

The Effect of Mercury on Acto-Myosin Motility *in vitro*

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Introduction

Mercury is a toxic heavy metal that is often found as a contaminant in our environment. The toxicity of mercury is believed to be a product of its interaction with sulphhydryl (SH) groups – essential for the normal function of many proteins [Clarkson, 1972 *Annu. Rev. Pharmacol. Toxicol.* **12**: 375-406]. In a previous study we demonstrated that mercury had a significant effect on the interaction of actin and cofilin [Kekic and dos Remedios, 1999 *Electrophoresis.* **20**(10):2053-8]. Actin is an abundant protein and is found in virtually all eukaryotic cells. It plays vital roles in a large number of cellular activities and is one of the major components in muscle – interacting with myosin to produce muscle contraction. Cofilin is an important actin binding protein (ABP) that plays a major regulatory role with actin (for a comprehensive review of this field see upcoming review by dos Remedios *et al*: *Actin Binding Proteins and Regulation of Cytoskeleton Microfilaments*). Actin and cofilin form a 1:1 complex when mixed *in vitro* and this complex formation can be readily monitored using native polyacrylamide gel electrophoresis (native-PAGE). By increasing the concentration of Mercuric Acetate it was possible to disrupt the formation of an actin:cofilin complex (see Fig. 1 below).

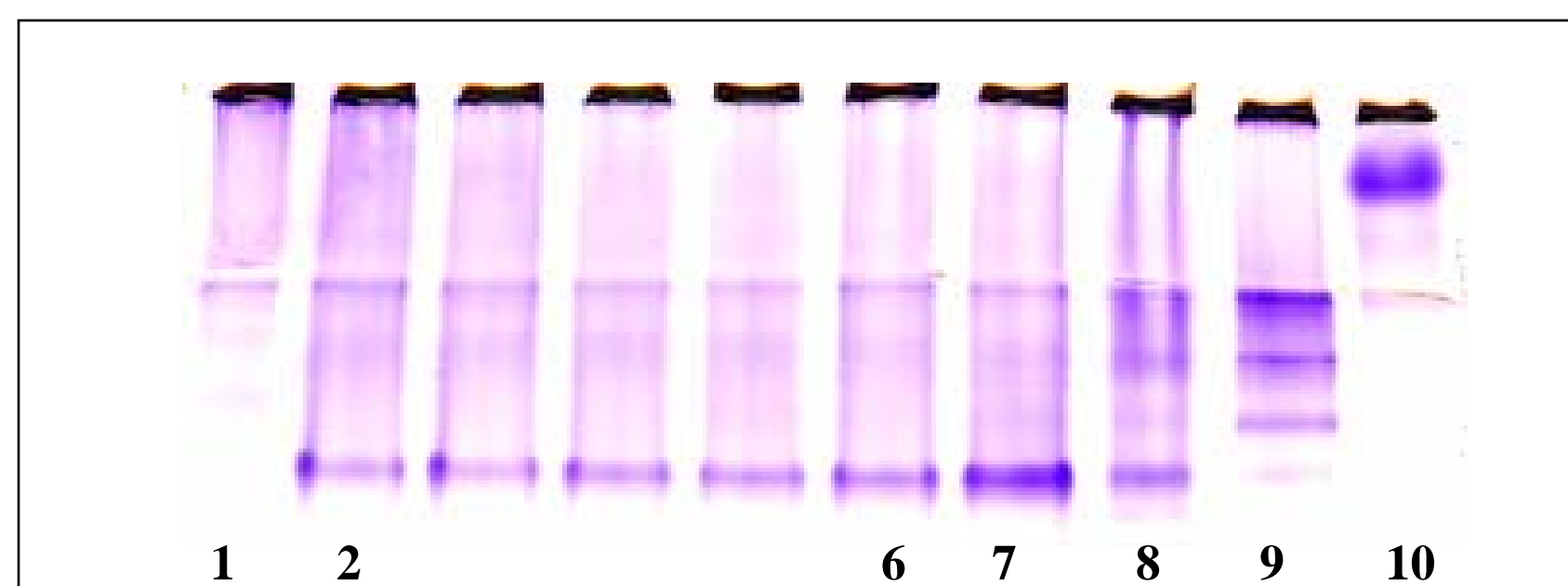


Fig. 1 12% Native-PAGE showing the effects of mercury on the actin:cofilin complex. Lane 1 is g-actin alone and lane 10 is cofilin alone. The actin:cofilin complex is shown in lane 2. Lanes 6-9 contain increasing levels of mercury (0.002-0.02 µg/L). The actin:cofilin complex has been disrupted in lane 9 (0.02 mg/L mercury)

