The Preparation of Microtools for the Micromanipulator

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SUMMARY: Tools required for the more delicate processes of micromanipulation are not commercially available and existing equipment for their making is expensive. Some cheaper equipment is described, comprising a gas forge and an electric forge which together suffice to prepare the tools needed for isolating single cells, for injecting and dissecting living tissue and living cells, and for determining their electrical potential.

A simple hanging-drop micromanipulation chamber is described.

Though a considerable amount of information is available on the technique of micromanipulation, notably in American and German publications, only those of Schouten (1934) and de Fonbrune (1937) give a detailed account of the equipment needed for making the tools used in the more delicate processes of micromanipulation, the dissection of cell structures and the injection of liquids into living microscopic cells. As neither of these publications is readily accessible, our observations on this subject may be of interest. Our methods for making microtools are based essentially on the work of both Schouten and de Fonbrune, but are simplified by the use of a more standardized equipment, which should be cheaper to construct than the forge designed by de Fonbrune. The design of one of the most frequently used manipulators is based on the work of Chambers, whose paper appeared in 1922. Schouten's work on micromanipulation, to the perfection of which he has devoted most of his scientific life, was begun as early as 1899, making him the undoubted originator of this fascinating technique, which to-day has acquired great importance in the exploration of the genetic behaviour of micro-organisms.

The preparation of the microtools requires two forges, one heated by gas, the other electrically.

The construction of the gas forge

The gas forge consists of a bar or bed $11\frac{1}{2}$ in. long by $\frac{3}{8}$ in. square, mounted on supports, upon which are placed two sliding members and two fixed supports. It is made of brass and where convenient, all joints are silver soldered.

Text-figs. 1-5 show the general arrangements of the forge; the enlarged views show the construction of the sliding members and the supports.

The spring-loaded head (Text-fig. 3) is the most important part of the instrument. It consists of a tubular section 3 in. long housing the spring and stem for applying tension to the glass tube. The whole head is movable along the bar or bed to the extent allowed by the slot *B* (Text-fig. 3) and is fixed in its required position by the knurled screw *A* (Text-fig. 2).

The spring is approximately $3\frac{1}{2}$ in. long and consists of 60 turns of no. 11 m.w.g. wire wound on a $\frac{7}{16}$ in. former. The stem is a $\frac{1}{8}$ in. diameter rod with a $\frac{3}{16}$ in. diameter head, screwed at the opposite end to carry the chuck *A* (Text-fig. 3). At its tip the chuck is provided with a shallow counter-sink to centre the glass tube when it is attached with sealing wax.

Text-fig. 1

Text-fig. 2

The two fixed supports (Text-fig. 4) are secured to the bar by counter-sunk screws and are in two parts, the fixed central pillars, and the knurled outer sleeves. The central pillars have a U-shaped groove at the top of which rests the glass tube. The knurled outer sleeves have a bayonette clip arrangement at their top edge and slide freely on the central pillars. The top edge of the sleeve is located just below the root of the U-shaped groove in the central pillar. When the glass has been placed in the grooves the outer sleeves are lifted and rotated. The glass tube is then engaged in the bayonette clips. The weight of the sleeve is sufficient to steady the tube for further manipulation.

The other slidable member is the adjustable table (Text-fig. 5). Its sliding movement is limited by the length of the slot *B* (Text-fig. 2) cut through the bar, and by the screw which passes through the slot into the base of the table. The table can be fixed where desired by the knurled screw *C* (Text-fig. 2).

Rising or falling adjustment to the table is made by screwing the table up or down on the stem *C* (Text-fig. 5). A very small amount of movement is needed, in fact once the table has been set at the correct height further adjustment is not necessary unless the glass tube is bent or of some special shape.

As shown, the table is spring-loaded. This is not absolutely necessary, but it adds greatly to the stability of the table.

The gas microburner

The microburner is a simple burner having as its chief feature a very small jet. Some elaboration has been made in the construction of the jet and has proved worthwhile. The burner is built up in brass and silver soldered; the large diagram (Text-fig. 6) shows the construction in detail. The jet *A* (Text-fig. 6) is screwed into the stem *B* and seats on the conical face *C* to form a seal below the hole *D*. The screw *E*, with the needlepoint, has a groove cut in it to allow the gas to pass up to the valve seat *F* where it is restricted before passing on to the small hole at the tip. This construction provides a means of regulating the size of the flame. The adjustment to the worker's need is soon found. Below the screw *E* is a small plug of cotton-wool to filter the gas and prevent any foreign particles entering and blocking the jet.

