# Immobilization of living specimens for microscopic observation

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**Abstract:** One major challenge for high magnification, high resolution microscopy of living organisms is proper immobilization of an actively moving specimen for observations and manipulations. A range of techniques, using chemical, physical, or mechanical means of immobilization of specimens has been described and published. We briefly review and evaluate many of these techniques. We discuss mechanical immobilization devices that hold the specimen between precisely controlled glass surfaces for the desired observation. These devices, known as rotocompressors or microcompressors, involve precision lowering of a coverslip until the specimen is trapped. Careful manipulation can gently flatten the specimen for extended times of observation. The coverslip can then be released and the specimen recovered for later observations or for culturing. Microfluidics technology can be combined with mechanical compression of the specimen permitting controlled amounts of chemicals or media to be applied while the specimen is under observation.

**Keywords:** immobilization; rotocompressor; microcompressor; compressorium; microfluidics; total internal reflection fluorescence microscopy

## 1. Introduction

From the earliest days of light microscopy, a major challenge has been the development of some kind of adequate, non-destructive immobilization techniques for careful examination of active, living biological specimens. Although some organisms or cells are sessile, many more, especially protists and small metazoa, are active swimmers or crawlers, making extended observations or manipulations difficult or impossible. A brief overview of the range of traditional and newer techniques that have been developed to capture and immobilize specimens is presented here. In this article, we will also very briefly discuss the advantages and disadvantages of many of these techniques. Emphasis will be made on recent designs and applications combining immobilization with microfluidics technology to permit a change of medium or application of drugs or chemoattractants to the specimen while it is being observed. Effluent from cells that are being trapped can also be collected after treatments for various chemical or mass spectrometry analyses. Additionally, new techniques, like total internal reflection fluorescence microscopy (TIRFM), can be enhanced by the gentle flattening of a cell. For comprehensive reviews of the literature, the reader is encouraged to consult with several of the references cited in this article.

## 2. Criteria for Immobilization

The process of capture and immobilization of a specimen involves a number of criteria and factors that conflict with one another, so any chosen technique must represent a compromise among those criteria. Immobilization of biological specimens can often introduce artifacts into the regular behaviour and physiology of those specimens, so the researcher must perform appropriate controls, and exercise caution in application and interpretation of any results from these techniques. Nevertheless, carefully applied, images and data of very high quality can be acquired. Several criteria for successful immobilization can be identified, and these criteria might influence the choice of technique used to hold the specimen.

Immobilization of an active, living specimen could possibly introduce some kind of changes to the organism's physiology and/or behaviour, but one should expect that the technique be as **benign as possible,** introducing a minimal amount of disruption. Assessment of the extent of effects upon the specimen might be necessary to validate the observations obtained. The researcher might also seek to use a few different methods of immobilization, to determine if the results from one technique are comparable to those from other techniques.

After observation, the researcher might want to recover the specimen from the microscope and keep it for later observations or for culturing. Therefore, the effects of the immobilization process should be **readily reversible,** so that the specimen can be accessed by the researcher, and should be able to recover quickly and return to a more normal culture state. Different methods reviewed here do have different degrees of reversibility, depending on the species being observed.

Whatever technique is selected, it should not interfere with the **optical characteristics** of the microscope in use. Ideally, the immobilization technique should enhance the performance of the optics to produce better images of the

 

specimen. Some of the techniques reviewed here do so by flattening the specimen between two nearly-parallel surfaces, through which the optical path of the imaging system passes.

The immobilization technique of choice would be especially valuable if it could be **combined with other possible manipulations** of the specimen. The possible combination of immobilization with microfluidics systems (see below for further applications), or some optical treatments, such as laser optical force traps (laser tweezers), or laser surgery, would therefore become very powerful tools for manipulation and analysis of specimens.

Ultimately, the researcher must consider a range of possible techniques for use in a particular investigation and a particular species under study, and select the techniques of appropriate utility. No technique is going to fully satisfy all the criteria listed, so any approach must include some kind of compromise among competing priorities. A variety of techniques, using different mechanisms for immobilization, might be applied to increase one's confidence that the results obtained are not the result of artifacts arising from a single observation protocol used.

## 3. Techniques of Immobilization

### 3.1 Chemical

Depending on the species being observed, a long list of chemicals has been identified as agents inducing torpor, anaesthesia, or paralysis to immobilize the specimen. For examples, many protistans can be immobilized by exposure to measured concentrations of nickel or copper ions to interfere with their ciliary/flagellar activity, and many multicellular species can be anaesthetized by various chemical agents. A comprehensive review of these techniques cannot be done in this short article. The interested reader can refer to the extensive lists and reviews by Delly [1; 2] for details and primary references concerning these chemicals, the specimens on which they are used, and their effects.