The motor domain of myosin contains 2 reactive SH residues (SH1 and SH2) which are considered critical for the proper functioning of myosin. These are the only 2 SH residues exposed to solvent in this region of myosin [Barany and Barany, 1959 *Biochim. Biophys. Acta.* **35**: 293-305]. Very early observations showed that organomercurial modification of SH1 resulted in a decrease of ATPase activity and modification of SH2 inhibited the ATPase activity completely [Kielley and Bradley, 1956 *J. Biol. Chem.* **208**: 653-659] In fact, myosin ATPase activity has been shown to be completely blocked by the presence of as little as 1 µM HgCl₂ [Vassallo *et al.* 1999 *Toxicol. Appl. Pharmacol.* **156**:113-118]. Thiol protective agents such as DTT are shown to guard the myosin ATPase from the damaging effects of mercury. Furthermore, it has been demonstrated that even slight modification to either of the SH groups by various compounds resulted in complete loss of any sliding motility [Tiepold *et al.* 2000 *Biochemistry* **39**(6): 1305-1315]

In this study we will detail the process required to set up a reliable (control) acto/myosin *in vitro* motility assay. In addition, we will examine the effects of various concentrations of mercury (0 – 100 µM) on the sliding velocities actin filaments under several different conditions – including in the presence and absence of DTT. In 3 different experiments mercury will be introduced into the assay in one of the following ways: 1. Myosin will be incubated with mercury prior to its introduction into the assay and the rest of the assay will proceed as per the control (+/- DTT). 2. Actin filaments will be incubated with mercury prior to their introduction to the assay (+/- DTT). 3. Mercury will be added to the motility buffer prior to its introduction into the assay (+/- DTT).

Methods

Protein Preparation:

Myosin is prepared from rabbit back muscle using a method adapted from: Margossian and Lowey, 1982 *Methods in Enzymology* **85**:55. It is critical that the muscle is dissected within 30 min of the rabbit has been euthanized.

HMM is digested from freshly prepared myosin using a-chymotrypsin using the method of Margossian and Lowey, 1982 *Methods in Enzymology* **85**:61. Once prepared, the HMM can be snap-frozen in liquid nitrogen and stored for up to 30 days at -80°C.

Actin is prepared using the method of Spudich and Watt, 1971 *J. Biol. Chem.* **246**:4866 as modified by Barden and Dos Remedios, 1984 *J. Biochem.* **96**:913.

Preparing for Motility Assay (Adapted from: Sellers *et al.*, 1993 in *Methods in Cell Biology (Motility Assays for Motor Proteins)* **39**:24)

Buffers Used:

A. 4 mM Imidazole pH 7.0, 2 mM MgCl₂, 0.1 mM EGTA, 3 mM Na₃N and 1 mM DTT.
B. 0.5 M NaCl, 10 mM MOPS pH 7.0, 0.1 mM EGTA and 1 mM DTT.
C. 20 mM KCl, 10 mM MOPS pH 7.2, 5 mM MgCl₂, 0.1 mM EGTA and 10 mM DTT.
M. 20 mM KCl, 10 mM MOPS pH 7.2, 5 mM MgCl₂, 0.1 mM EGTA, 1 mM MgATP and 10 mM DTT. The following are used to inhibit photobleaching: 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase (Sigma cat # G-7016) and 0.02 mg/ml catalase (Sigma cat # C-100)

Labelling Actin with Rhodamine Phalloidin (Molecular Probes cat # R-415)

1. Prepare a solution of 20 mM F-actin in buffer A.
2. Add 1.5 ml of methanol to the entire contents of R-415. This gives a concentration of 6.6 mM rhodamine phalloidin. Place 30 ml of this into an Eppendorf tube and dry with a Speed Vac-this usually takes 3-4 hours on low heat.
3. Redissolve with ~3 ml of methanol and add 90 ml of buffer A.
4. Add 10 ml of the 20 mM F-actin solution to this and gently vortex. For best results leave this overnight on ice. This "stock" is generally good for use for 2-3 weeks if stored on ice.
5. Dilute the stock into buffer C on the morning of the assay to a concentration of 10-20 nm (10 nm seems best.)

