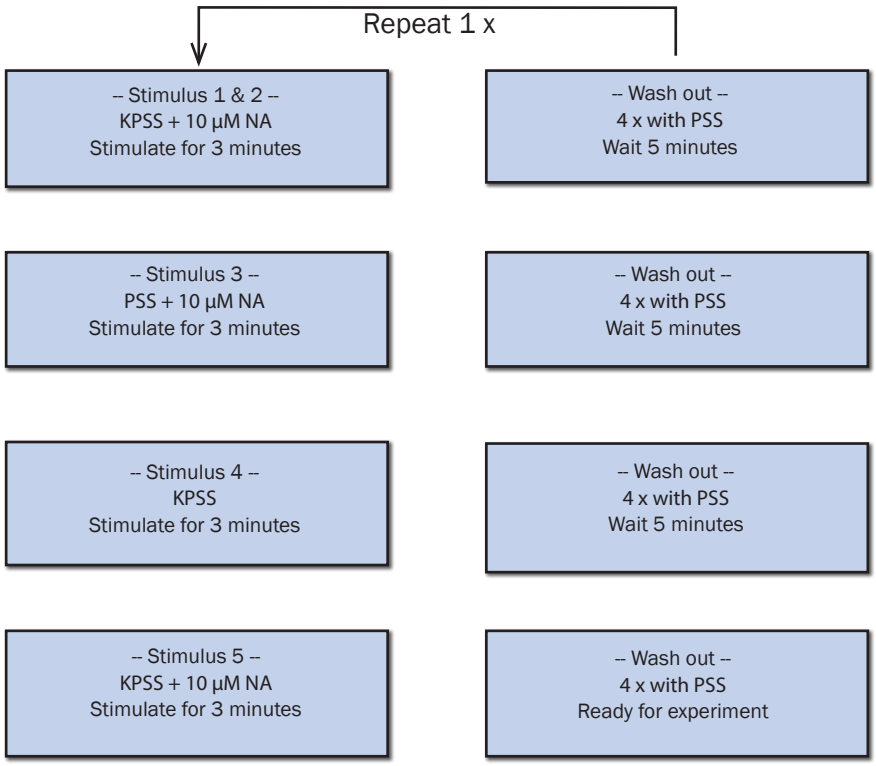
2. Check that responses to different types of stimuli are normal in appearance and thereby ensuring that the functionality of the vessel segment has not been damaged during the dissection or mounting procedures.

3. Ensure that the tension development gives an effective active pressure that is above the chosen accepted value (usually 13.3 kPa = 100 mmHg).

The standard start is performed after the vessel segment has been heated, equilibrated and normalized. The present procedure is suitable for rat mesenteric arteries. Another procedure may be needed for other animal species and tissue or vessel types.

### 3.3.1 Principles of the standard start procedure

The standard start procedure consists of a series of five stimuli and washout periods. The first two stimuli are performed using a mixture of KPSS and 10 µM noradrenaline to give a maximum contractile response. The third stimulus is performed using a mixture of PSS and 10 µM noradrenaline to give a maximum pure agonist mediated (α-adrenoceptor) contraction. The fourth stimulus is performed using KPSS to give a depolarising contractile response (this stimulus also includes a component from neurally released noradrenaline). The final stimulus is performed using a mixture of PSS and 10 µM noradrenaline. All solutions are preheated to 37°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$ before use. Instructions for making the necessary solutions are described in appendix 1.




## 3.4 Endothelium function

The reasons for checking endothelium function may include:

1. To check whether the relaxing function of the endothelium is intact. The procedure is performed to make sure that the endothelium is not damaged during the dissection or mounting procedure.

2. If an experiment requires removal of the endothelium this procedure is useful to check whether the endothelial cells were successfully removed.

The procedure can be performed after the vessel segment has been heated, equilibrated and normalized. Preferably the procedure should be done after performing a standard start to make sure that the vessel segment is viable.

The present procedure is for use with rat mesenteric arteries. Another procedure may be needed for other animal species and tissue or vessel types.

### 3.4.1 Principles of checking endothelium function

Stimulating a vessel segment with acetylcholine causes a release of nitric oxide (NO, also known as EDRF) from the endothelium cells and subsequent relaxation of the vascular smooth muscle cells. If the endothelium is undamaged by the dissection and mounting procedures, then a substantial relaxation will occur. With complete removal or damaged endothelium, a partial relaxation or no relaxation to acetylcholine is observed.