Text-fig. 3

Text-fig. 4

Common to
Figs. 3-5.

Text-fig. 5

By unscrewing the jet from the conical face *C* for a few moments, air trapped in the line is allowed to escape by the hole *D*. This makes it possible to light the burner almost immediately on turning on the gas. If this is not done, the time required to get rid of any air through the jet is considerable. It will be noticed that it is not necessary to make provision for primary air with this burner.

The size and general construction of the burner can be made to suit the individual worker, the only detail of importance is the size of the final hole in the jet which should be in the order of 0.01 in.

The gas forge is needed to reduce the diameter of the commercially available glass tubing as a first step in the fashioning of microtools. The original diameter of this tubing is governed by the size of the holder of the micromanipulator (Pl. 1, fig. 1) into which the glass tubing carrying the final microtool has eventually to be fitted.

When the gas-heated forge is used, a length of commercial glass tubing, usually of soft soda glass and of a diameter suitable to be held in the manipulator holder, is placed on the two central pillars of the forge. One or both of

these is then locked. The two ends of the tubing are next sealed with sealing wax to the spring-loaded head and the surface of the table. When the sealing wax has solidified, tension is brought to bear on the glass tubing by moving the table to the right and clamping it there by means of the appropriate screw. This movement contracts the spring within the head. This, in its turn, can be moved in a horizontal direction to increase the tension on the glass tubing if needed.

Text-fig. 6. Cross-section of gas microburner. $\times 1.4$.

The microburner is now applied to the glass tubing, between the head and the first pillar and the glass is softened until the pull of the contracted spring in the head overcomes the tension of the glass. When this point has been reached the recoiling spring elongates the glass tubing to an extent which depends on the recoil of the spring and on the temperature of the glass.

The glass tubing is next cut with a pair of scissors at its narrowest point and lifted from the table. The end covered with sealing wax is broken off and the fracture trimmed in the microburner. When cool the tube is inserted and fixed in the manipulator holder and is then ready for the making of the more delicate microtools.

An illustration of some of these tools is given in Text-fig. 7. A detailed description of making these tools is given below. Coarser pipettes and tools for fracturing sporangia can be made wholly in the gas forge; the more delicate microtools only in the electrical forge.

The electrical forge

The component parts of the electrical forge are shown in Text-fig. 8.

The platinum filament, inserted in an insulated holder, is designed for clamping to one of the two manipulator hands, since the finer microtools are

made entirely under microscopic control. The heater is wired to a 12 V. transformer, and to a resistance by which the current to the filament is regulated. The insulated holder consists of a brass rod on which is mounted an ebonite block *A*, and an ebonite guide *B*, both of which are drilled to accommodate the conductor rods of the platinum filament. The holes in the guide block *B* allow the conductor rods, which are of 16 s.w.g. copper wire, to slide through freely and pass on through the holes in the terminal block *A*. In the terminal block the holes are of such clearance that when the terminal studs 1 and 2 are locked,

Text-fig. 7. Glass microtools.

and the knurled clamping screws 3 are adjusted, the conductor rods are held between them. The glass tube *C* protects the copper conductor from accidental contact with the manipulator. The platinum filament is easily constructed, very robust, and convenient to handle. The two 16 s.w.g. copper conductor rods *D* are flattened at one end to about half their thickness for a distance of approximately $\frac{3}{16}$ in. and clipped to neat spear points. They are inserted into the holes drilled in the small ebonite block 5 and the flats of the spear points set in the same plane and at the requisite distance from the block. A small blowpipe flame is carefully applied to the copper rods at a safe distance from the ebonite. The heat conducted along the copper will soon cause the ebonite round the copper to melt and at that point the heat is carefully quenched in water. When cold the copper rods will be found to be securely anchored in the ebonite which forms a very convenient grip when handling the completed filaments.

All that now remains to be done is to solder the platinum wire filament *E* on to the conductors. The filament is 0.20 mm. wire, 19 mm. long; it is attached

with ordinary tinman's solder. Although the solder does not readily wet the platinum, a sound connexion is easily achieved. Work can be done with the filament at almost white heat without fear of melting the solder since the heavy gauge of the conductors quickly dissipates the heat.