Preparing the Assay Chamber

Nitrocellulose coating a coverslip: Coverslips (either 18 x 18 mm or 22 x 22 mm) are cleaned with 70% ethanol and a lint free tissue. Same applies for the glass slides. Take a 2 L (or bigger) beaker and fill to the rim with deionized water. Take a lint free tissue and swipe the surface of the water to remove any dust etc. Add 1 drop of nitrocellulose (Fullam NY, 1% nitrocellulose in anyl acetate, ultra-pure cat # 11180). Leave for ~ 1 min to dry and then gently place the coverslip on the surface. Leave this for 1 min and then remove the coverslip by grabbing a corner with forceps and pushing down into the beaker and inverting the coverslip under water. Remove coverslip from the beaker and leave to air dry (~1 hour). The assay chamber is constructed as shown in Fig. 2.

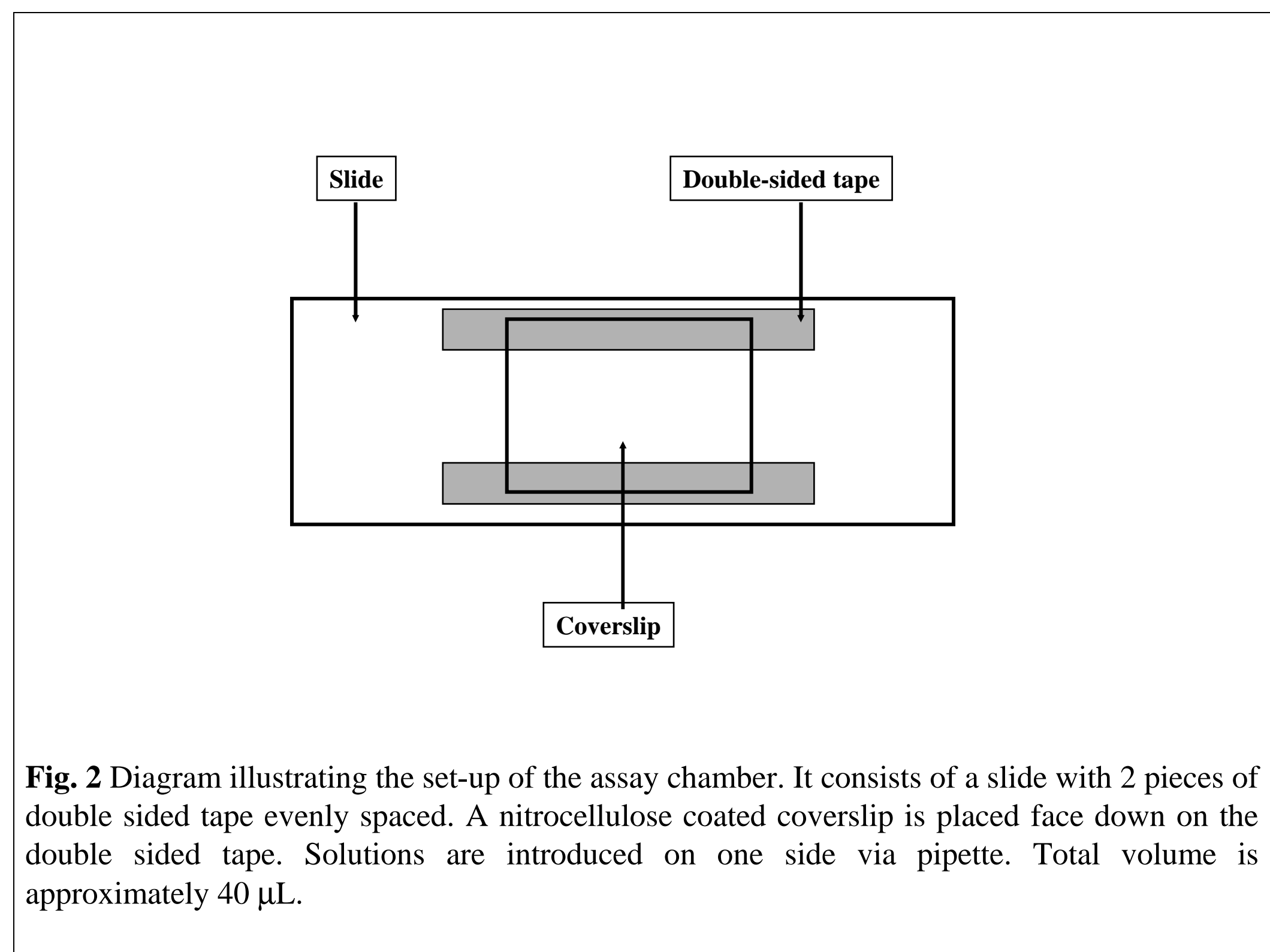


Fig. 2 Diagram illustrating the set-up of the assay chamber. It consists of a slide with 2 pieces of double sided tape evenly spaced. A nitrocellulose coated coverslip is placed face down on the double sided tape. Solutions are introduced on one side via pipette. Total volume is approximately 40 µL.

HMM is prepared by taking a 1 mg/ml stock and adding an equimolar amount of F-actin. Leave this to incubate on ice for ~10 min and then Add 2 mM MgATP. Spin at 100000 rpm for 10-15 min at 4°C. This should remove any "dead" HMM from the solution.

1. Dilute the stock HMM into buffer C - 0.05-0.1 mg/ml
2. Introduce the HMM into the flow cell. Keep the slide on an angle of 20-30° to help with flow. Incubate for 1-2 min.
3. Wash the flow cell with a solution of 1 mg/ml BSA in buffer C. Incubate for 1-2 min.
4. Wash with 2 vol of a solution of 5 µM unlabelled F-actin in buffer C (sheared by passing through a 26 gauge needle). Incubate for 2 min.
5. Wash with 2-3 vol of 1.5 mM MgATP in buffer C, immediately followed by 2-3 vol of buffer C.
6. Add 2 vol of the 10-20 nm rhodamine-phalloidin-actin and incubate for 1-2 min.
7. Add 2 vol buffer M to start the reaction.