It is important to note that the amount of NO or EDRF in a vessel is often dependent upon its size. In certain vessels, endothelium-derived hyperpolarizing factor (EDHF) can contribute more or less than EDRF, and in other vessels the same stimulation with ACh can promote release of endothelium-derived contracting factor (EDCF). Therefore, it is important to check the existing literature in order to determine the expected response in your particular vessel with the given concentration of agonist.

## 3.5 In vitro experiment 1: Noradrenaline contractile response

The purpose of the present protocol is to determine the sensitivity of rat mesenteric small arteries to the vasoconstrictor noradrenaline/norepinephrine with a cumulative concentration-response curve.

### 3.5.1 Background

Noradrenaline (norepinephrine) causes contraction of mesenteric small arteries through activation of α-adrenoceptors whereas noradrenaline activation of β-adrenoceptors causes vasodilatation. As the purpose is to determine the contraction sensitivity to noradrenaline, the vasodilatory effect of noradrenaline is eliminated throughout the experiment by the constant presence of the β-adrenoceptor antagonist, propranolol.

Rat mesenteric arteries are densely innervated by sympathetic nerves, which have a highly efficient reuptake mechanism that removes noradrenaline from the neuromuscular junction. The reuptake mechanism will create a concentration gradient between the solution around the vessel segment and the receptors on the smooth muscle. To correctly determine the sensitivity to noradrenaline it is necessary to eliminate this concentration gradient by performing the experiment in the presence of cocaine to block the noradrenaline reuptake.

To determine the sensitivity to noradrenaline the vessel segment is exposed to increasing concentrations of noradrenaline. Each concentration is applied until a steady response has been reached and then the next concentration is applied. When the vessel segment is fully contracted or does not response more upon increasing the noradrenaline concentration, the experiment is ended.

### 3.5.2 Protocol

Prepare the following stock solutions:
Noradrenaline: $10^{-4}$, $10^{-3}$, $10^{-2}$ M
Propranolol: $10^{-3}$ M
Cocaine: $10^{-3}$ M

1. Mount and normalize the vessels as described in chapter 3.1 and 3.2.

2. Perform a standard start as described in chapter 3.3.

3. Incubate the vessel segment in 1 µM propranolol (add 5 µL of $10^{-3}$ M to 5 mL PSS in chamber) and 3 µM cocaine (add 15 µL of $10^{-3}$ M to 5 mL PSS in chamber) for at least 10 minutes.

4. Add increasing concentrations of noradrenaline into the bath (use the table below as a guideline). Wait for a stable contractile response or a standard time such as 2 minutes between each application.

| [NA] in chamber (µM)* | Volume of stock solution to add to chamber |
|---|---|
| 0.1 | 5 µL of $10^{-4}$ M |
| 0.3 | 1 µL of $10^{-3}$ M |
| 0.5 | 1 µL of $10^{-3}$ M |
| 1 | 2.5 µL of $10^{-3}$ M |
| 1.3 | 1.5 µL of $10^{-3}$ M |
| 1.5 | 1 µL of $10^{-3}$ M |
| 3 | 7.5 µL of $10^{-3}$ M |
| 5 | 1 µL of $10^{-2}$ M |
| 10 | 2.5 µL of $10^{-2}$ M |
| **In calculating the [NA] in the chamber, the applied volume of noradrenaline is ignored.* | |

## 3.6 In vitro experiment 2: Acetylcholine relaxation curve

The purpose of the present protocol is to determine the sensitivity of the endothelium dependent vasodilator acetylcholine in noradrenaline pre-contracted rat mesenteric small arteries.

### 3.6.1 Background

Acetylcholine causes relaxation of rat mesenteric small arteries by activating of muscarinic M3 receptors at the endothelial cell layer leading to release of endothelium-derived relaxing factors.

Rat mesenteric arteries do not show spontaneous tone in the Tissue Bath, which is why it is necessary to first induce a contraction to be able to observe the relaxation to acetylcholine. In this protocol the contraction is induced by noradrenaline. The required concentration of noradrenaline needs to be optimized since a too low concentration makes it impossible to evaluate the relaxation. On the other hand it may be difficult to relax super maximally contracted arteries, which may lead to an underestimation of the sensitivity to acetylcholine. Therefore it is recommended to apply a concentration of noradrenaline inducing 60-70% of maximal contraction response. In practice this concentration is found by performing a noradrenaline concentration-response curve as described in the previous section.