The general problem with chemical immobilization techniques is that, essentially by definition, one has applied some kind of molecular/biochemical disruption to the physiology and molecular activity of the cell. Some of the preparations are toxic, and the specimen could be slowly dying as one observes it. For some observations, such as basic morphology descriptions, these techniques might be appropriate, but for physiology or biochemical studies, the chemical used might be a completely inappropriate choice [e.g., 16]. Therefore, the possibility of treatment artifacts, and also the issue of reversibility of the immobilization effect, could very significant to the technique used.

### 3.2 Physical

A range of physical manipulations to the specimen can be used to immobilize it for microscopic observations. These include adjustments to the viscosity of the medium the specimen is in, trapping the specimen in a maze of fibers, and a diverse series of more exotic techniques.

Viscosity-based slowing or immobilization of specimens has been a well-established and widely used approach for many years. A number of proprietary products are available from biological supply houses, and recipes for one's own preparations using methylcellulose or polyvinyl alcohol are easily obtained [1, 4]. The idea is to mix the specimen into this viscous substance, making rapid motions difficult or impossible. The viscous substance must be as biologically inert as possible, non-toxic, and also as non-osmotic as possible, to minimize any detrimental effects on the specimen. At an extreme, some workers have embedded the specimen in solidified agar or gelatin [e.g., 17]. Reversibility would involve retrieving the specimen from the viscous medium, if that is possible [8].

Another physical approach involves placing a shredded piece of lens or filter paper, or various fibers, natural or synthetic, on the slide along with the specimens. Scanning with a low power objective can allow the observer to identify specimens that are trapped within a network of the fibrous material and limited to a small area [1]. Although the technique is simple, it is difficult to control the placement and entrapment of a particular specimen, much of the specimen might be obscured by the random array of fibers, and retrieval of the specimen could be problematic.

A group of unusual or exotic physical immobilization techniques have also been described in the literature [1, 3]. These include, for example, feeding the specimen magnetic particles and then immobilizing the specimen by applying a magnetic field to the microscope stage [e.g., 9, 18]. Some organisms can be immobilized by exposure to antibodies homologous to their ciliary proteins, binding the cilia together so that they cannot beat [7]. Specimens can also be "glued" to the slide using biologically compatible adhesive substances such as protamine sulphate or polylysine [3]. These latter techniques obviously make recovery of the specimen very difficult, and some of these immobilization methods have been documented to distort some cellular processes [10].

### 3.3 Mechanical

At its simplest, mechanical immobilization involves drying down a wet mount until the coverslip presses down on the specimen, trapping it between the slide and coverslip [5, 6]. One usually has a few minutes to observe the specimen before continued drying of the mount eventually crushes the specimen. Although this approach is fast and simple for quick looks at the specimen, requiring no special equipment or supplies, it is not appropriate for longer-term studies, nor

 

is recovery of the specimen for subsequent handling or culturing easy. Various modifications to this basic technique have been published [reviews: 1, 3]. All of these techniques distort the specimen to immobilize it, and recovery might be problematic.

More sophisticated mechanical immobilization techniques involve the use of special devices that allow controlled capture of the specimen by precision flattening between a slide and coverslip. This kind of device is known by different names: compressorium, rotocompressor, or microcompressor, to list just a few. Versions of these devices date back to the 19th Century, and all of them involve some kind of threaded micrometer assembly that brings a coverslip into close proximity to the surface of the slide, trapping the specimen between the two. Properly used, these devices permit delicate capture of a specimen, extended times of immobilization of the specimen for observations, and allow for the specimen to be released and recovered after observations. There can often be an added benefit from flattening the specimen, because fluorescence from cellular components in out-of-focus fields is reduced. Additionally, the chance of capturing images of multiple organelles or structures in the same z plane are dramatically increased. This can be very beneficial for fluorescent imaging and especially TIRFM (see below).

An early model of a mechanical immobilization device is known as the Rousselet Compressorium [1, 11]; more recently, Taylor modernized this design to construct the Taylor Microcompressor [12, 13], which includes several improvements in the ease of use of this kind of instrument.

Asa Schaeffer designed a device he called a rotocompressor, which combined a coverslip holder and the micrometer vertical distance control into one unit. He sold these devices in the 1940s and 1950s. His basic design also stimulated a series of improvements by other workers of this device's usefulness [reviews and discussions: 3, 14, 15]. An advantage of the Schaeffer type of rotocompressors is their low profile, permitting better access and control while the unit is on a microscope stage under a multiple objective nosepiece. The Aufderheide Compressor [3, 14] features a slip fit coverslip holder, which allows quick loading and unloading and recovery of the specimen without altering the micrometer settings for the coverslip clearance from the slide surface. While the traditional designs have been for upright microscope stages, a recently developed version has been fabricated that contains several features found in the Aufderheide compressor. This mechanical microcompressor is readily useable on either upright or inverted design microscopes [24]. Traditional designs have been based on machined metal and glass components, but newer designs can now make use of precision molded polymers and potentially new 3D printing techniques for components of the unit.