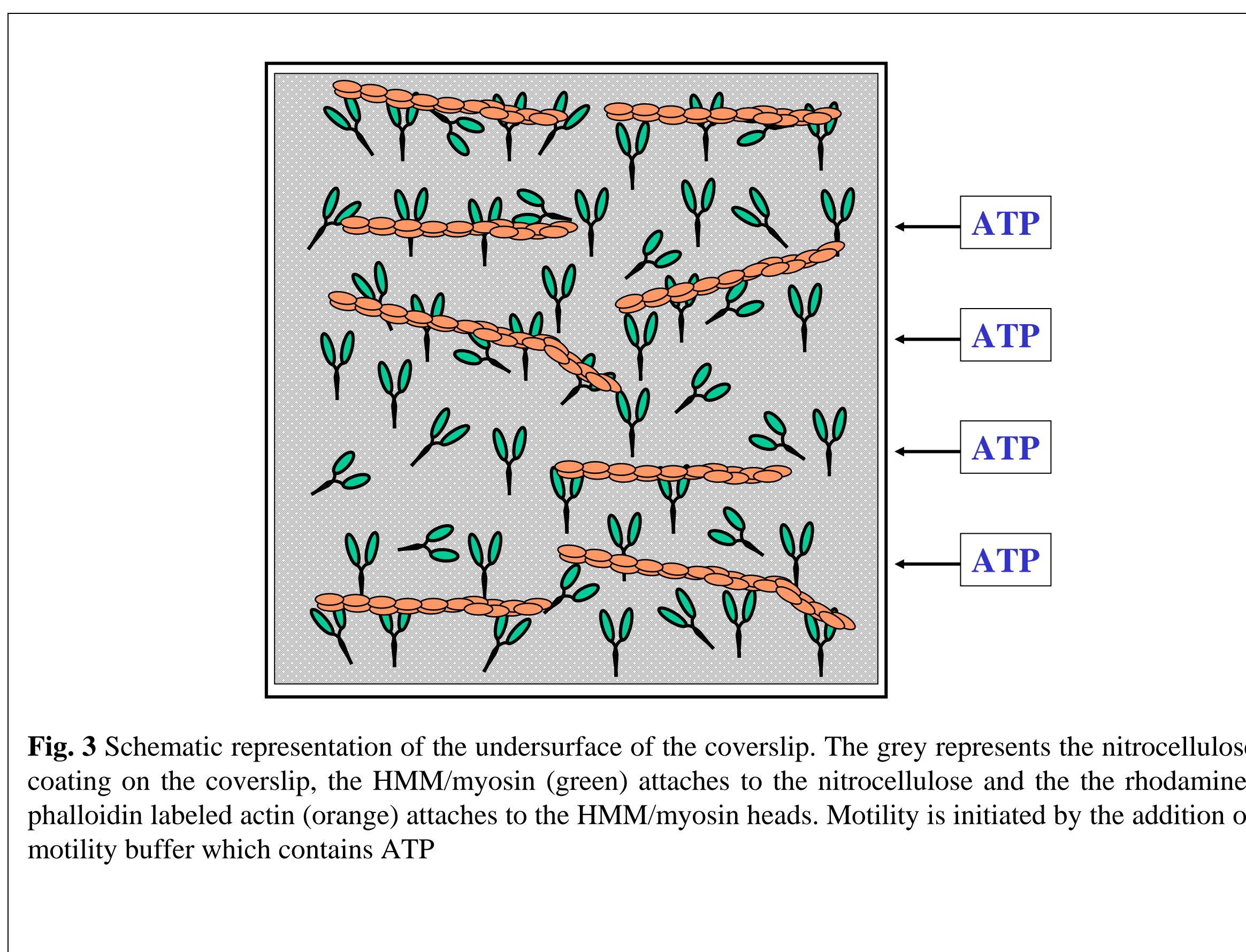


Fig. 3 Schematic representation of the undersurface of the coverslip. The grey represents the nitrocellulose coating on the coverslip, the HMM/myosin (green) attaches to the nitrocellulose and the rhodamine-phalloidin labeled actin (orange) attaches to the HMM/myosin heads. Motility is initiated by the addition of motility buffer which contains ATP

The above details the procedures for a control assay. The effects of mercury on motility will be examined in the following experiments. A 0.01 M mercury stock was prepared from HgCl₂ taking into account the molecular mass of the Cl.

- 1.HMM will be incubated for 30 min with mercury (100 µM) prior to its introduction into the assay and the rest of the assay will proceed as per the control (+/- DTT).
2. Actin filaments will be incubated for 30 min with mercury (100 µM) prior to their introduction to the assay (+/- DTT).
3. Mercury (100 µM) will be added to the motility buffer prior to its introduction into the assay (+/- DTT).

Microscopy and Measurements

Microscopy was performed on a NIKON ECLIPSE E800 - VFM, X100 oil immersion.

Images were captured using a SENSICAM PCO 12bit CCD camera. The resulting images were animated using Jasc Animation Shop V3.0.