The vessel segment is exposed to the noradrenaline concentration and when the response has stabilised, increasing concentrations of acetylcholine are added to relax the vessel. Each concentration is applied until a steady response has been reached and then the next concentration is applied. When the vessel segment is either fully relaxed or does not relax more upon increasing the acetylcholine concentration, the experiment is ended.

### 3.6.2 Protocol

Prepare the following stock solutions:
Acetylcholine: $10^{-4}$, $10^{-3}$, $10^{-2}$ M
Noradrenaline: $10^{-2}$ M

1. Mount and normalize the vessels as described in chapter 3.1 and 3.2.

2. Perform a standard start and check the vessel segment for endothelium function, as described in chapter 3.3 and 3.4.

3. Add noradrenaline to obtain a response around 60% of maximum (determined from the previous noradrenaline concentration-response curve). When the contractile response is stable, add increasing concentrations of acetylcholine to the chamber, using the table below as a guideline. Wait for a stable contractile response or a standard time such as two minutes between each application.

| [ACh] in chamber (µM)* | Volume of stock solution to add to chamber |
|---|---|
| 0.1 | 5 µL of $10^{-4}$ M |
| 0.3 | 1 µL of $10^{-3}$ M |
| 0.5 | 1 µL of $10^{-3}$ M |
| 1 | 2.5 µL of $10^{-3}$ M |
| 1.3 | 1.5 µL of $10^{-3}$ M |
| 1.5 | 1 µL of $10^{-3}$ M |
| 3 | 7.5 µL of $10^{-3}$ M |
| 5 | 1 µL of $10^{-2}$ M |
| 10 | 2.5 µL of $10^{-2}$ M |
| **In calculating the [ACh] in the chamber, the applied volume of ACh is ignored.* | |

# CHAPTER 4 - CLEANING AND MAINTENANCE

## 4.1 Cleaning the Tissue Bath

DMT STRONGLY RECOMMENDS THAT THE TISSUE BATH AND SURROUNDING AREAS ARE CLEANED AFTER EACH EXPERIMENT.

At the end of each experiment, use the following procedure to clean the Tissue Bath.

1. Fill the chamber to the edge with an 8% acetic acid solution and allow it to work for a few minutes to dissolve calcium deposits and other salt build-up. Use a cotton-tipped applicator to mechanically clean all chamber surfaces.

2. Remove the acetic acid and wash the chamber and supports several times with double distilled water.

3. If any kind of hydrophobic reagents have been used which might be difficult to remove using steps 1) and 2), then try incubating the chamber and supports with 96% ethanol or a weak detergent solution (i.e. 0.1% triton-100).

4. To remove more resistant or toxic chemicals, incubate the chamber and supports with 1M HCl for up to 1 hour. In exceptional cases, incubate the chamber and supports with no stronger than a 3M $HNO_3$ solution for about 15 minutes.

5. Wash the chamber and supports several times with double distilled water

6. If acids such as 1M HCl and 3M $HNO_3$ are used to clean the chambers, make sure ALL surfaces are thoroughly dried after copious washes with double distilled water. Any residual acid will cause corrosion of the stainless steel pins.

To prevent the tubing from becoming blocked with buffer salt deposits after an experiment, remove the chamber cover from the Tissue Bath and turn on the vacuum and press the vacuum valve for about 10 seconds by holding down the valve button(s). Turn off the vacuum and gas supply. Remove any water or buffer remaining in the chamber or on the tubing using absorbent paper.

IMPORTANT NOTES

BE VERY CAREFUL USING HCL OR HNO3 BECAUSE THESE ACIDS MAY CAUSE EXTREME DAMAGE TO THE STAINLESS STEEL CHAMBERS AND SUPPORTS. DO NOT USE BLEACH TO CLEAN THE CHAMBERS. REPEATED USE OF CHLORINATED SOLUTIONS SUCH AS BLEACH AND HCL WILL CAUSE DAMAGE TO THE STAINLESS STEEL PARTS OF YOUR TISSUE BATH. AVOID USING THEM IF AT ALL POSSIBLE.

AFTER CLEANING, ALWAYS CHECK THAT THE GREASE AROUND THE TRANSDUCER PIN IS SUFFICIENT TO KEEP THE BUFFER AND WATER FROM ENTERING THE TRANSDUCER HOUSING (SEE FIGURE 4.1).