Considerable skill is required to weld the tip on the filament to form the anvil illustrated in Text-fig. 8, *F* and *G*. After the filaments have been constructed the tip is hammer welded as follows:

A piece of platinum wire 1 in. long is soft soldered to a 3 in. length of 16 s.w.g. copper wire to give it some stability and to make manipulation easier. The

Text-fig. 8

completed filament and the 1 in. length of platinum wire are set up for welding on a steel plate, preferably hardened and polished. When the filament is laid on the steel plate the ebonite block will prevent the platinum from touching the surface of the steel. As the distance between the platinum wire and the plate is too great it should be reduced to about $\frac{1}{32}$ in. by gently bending the wire downwards. The tip of the 1 in. length of platinum wire is now carefully laid on the top of the platinum filament at the point where the weld is to be made, noting that the $\frac{1}{32}$ in. gap between platinum wire and steel plate is retained. Small metal blocks may be used to hold the parts in their correct position during the subsequent welding operation.

A small hot blowpipe flame is next directed on to the point where the weld is to be made and, the correct welding temperature having been reached, a carefully directed tap with a small hammerpoint completes the union. All that now remains is to snip off the anvil tip at the correct length, approximately 3 mm.; the remainder of the 1 in. length of platinum wire can be used to produce more anvils.

When the electrical forge is to be used, its heater is first clamped into position on one of the manipulator's two hands, for right handed workers preferably in the right hand of the manipulator.

In the left hand is inserted a holder with drawn out glass tubing. The tip of the glass tubing and of the platinum filament are next centred opposite each other and directly under the low objective of the microscope so that they may be viewed simultaneously while the tool is being shaped. A magnification of approximately $\times 100$ is convenient for the fashioning of most tools.

The filament of the heater can be replaced by others of different size or design. They include types which hold a glass or metal bead at their tip and others, anvils, with a short platinum rod welded to their tip (Text-fig. 8, *F* and *G*). The latter serve as anvils in the making of microloops and in moulding the base, or handle, of most microtools used in hanging-drop preparations. Metal and glass beads can be fused to the filament by heating it to a bright glow and touching a thin metal or glass rod with it. The glass or metal will melt and collect as a droplet at the tip of the filament. The ensemble for making microtools is set out in Pl. 1, fig. 2.

Microneedles

A platinum filament, holding a glass bead at its tip, is inserted into the heater and centred opposite the drawn glass tubing within the low-power optical system.

By adjusting the resistance of the electrical forge the glass bead is softened to the consistency of thick treacle. The glass tubing in the left hand of the manipulator is now moved towards the molten glass bead and fused to it.

The two manipulator hands are next moved away from each other in a horizontal direction. The pace of this movement, which can be learned only by experience, must be such that the glass rod withdrawn from the glass bead by the pulling movement is maintained at a uniform diameter. The temperatures of the bead and the rate of pull will govern the diameter. When the right length of glass rod has been withdrawn, the heat is cut off. The pull exercised on the rod by contraction of the cooling glass bead is normally sufficient to fracture the glass rod at its point of least resistance, that is nearest the glass bead, if care has been taken to draw the glass rod slightly thinner at this point. When the cooling glass bead does not exercise sufficient pull, the break is made by an extra pull from the two manipulator hands. The resulting rod serves as the handle of the needle. The needle point is made by remelting the glass bead and fusing the handle and the bead. A sharp pull of the manipulator hands draws the handle into the needle point. It is now ready for use if a straight instrument is required. For work on cells suspended in a hanging drop and sealed from evaporation by an overlying layer of liquid paraffin, the handle of the needle must be bent to an angle of approximately 45° , followed by a deflection of the needle itself almost back to the horizontal (Text-fig. 7).

An anvil is inserted in the heater in place of the U-shaped platinum filament and the heater clamped in the right manipulator hand in such a way that the upright piece of platinum wire—the actual anvil—is centred in the microscopic field, just below the handle of the needle. Its top should be some 5μ . higher in the optical field than the handle. The anvil is heated to a temperature high

enough to soften the glass of the handle without melting it, and is brought carefully into contact with it. The anvil should be sufficiently sturdy to withstand deformation when pressed against the softening glass handle. The required pressure is obtained by moving the screw of the right manipulator hand governing the optical upward movement and at the same time turning the screw of this hand responsible for the optical forward and backward thrust. As a result the anvil will slide slowly forward and backward along the lower side of the handle, gradually warming and softening it. At the same time the pressure of the anvil exercised on the handle will force the softening handle upwards and out of the straight position. When the handle is correctly bent the anvil is moved from below the handle to above the base of the needle point and is brought into contact with this side of the needle to bend it back towards the horizontal. The procedure now is exactly the same as before except that greater care must be taken in the application of heat and pressure to the thinner needle.