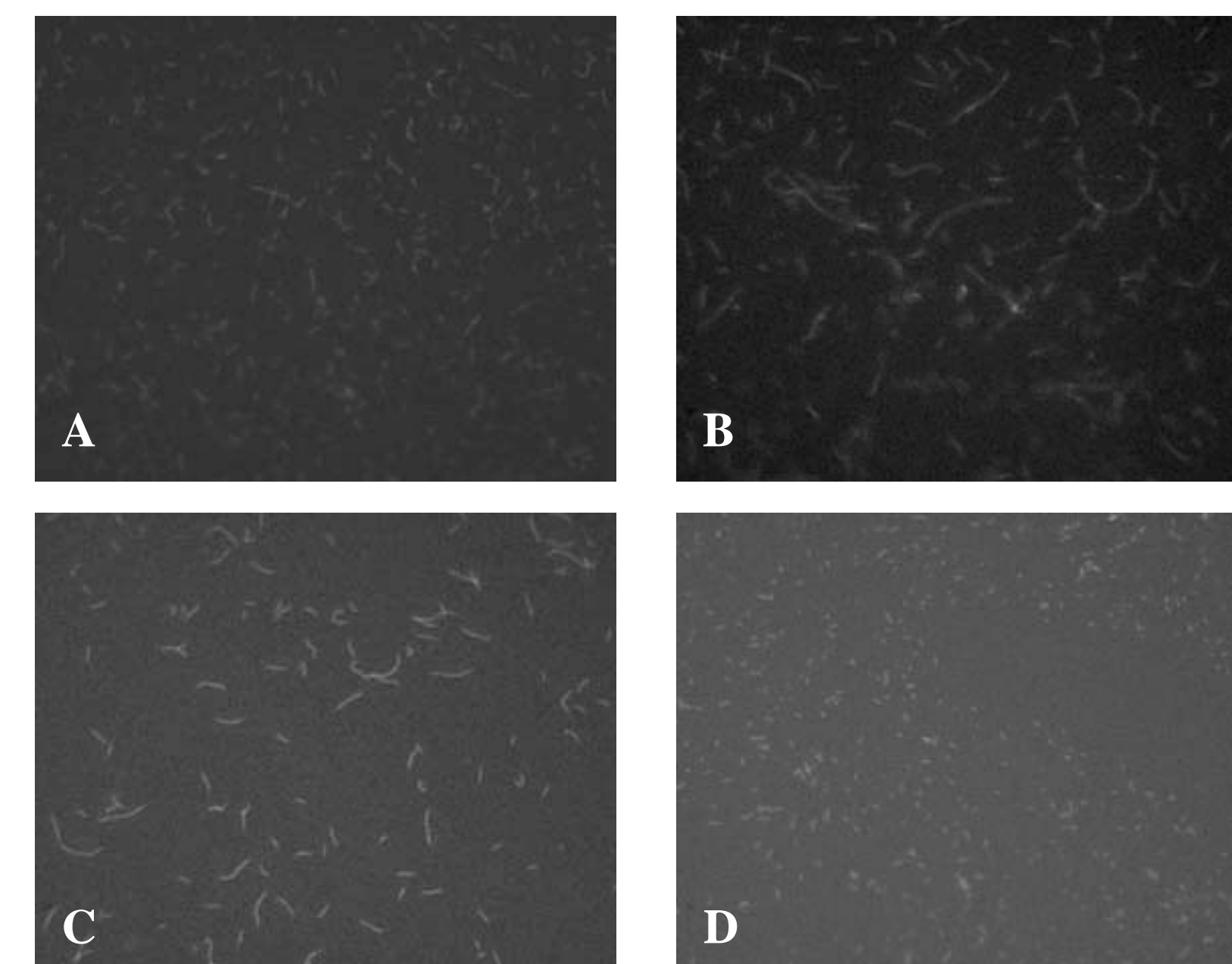
Velocity and filament length measurements were performed using Retrac-2 rev7

Results

The results are detailed in table 1. DTT was removed from all buffers in the relevant assays.

Sample	Motility	Observations
Control +/- DTT	Yes	Normal motility (~4 µm/s). Sample -DTT photo bleached quicker. (See A – Fig 4)
HMM + 100 µM Hg + DTT	Yes	Normal motility.
HMM + 100 µM Hg - DTT	No	Motility completely abolished. Does not return upon addition of DTT. (See B – Fig 4)
Actin + 100 mM Hg + DTT	Yes	Normal motility. Filaments have a significantly longer average length. (See C – Fig 4)
Buffer M + 100 mM Hg + DTT	Yes	Normal motility
Buffer M + 100 mM Hg - DTT	Marginal	Majority of filaments are not moving. (See D – Fig 4)

Selected images from these assays are shown below in Fig 4. The relevant "movie" may be viewed on the laptop computer.



Discussion

An *in vitro* motility assay is a powerful tool for studying the interaction of actin and myosin under various experimental conditions. In this study we have detailed how to routinely perform such an assay and have extended this to include some interesting experimental observations.

The most significant result arising from this work is that mercury has a detrimental effect on acto/myosin motility when no DTT (thiol protective reagent) is present (as seen with the HMM incubated with Hg and also when Hg was introduced in buffer M). Interestingly, the effect of mercury cannot be reversed by adding DTT (this was seen with the HMM incubated with 100 µM Hg). This suggests that mercury has an irreversible effect on the force producing properties of myosin. Further experiments will need to be completed to measure the effects of the myosin ATPase.

Also of interest was the increased filament length seen when actin was incubated with 100 µM Hg. This suggests that mercury may have an effect on the polymerisation of actin filaments or perhaps a filament stabilising effect.

Quite clearly, there needs to be more analysis of the current data and this has not been completed due to time constraints. As mentioned, future experiments measuring myosin ATPase will be performed as will assays involving different heavy metals and environmental pollutants.

Acknowledgements

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