If red or brown discolorations appear on the chamber sides or on the supports, the following cleaning procedure will work in most cases:

7. Incubate the chamber and supports for 30 minutes with 2mM T-1210 Tetrakis- (2-pyridylmethyl)- ethylenediamine solution dissolved in double distilled water.

8. Use a cotton-tip applicator to mechanically clean all the affected surfaces during the last 15 minutes of the incubation period.

9. Wash the chamber and supports several times with double distilled water.

10. Incubate the chamber with 96% ethanol for 10 minutes while continuing the mechanical cleaning with a cotton-tip applicator.

11. Remove the ethanol solution and wash a few times with double distilled water. Incubate the chamber and supports with an 8% acetic acid solution for 10 minutes and continue the mechanical cleaning with a swab-stick

12. Wash the chamber and supports several times with double distilled water..

13. Dry the surfaces using absorbent paper or cotton-tip applicators.

IMPORTANT NOTES
IN EXCEPTIONAL CASES, THE SUPPORTS MAY NEED TO BE REMOVED FROM THE CHAMBER AND CLEANED INDIVIDUALLY TO ASSURE PROPER CLEANING OF ALL SURFACES. NEVER SOAK THE SUPPORTS IN ANYTHING STRONGER THAN 8% ACETIC ACID FOR EXTENDED PERIODS OF TIME (I.E. SEVERAL HOURS OR OVERNIGHT)!

## 4.2 Maintenance of the force transducer

The force transducer is the most delicate and fragile component of the Tissue Bath. Extreme care should be used when handling or touching the force transducers. As a part of daily maintenance, inspect the grease around the transducer pin extending from the transducer housing pinhole before starting any experiment, see figure 4.1 below. Insufficient grease in this area will allow buffer and water to enter the transducer housing and causing damage to the force transducer.

IMPORTANT NOTES
DMT RECOMMENDS THAT THE HIGH VACUUM GREASE SEALING THE TRANSDUCER PINHOLE IS CHECKED AND SEALED AT LEAST ONCE A WEEK, ESPECIALLY IF THE TISSUE BATH IS USED FREQUENTLY.

DMT TAKES NO RESPONSIBILITY FOR THE USE OF ANY OTHER KINDS OF HIGH VACUUM GREASE OTHER THAN THE ONE AVAILABLE FROM DMT.

DMT TAKES NO RESPONSIBILITY FOR ANY KIND OF DAMAGE TO THE FORCE TRANSDUCERS.

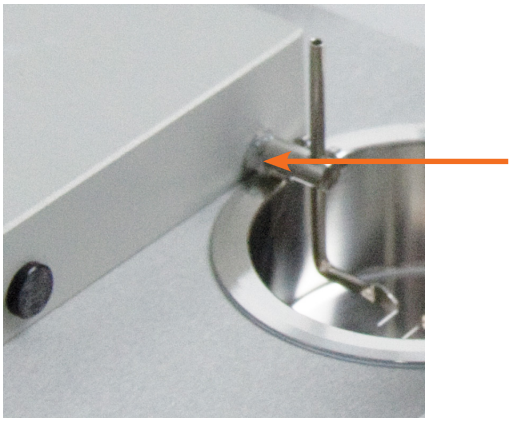


Figure 4.1 *Close-up of transducer pin from outside.*

*The arrow indicates the place that the grease needs to be applied to prevent water and buffer from damaging the transducer.*

### 4.2.1 Checking the force transducer

The force transducer is a strain gauge connected to a Wheatstone bridge. The force transducers for each chamber are housed in a separate, protective compartment. While the protective cover offers some mechanical protection for the force transducers, they are still very vulnerable to applied forces exceeding 1 Newton (100 grams) or fluid running into the transducer compartment due to insufficient greasing of the transducer pinhole.

If the force readings on the Multi Interface appear unstable or noisy, then first check that the chambers are connected properly to the Multi Interface and that the chambers are plugged all the way into the Multi Interface. If the force reading(s) are still unstable or noisy, then perform a new calibration as described in chapter 3.5.1 in Multi Myograph System User Manual.

During the new calibration, monitor the relative force reading values in the Force Calibration sub-menu on the Multi Interface (Steps 4 and 5 of the force calibration procedure). The normal operating values for the force transducer during calibration should be between 3000 and 3500.