Microloops

A handle is made as described for needles. The tip of this handle is fused to the glass bead to make a second, shorter and thinner handle. This should have a length of 40 or 50 μ .; it is broken off from the bead by switching off the current.

An anvil is now centred in the microscopic field immediately opposite the tip of this rod. The top of the anvil should rise no more than 1 μ . above the optical height of the short rod. The anvil is gently heated and the short glass rod thrust forward against it by a rapid movement of the appropriate left manipulator hand, until it hits the anvil. If the latter has been sufficiently heated, but not overheated, it will be found that the tip of the short rod bends slightly upwards or sideways. By repeating the forward thrust of the left manipulator hand, the tip of the short rod gradually shapes itself into a loop, the diameter of which is governed by the heat applied to the anvil and by the vigour of the thrust. Once the loop has been closed, the holder in the left manipulator hand is rotated until the loop is viewed sideways and the anvil is moved below the thicker handle where it is used to bend the handle as already described for microneedles.

Microknives

The procedure for microknives is the same as that for microloops, up to and including the drawing out of a thin glass rod at the tip of the handle. This rod should not be more than 12–20 μ . long. The two bends in handle and thinner rod are next made with the anvil.

The holder of the glass tubing is then rotated in its clamp until the thin glass rod is viewed horizontally in the optical field. The anvil is replaced by a filament with a glass bead and this is fused to the thin glass rod. By a sharp pull the thin glass rod is separated from the molten bead, leaving at its tip a

short and exceedingly fine rodlet. The diameter of this rodlet, which should not exceed 3μ ., is usually less than 0.1μ . at its end point, and is sharp enough to cut the cell membrane of yeasts and bacteria.

Micropipettes

Micropipettes are drawn out to the desired diameter in the gas forge by moving the adjustable table as far to the right as the contractable spring will allow, and by heating a wider area of the glass tubing than usual. A hot flame is usually required. Recoil of the spring draws out the pipette. The tip of the glass tubing is severed from the micropipette, which is next shaped in the electrical forge. The holder with the straight pipette is clamped in the left manipulator hand and the heater with a U-shaped filament in the right. Both pipette and filament are now centred in the microscopic field, the heater well below the optical plane of the pipette and some 40μ . to the left of its tip. The filament is heated sufficiently to soften the glass of the pipette at the desired point and to allow the last 40μ . of the pipette to drop downwards until the desired angle is formed. Then the current is cut and the pipette raised within the optical field by rotating its holder, until the bent part is viewed in the optical field in a more or less vertical position.

The filament is now moved below the stem of the pipette some 80μ . behind the bend and some 40μ . below it, and heated sufficiently to soften the glass of the pipette. The softened tip of the pipette is allowed to sink towards the heater until it is almost parallel with the stem when the current is cut.

Micro-electrodes

For making a micro-electrode a fine gauge copper or platinum wire of approximately 0.1 mm. diameter is first passed through the aperture of a standard size glass tube, which is then sealed to the head and the table of the gas forge. The wire at this end should be long enough to allow for the extension of the glass tubing during drawing and to leave enough for an electrical connexion.

When the glass tubing and the wire are inserted in the gas forge and tension applied by sliding the table to the right, the glass is heated in the microburner and drawn out in the usual manner. Instead of cutting the head end of the drawn tubing with scissors, as with other microtools, the whole of the glass tube is lifted from the forge, and the glass at the constricted end broken by gentle tapping with a pocket knife. The fragments of glass are removed from the exposed wire, the latter firmly sealed to the remaining tip of the glass tubing and all but the last 80 to 100μ . of the exposed wire cut away.

The electrode is now inserted into the left-hand manipulator holder and optically centred opposite a U-shaped filament heater. At the tip of this filament has been fused a bead of tin or solder.

When the electrode and the filament have been centred, the metal bead is melted and the wire fused to it. By maintaining the metal bead at a suitable

temperature and by withdrawing the end of the electrode from it in a horizontal direction a metal rod is formed at the end of the wire, which can be drawn out into a point fine enough to penetrate a living cell.