### 3.4 Microfluidics applied to rotocompressor designs.

With the arrival of microfluidics, experimenters are making use of devices that contain microchannels for generating mechanical force to trap cells and specimens. These channels can be on the order of the physical scale of biological cells and have numerous applications for cell culture as well as single cell analysis [19–21]. Typically these devices are microfabricated using polydimethylsiloxane (PDMS). PDMS is a flexible polymer that allows oxygen diffusion and can be used to create devices with small (micron-sized) and large features [22]. Microfluidic cell culture environments allow the experimenter to interact directly with the specimen, because fluids can be applied to the specimen and retrieved from the return ports while a sample is under observation.

In addition, several groups have taken advantage of the capability of custom-designed structures of PDMS to trap specimens and cells. These designs have PDMS ceilings or walls that can be gradually lowered or expanded by pumping in water or air in a channel or chamber that is on the opposite side as the specimen. This expansion can be controlled and provide trapping capacity. This has been used with some success to immobilize the nematode *Caenorhabditis elegans* [23]. Many of these techniques use closed microfluidic devices that often require a sophisticated specimen loading process, or they are difficult to keep in working condition and free of air bubbles. It would therefore be valuable to have a microfluidic mechanical compression device that allows easy cell and specimen loading and unloading as well as reliable immobilization and microscopic optical performance.

A new mechanical microcompressor, using some of the advanced features from the Aufderheide rotocompressor design, has been fitted with microfluidics, and now works on a variety of upright and inverted microscopes [24]. This mechanical microcompressor incorporates PDMS components to create microfluidics reservoirs and channels and custom holding platforms unique to the specimen, as well as recently developed glass machining technologies to integrate microfluidics into the microcompressor glass components [25, 26]. The combination of microfluidics, provided by PDMS, with the glass components, permits high resolution optical performance along with the ability to control the fluid surrounding the immobilized specimen. This device has the delicate immobilization control of earlier devices: it can immobilize specimens as small as a bacterium or as large as a *C. elegans* worm. The open nature of the device's microfluidics system allow the experimenters to position and hold the worms in a custom-designed trapping structure or "bed" microfabricated using PDMS. This physically confines the animal in a small containment vessel and requires less physical force for immobilization. This would be expected to limit any possible artifacts generated by over-compression. Worms can also be trapped and released continuously to minimize any possible physiological stress that might be caused by mechanical compression. In addition, larvae of the fly *Drosophila melanogaster* have been oriented and imaged in the device.

 

At the other end of the range of sizes within the device's capabilities, similar extended immobilization and media change experiments were performed on the yeast *Saccharomyces cerevisiae*. This demonstrates that combining the microcompressor and microfluidics technologies still permits use of the benefits of both capabilities. Yeast cells can be cultured continuously for hours, and possibly days, since budding off of new progeny could be observed. Because new progeny yeast cells are too small to be trapped, they are swept away by the microfluidic current and could be analysed for proteomic or metabolic studies by mass spectrometry. In addition, effluent could be collected during the life cycle of an organism or after various treatments. The low ceiling height of the device make this quite attractive since the fluid volumes are tiny and would increase the signal to noise for identification of various released or secreted factors.

The authors also mixed identically sized polystyrene beads and found that they could control the immobilization distance between the glass interfaces across very large areas of the imaging field. Addition of beads to the system could be useful for rapid immobilization and has potential applications for the high throughput analysis of compressed specimens.

Lastly, a recently demonstrated advantage that comes from mechanical compression is the increasing size of, and stability of, the "footprint" of cells during total internal reflection fluorescence microscopy (TIRFM) imaging. During TIRFM, an evanescent wave excites fluorophores approximately 150 nm into the cell [27]. This has become a very attractive technique for analyzing plasma membrane components and signalling mechanisms and dynamics. Because the excitation does not illuminate fluorophores deep within the cell, the signal to noise for the area of illumination using this technique is quite high. Recent advances in the collection efficiency of intensified cameras have made the imaging of signal molecules a reality and this is rapidly becoming an area of intense focus. One relatively simple technique that has been used to flatten cells during TIRFM imaging is the agar overlay [28, 29]. While the agar overlay technique can be difficult to control or manipulate, the mechanical microcompressor described above can be used for precision compression of cells for TIRFM in real time. This is particularly useful for imaging cells in response to ligand. Many cells, such as the amoeba *Dictyostelium discoideum,* will "cringe" and change their morphology when stimulated. This leads to a loss of signal since the TIRFM excitation is heavily dependent on the distance from the coverslip. The gentle flattening of the cell helps prevent this lifting and will provide a more accurate fluorescent signal, thus limiting artifacts.

## 4. Conclusions

No immobilization technique is fully free of problems or artifacts [1, 3]. The researcher must select the technique or techniques that best support his/her microscopic configuration, the organisms being studied, the nature of the investigation being done, and the optical systems being employed. Nevertheless, recent developments in immobilization technology and construction allows the creation of unique devices that potentially permit precise and delicate trapping of specimens combined with sophisticated abilities to change the fluid environment of the trapped specimen.

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