- If the value is 0, a single digit, or a three digit number, the force transducer is broken and needs to be replaced.
- If the value is less than 2000 or greater than 4500, the force transducer has been broken and needs to be shipped to DMT for further test and a temperature compensation.
- If the message “OFF” is displayed on the main page of the Multi Interface, even though the Tissue Bath is plugged in at the rear of the Multi Interface, the force transducer is broken and needs to be replaced. In addition, if the force reading(s) appear yellow in color, cannot be reset to zero, AND the transducer cannot be recalibrated, the force transducer is broken and needs to be replaced.

If any other problems related to the force transducer are encountered, please contact DMT for advice or further instructions.

### 4.2.1 Force transducer replacement

If the force transducer breaks and needs to be replaced, follow this step-by-step replacement procedure carefully:

1. Remove the pin from the transducer pin coming out of the transducer house.
2. Disconnect the Tissue Bath from the Multi Interface.
3. Turn the Tissue Bath upside down and remove the transducer housing by loosening the two screws “A” and “B” as illustrated in figure 4.2 below.
4. The replacement transducer will be shipped with the new transducer inside a new transducer house.
5. Place a small amount of vacuum grease (clear or whitish grease) around the bottom of the transducer housing to seal the transducer housing when set back in place, see figure 4.3
6. Carefully realign the transducer housing with the new transducer on the Tissue Bath and reinsert the Allen screws through the bottom of the Tissue Bath.
7. Tighten the screws and place some vacuum grease around the transducer pin that protrudes from the transducer housing, see figure 4.3. Make sure that the hole is completely sealed to prevent buffer solution or water from entering the transducer housing and damaging the new force transducer.

IMPORTANT NOTE
CALIBRATE THE NEW FORCE TRANSDUCER BEFORE PERFORMING A NEW EXPERIMENT.


Figure 4.2 *The two screws that secure the transducer house to the unit*

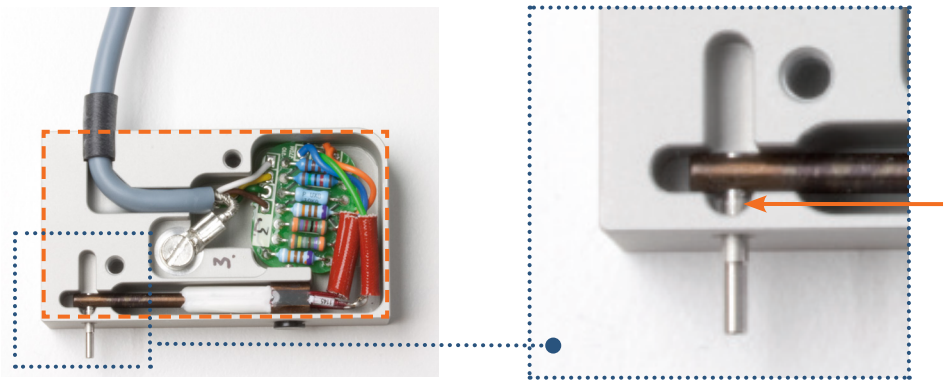


Figure 4.3 *Inside the transducer housing and close-up of transducer pin.*

*The orange arrow indicates the place that the vacuum grease needs to be applied to prevent water and buffer from damaging the transducer*

## 4.3 Maintenance of the linear slides

Check the linear slides (under the black covers) for grease at least once a week. In case of insufficient lubrication, grease the slides with the “Grease for Linear Slides” included with your system. See figure 4.4.

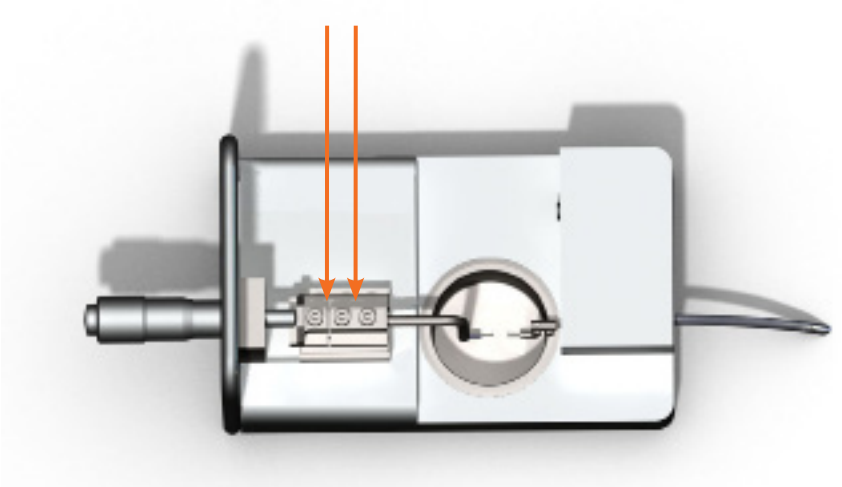


Figure 4.4 *The areas where linear slide grease may be applied for smooth micropositioner movement*