The bending of the wire and its point into shape for work in the hanging drop is done without the application of heat with the anvil as described for making microloops.

It remains to be added that, before a glass microtool is used, it should be sterilized by dipping into concentrated sulphuric acid, rinsing in sterile distilled water, immediately transferring to concentrated ammonia, specific gravity 0.880, and again washing in sterile distilled water.

Hanging-drop chamber

Though not strictly a microtool it is appropriate to describe the type of chamber used by one of us (A. C. T.) for work in hanging drops. It differs in several respects from the chambers usually recommended and offers greater freedom of movement.

The chamber consists of a microscope slide 3×1 in. to the centre of which is cemented an aluminium cylinder, 20 mm. high and 20 mm. in diameter. The metal is 3 mm. thick. Three slits are cut in the upper rim of the cylinder to a depth of 15 mm.; one slit is 12 mm. wide and two, facing each other at the side of the wider slit, are 8 mm. in width.

The base of the cylinder is joined to the microscope slide with sealing wax or paraffin wax and its top edge covered with a thin layer of vaseline.

In use the chamber is inserted into the moving stage of the microscope so that the major slit faces the light source, and the two side slits the two hands of the manipulator. The chamber is centred under the optical system of the microscope, and a microtool in its holder is inserted into each side slit of the chamber and clamped to the left and right hands of the manipulator. The tools are next focused in the centre of the optical field by movements of the manipulator hands. For certain work, such as the isolation of a single cell, only one microtool is required. For other, such as electrode work and the perforation or dissection of a cell, two and sometimes three tools are required. When three tools are needed, the third is inserted through the slit facing the source of light, and an extra manipulator hand will then have to be provided. The height of the chamber makes it difficult to focus the light source on the hanging drop, but sufficient light can be obtained by a 100 c.p. lamp passing through the Abbé condenser to make it possible to work with a 4 mm. objective. Cells of the organism with which it is intended to work are suspended in a hanging drop on a coverslip of appropriate dimensions and thickness.

It is important first to clean the coverslip carefully before use. Before placing the hanging drop, or drops, on the underside of the coverslip a trace of vaseline is rubbed over this surface and as far as possible cleaned off again with a clean cloth, free from fluff. Complete removal of the vaseline is not possible by such treatment, and is not desirable, for sufficient should be left on to prevent the edge of the hanging drop from running. Schouten (1934)

Fig. 1

Fig. 2

recommends the triglyceryl ester of lauric acid for this purpose and states that it is better than vaseline; but, like Barber (1914) the writers have always found vaseline satisfactory.

For the isolation of a single cell at least two minute hanging drops of a suitable nutrient medium are placed on the greased underside of the flamed coverslip, about 0.5 mm. from each other. A very fine platinum loop is used, and each droplet should not exceed 50μ . in diameter. Before the droplets have time to evaporate, a trace of fresh culture of the test organism is transferred to one of the droplets and partially mixed with the medium in the droplet. It is important that parts of the droplet should be free from cells. When the mixing has been too thorough, or the cells introduced too numerous, the isolation of a single cell becomes difficult.

Both or all the droplets are enclosed within a drop of sterile liquid paraffin, using a large platinum loop. The paraffin cover prevents the evaporation of the droplets during work and subsequently protects them from contamination.

For the actual isolation or other manipulation the coverslip with the droplets on its underside is gently pressed on the vaselined top of the chamber. The edge of the droplet containing cells is centred and focused and a microloop plunged into the droplet. When a suitable cell has been caught within the loop the stage of the microscope is moved in the direction of the second droplet, to bring the microloop into the second droplet where it is raised and lowered until the cell is released.

The next stage in the isolation consists in the transfer of the coverslip from the chamber to an ordinary moist chamber at the bottom of which is placed a droplet of sterile water. The slip is sealed to the top of this chamber with vaseline and the chamber then incubated, preferably at room temperature, if this allows the isolated cell to grow. The use of higher temperatures demands careful control of the humidity within the moist chamber and makes it necessary to open the chamber when it is removed from the incubator to a lower temperature for microscopic observation.

As soon as macroscopic growth is visible, the coverslip is lifted from the chamber and the growth transferred in a fine platinum loop to a suitable solid or liquid medium.

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