# APPENDIX 1 - BUFFER RECIPES

## Physiological Saline Solution (PSS)

### 1x PSS:

| Chemical | Mol.Wt | mM | g/0.5L | g/L | g/2L | g/4L |
|---|---|---|---|---|---|---|
| NaCl | (58.45) | 130 | 3.799 | 7.598 | 15.20 | 30.39 |
| KCl | (74.557) | 4.7 | 0.175 | 0.35 | 0.70 | 1.40 |
| $KH_2PO_4$ | (136.09) | 1.18 | 0.08 | 0.16 | 0.32 | 0.64 |
| $MgSO_4\ 7H_2O$ | (246.498) | 1.17 | 0.145 | 0.29 | 0.58 | 1.16 |
| $NaHCO_3$ | (84.01) | 24.9 | 1.05 | 2.10 | 4.18 | 8.37 |
| Glucose | (180.16) | 5.5 | 0.5 | 1.00 | 2.00 | 4.00 |
| EDTA | (380) | 0.026 | 0.005 | 0.01 | 0.02 | 0.04 |
| $CaCl_2$ | (110.99) | 1.6 | 0.8mL | 1.6mL | 3.2mL | 6.4mL |

1. Make a 1.0M solution of $CaCl_2$ (110.99) in double-distilled $H_2O$. Filter-sterilize the calcium solution through a 0.22 µm filter. The sterilized solution can be stored in the refrigerator for up to 3 months.

2. Dissolve all the chemicals except the $CaCl_2$ in approximately 80% of the desired final volume of double distilled $H_2O$ while being constantly stirred. For example, if 1 litre of PSS is to be made, then dissolve all the chemicals in 800mL of double distilled $H_2O$.

3. Add the appropriate volume of 1.0M $CaCl_2$ for the total volume of PSS being made (for example, 1.6mL of 1.0M $CaCl_2$ for 1 litre of buffer). Continue to stir the PSS while the $CaCl_2$ is being added.

4. Bring the solution up to the final volume with double-distilled $H_2O$. Continue to stir the solution until the EDTA is fully dissolved. This takes about 15 minutes at room temperature.

5. Aerate the solution with carbogen (95% $O_2$ + 5% $CO_2$) for about 20 minutes.

### 25x Concentrated PSS:

| Chemical | Mol.Wt | mM | g/0.5L | g/L | g/2L | g/4L |
|---|---|---|---|---|---|---|
| NaCl | (58.45) | 3250 | 94.98 | 189.96 | 379.92 | 759.84 |
| KCl | (74.557) | 117.5 | 4.375 | 8.75 | 17.5 | 35.0 |
| $KH_2PO_4$ | (136.09) | 29.5 | 2.0 | 4.0 | 8.0 | 16.0 |
| $MgSO_4\ 7H_2O$ | (246.498) | 29.25 | 3.625 | 7.25 | 14.5 | 29.0 |
| $NaHCO_3$ | (84.01) | 622.50 | 26.25 | 52.50 | 104.50 | 209.25 |
| Glucose | (180.16) | 137.50 | 12.50 | 25.00 | 50.00 | 100.00 |
| EDTA | (380) | 0.65 | 0.125 | 0.25 | 0.50 | 1.0 |
| $CaCl_2$ | (110.99) | 40 | 20mL | 40mL | 80mL | 160mL |

1. Make a 1.0M solution of $CaCl_2$ (110.99) in double-distilled $H_2O$. Filter-sterilize the calcium solution through a 0.22 µm filter. The sterilized solution can be stored in the refrigerator for up to 3 months.

2. Dissolve all the chemicals except the NaHCO3, Glucose, and CaCL2, in approximately 80% of the desired final volume of double distilled $H_2O$ while being constantly stirred. For example, if 1 litre of PSS is to be made, then dissolve all the chemicals in 800mL of double distilled $H_2O$.

3. Bring the solution up to the final volume with double-distilled $H_2O$. Continue to stir the solution until the EDTA is fully dissolved. This takes about 15 minutes at room temperature.

Before use:

4. Dilute the 25 x PSS stock solution 1:25 using double distilled $H_2O$.

5. Add:
   1.091 g/L Glucose
   2.100 g/L $NaHCO_3$

6. Add the appropriate volume of 1.0M $CaCl_2$ for the total volume of PSS being made (for example, 1.6mL of 1.0M $CaCl_2$ for 1 litre of buffer). Continue to stir the PSS while the $CaCl_2$ is being added.

7. Bring the solution up to the final volume with double-distilled $H_2O$. Aerate the solution with carbogen (95%$O_2$ + 5%$CO_2$) for at least 20 minutes. If necessary wait further for the pH of the buffer to reach pH 7.4.

## High potassium Physiological Saline Solution (KPSS)

### 1x 60mM KPSS:

| Chemical | Mol.Wt | mM | g/0.5L | g/L | g/2L | g/4L |
|---|---|---|---|---|---|---|
| NaCl | (58.45) | 74.7 | 2.18 | 4.37 | 8.73 | 17.46 |
| KCl | (74.557) | 60 | 2.24 | 4.47 | 8.95 | 17.89 |
| $KH_2PO_4$ | (136.09) | 1.18 | 0.08 | 0.16 | 0.32 | 0.64 |
| $MgSO_4$ $7H_2O$ | (246.498) | 1.17 | 0.145 | 0.29 | 0.58 | 1.16 |
| $NaHCO_3$ | (84.01) | 24.9 | 1.05 | 2.10 | 4.18 | 8.37 |
| Glucose | (180.16) | 5.5 | 0.5 | 1.00 | 2.00 | 4.00 |
| EDTA | (380) | 0.026 | 0.005 | 0.01 | 0.02 | 0.04 |
| $CaCl_2$ | (110.99) | 1.6 | 0.8mL | 1.6mL | 3.2mL | 6.4mL |

1. Make a 1.0M solution of $CaCl_2$ (110.99) in double-distilled $H_2O$. Filter-sterilize the calcium solution through a 0.22 µm filter. The sterilized solution can be stored in the refrigerator for up to 3 months.

2. Dissolve all the chemicals except the $CaCl_2$ in approximately 80% of the desired final volume of double distilled $H_2O$ while being constantly stirred. For example, if 1 litre of PSS is to be made, then dissolve all the chemicals in 800mL of double distilled $H_2O$.

3. Add the appropriate volume of 1.0M $CaCl_2$ for the total volume of PSS being made (for example, 1.6mL of 1.0M $CaCl_2$ for 1 litre of buffer). Continue to stir the PSS while the $CaCl_2$ is being added.

4. Bring the solution up to the final volume with double-distilled $H_2O$. Continue to stir the solution until the EDTA is fully dissolved. This takes about 15 minutes at room temperature.

5. Aerate the solution with carbogen (95% $O_2$ + 5% $CO_2$) for about 20 minutes.

# APPENDIX 2 - NORMALIZATION THEORY

The importance of making a normalization before initiating an experiment with any tubular tissue segment is described in chapter 3.2. In this appendix the mathematical rationale and calculations underlying the normalization procedure are described in detail.

## Mathematical calculations

Let $(X_i, Y_i)$ be the pair of values representing the micrometer reading (see appendix 3) and force reading respectively characterizing each step in the normalization procedure. $Y_0$ is the force reading at the start position of the normalization procedure where the wires are just separated and the force reading is approximately zero. Then, given that tension on the vessel is equal to force divided by wall length, the wall tension at the i-th micrometer reading is calculated by:

$$T_i = \frac{(Y_i - Y_0)}{2\delta \bullet (a_1 - a_2)}$$

where δ is the microscope eyepiece reticule calibration factor in mm per division and $a_1$ and $a_2$ are the vessel end points when measuring the length of the mounted vessel segment.

The internal circumference of the mounted vessel at the i-th reading is calculated by:

$$IC_i = IC_0 + (2 \bullet (X_i - X_0))$$

where $IC_0$ is the internal circumference of the mounted vessel when the wires are just separated and is given by:

$$IC_0 = (2 + \pi) \bullet d$$

where d is the wire diameter. For 40 µm wires, $IC_0$ = 205.6 µm.

Using the Laplace relation, the effective pressure $P_i$ is calculated for each pair of readings. The effective pressure is an estimate of the internal pressure, which is necessary to extend the vessel to the measured internal circumference.

$$P_i = \frac{T_i}{\left(\frac{IC_i}{2\pi}\right)}$$

The stepwise distension is continued until the calculated effective pressure exceeds the target transmural pressure. The target value needs to be optimized for the individual tissue preparation (optimal active force as determined by the length-tension relationship for that tissue). For rat mesenteric arteries the target transmural pressure is normally 100 mmHg (13.3 kPa):

$$T_{100\ mmHg} = 100\ mmHg \bullet \left(\frac{IC}{2\pi}\right)$$

An exponential curve is fitted to the internal circumference pressure data as illustrated in figure 3.9. Now the isobar corresponding to 100 mmHg is used to calculate the $IC_{100}$ value from the point of interception between the function of the exponential curve and the function of the 100 mmHg isobar.

The normalised internal circumference $IC_1$ is calculated by multiplying the internal circumference corresponding to 100 mmHg, $IC_{100}$, by a factor k. The factor is for rat mesenteric arteries 0.9. Again, this value should be optimized for the particular tissue preparation being used by a length-tension curve.

$$IC_1 = k \bullet IC_{100}$$

The normalized internal (lumen) diameter is then calculated by:

$$d_1 = \frac{IC_1}{\pi}$$

The micrometer reading $X_1$ at which the internal circumference of the normalized vessel is set to is calculated by:

$$X_1 = X_0 + \frac{(IC_1 - IC_0)}{2}$$

# APPENDIX 3 - READING A MILLIMETRE MICROMETER

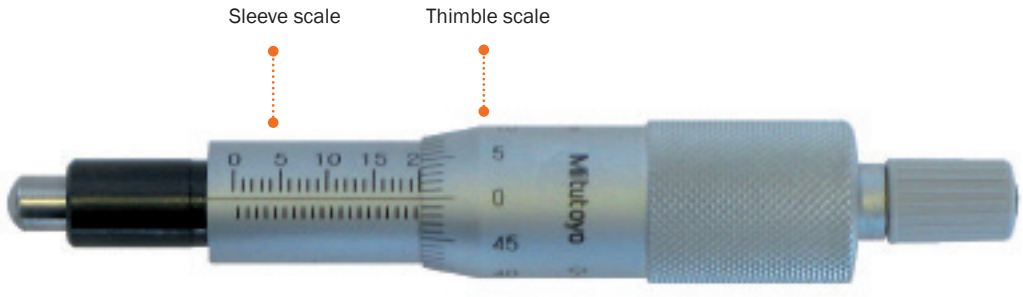



Figure A3.1 *Overview of the micrometer parts (actual reading 20000 µm = 20 mm)*

## Sleeve scale

The micrometer sleeve scale has a total length of 25 mm divided into 50 equal parts. Each part of a division above the horizontal line represents 1 mm, where each 5$^{th}$ line is marked by a longer line and a number designating the length in mm. Each division below the horizontal line is placed between each 1 mm mark (scale above the horizontal line) and represents 0.5 mm.

## Thimble scale

The thimble is divided into 50 equal parts, and one complete rotation of the thimble is indicated by the smallest division on the sleeve, which equals 0.5 mm. Each division on the thimble scale is 10 µm. If the thimble scale falls between two lines, then a number between 0 and 10 µm must be approximated.

## Example 1

1. Note that the thimble has stopped at a point beyond "10" on the sleeve indicating 10000 µm (10 mm).
2. Note that there is no mark completely visible between the 10 mm mark and the thimble.
3. Read the value on the thimble corresponding to the intersection with the horizontal line on the sleeve.

| | | |
|---|---|---|
| A. | Reading on sleeve: | 10000 µm |
| B. | No additional mark visible: | 0 µm |
| C. | Thimble reading: | 380 µm |
| | Total reading: | 10380 µm |


Figure A3.2 *Example 1: reading = 10380 µm*

## Example 2

1. Note that the thimble has stopped at a point beyond "16" on the sleeve indicating 16000 µm (16 mm).
2. Note that this time a mark is visible between the 16 mm mark and the thimble indication 500 µm.
3. Read the value on the thimble corresponding to the intersection with the horizontal line on the sleeve.

| | | |
|---|---|---|
| A. | Reading on sleeve: | 16000 µm |
| B. | One additional mark visible: | 500 µm |
| C. | Thimble reading: | 280 µm |
| | Total reading: | 16780 µm |


Figure A3.3 *Example 2: reading = 16780 µm*