SINGLE CELL TECHNIC

A PRESENTATION OF THE PIPETTE METHOD AS A ROUTINE LABORATORY PROCEDURE

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Procedures for preparing pure cultures from single bacterial cells which have been published during recent years are diverse and numerous. This mass of advice and description does not, however, seem to have appealed sufficiently to bacteriologists to warrant the introduction of a single cell method as a routine laboratory technic. The indifference which is still widely displayed toward the practice may be due in part to the conviction that some of the systems advocated are uncertain and may result in cultures from more than one organism. Antipathy to other systems arises from the expensive equipment required. In addition to these two objections there is a general feeling that any satisfactory single cell method can be acquired only with great difficulty, and used at the expense of much time even after the operator becomes skilful.

A worker wishing to master a single cell technic without previous experience in it will encounter in the literature on the subject many pitfalls which may well lead to the conclusion that single cell isolations are not only uncertain and expensive but also vexatious and laborious. He will find on the other hand the frequently recurring statement that once gained, the method in question is sure and rapid. It is the opinion of the authors that a negative or unsatisfactory result, when the method is tried, is due to omission of detail in many of the short descriptions and to underem-

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phasis of fundamental points in the more complete articles. It is certain that there are different methods, each sound and speedy, and each giving acceptable results in the hands of those familiar with them, but there has not come to our attention in print a full exposition of any one of these. The usual experience is the development of a new or modified method after time-consuming experiment in each of the laboratories where single cell cultures are required.

The inadequacy of existing information was evident in this laboratory when work on problems involving the question of variation demanded the use of cultures from single cells. Printed descriptions were followed word for word without success. Eventually a modified procedure was developed and used with absolute confidence for securing pure strains of spore-formers, lactobacilli, micrococci, coliform organisms, and other forms of small size requiring the making of examinations under a magnification of 950 diameters.

We venture to place our operations on record in detail with the hope of saving the time of those who may wish to perform single cell operations without making the annoying mistakes which many of the previous descriptions invite. Our instructions have already been followed without difficulty by other workers with successful results, and it has been found that once introduced into a laboratory, many uses are discovered for a single cell method of purifying cultures, frequently with a saving of time over plate, dilution or enrichment methods.

This procedure requires a minimum of special equipment. A viable culture can usually be started in less than half a day. The element of chance is reduced to a minimum. It is therefore felt that the particulars are of general interest, and that laboratories will adopt the single cell procedure for routine as soon as its utility and relative simplicity are generally realized.

It will be noted that the operation as we describe it contains nothing distinctly new. The essential points have been culled from some 30 articles on the subject, however, and certain of them have been refined for working under the oil immersion objective. Steps in the procedure as they appear under the microscope,

and which usually defy verbal description, are illustrated photographically.

EQUIPMENT, MATERIALS AND PRELIMINARY TECHNIC

Principle of method

The most acceptable single cell methods are those which permit the examination under the oil immersion lens of the organisms sought. Two schemes were introduced at about the same time for handling microörganisms on the underside of coverslips. Barber (1904) introduced a mechanically operated micropipette and subsequently enlarged upon his method (1907, 1914). Schouten (1905) devised a micromanipulator with which the organisms were transported by means of a mechanically operated glass-loop of micro dimensions. We arbitrarily decided to adopt the Barber micropipette system. It appealed to us as preferable for the isolation of the smaller organisms and spores, and in addition the making of pipettes is less difficult than the manufacture of the minute glass-loops for which Schouten's arrangement calls. The three separate steps in the micropipette method are the making of a minute hanging drop by means of a mechanically moved micropipette, the examination of this drop for number and type of organisms and the transfer of a chosen drop by means of another pipette into sterile liquid medium.

Apart from the early and now obsolete India ink suspension employed by Burri, nearly all schemes, other than those requiring pipettes, call for the isolation of the organism on solid medium and the making of transfers after a colony has developed from it. One may infer that many of those who still use this means choose it on account of fancied difficulties of the pipette method and that the Barber principle has not been given a fair trial. The pipette isolation is decidedly neater, and in our hands permits the expedition which is claimed as the chief advantage of the agar-block-colony plan.

Both Barber and Schouten controlled their pipettes by means of three lead screws operating in guides in three planes. The modern version of the screw-operated pipette holder is exemplified by the

device of Taylor (1923–25). This equipment although exact and serviceable is costly, and cumbersome for bacterial isolations requiring the constant changing of pipettes. We use the instrument described by Chambers (1922), a compact tool based on the lever principle, which is more flexible for the bacteriologist's purposes.

Special equipment

The micromanipulator is the "right-hand" single pipette holder of Chambers. This clamps on the left side of the microscope stage for operating the pipettes from the side into the open left end of the moisture chamber.

The only additional special equipment necessary is a long focus substage condenser. This is in order to provide sufficient illumination for the examinations, taking into account the fact that the moisture chamber is between the objective and the substage condenser. Our condenser focuses the light at a point approximately 20 mm. above the surface of the stage, that is, on the coverslip where the organisms to be examined are placed.

Microscope

The microscope is furnished with 10× ocular, 1.8 mm. (oil immersion), 4 and 16 mm. objectives. The oil immersion lens is used for the actual pipettings and examinations, and the other two objectives are used to watch the coverslip films and to facilitate the centering of the pipettes. Since the three objectives are frequently used in rapid succession, it will be found an advantage to have them accurately parfocalized.

The isolation moisture chamber is carried on an independent mechanical stage.

The microscope assembly is detailed by Kahn (1922), who presents the adaptation of the Chambers micromanipulator to bacterial isolations.

Lighting system

Despite the special substage condenser, the efficiency of an ordinary lighting system is greatly reduced by the gap which must be left between the objective and the condenser. We use a 100-

watt gas-filled bulb placed horizontally about 40 cm. from the microscope with the edge of the filament toward the instrument so that the centers of the filament and mirror are at the same height above the bench. A 500 cc. Florence flask filled with water is used between the bulb and the microscope to converge the light on the mirror and to exclude the heat rays. Some of those who have used this source of illumination prefer to soften the light by adding methylene blue to the water in the Florence flask.

The 100-watt bulb should be shielded in order to protect the eyes of the worker from all light not actually entering the water flask. This precaution facilitates the examinations under the oil-immersion lens by providing contrast between the illumination in the microscope and the light of the laboratory. We further sharpen this contrast by operating in a darkened laboratory and by connecting to a 2-way switch the microscope light and a desk light. The latter is used for preparing the pipettes and for the other steps in the procedure away from the microscope. In this way the organisms in the moisture chamber are exposed to light only when they are being examined, and this is the only illumination in the laboratory when the microscope is in use. As each examination is completed the microscope light is switched off and the desk light switched on simultaneously. In this way it is possible to work for several hours at a time with a minimum of eye strain.

Moisture chamber

Dimensions of 48 mm. long, 23 mm. wide and 20 mm. high, substantially as given by Kahn (1922), were found convenient for the moisture chamber. The walls, of thin window glass, were mounted on an ordinary microscope slide with Canada balsam. An additional piece of glass was placed flat on the slide in the open end, for stability and to prevent moisture on the floor of the chamber from running onto the stage. By constructing the chamber with its open end flush with the end of the slide the full lateral traverse of the mechanical stage can be utilized. After assembling, the chamber was baked carefully in the oven for several

hours. The sides and end were afterwards lined with black filter paper. Proper moisture conditions can be maintained in this way and the capillary tip of the pipette which refracts the light can be readily seen against the black background. The filter paper should be cut slightly lower on the side facing the operator than on the opposite side and end, so that the tip of the pipette is visible from the side.

The walls of the completed chamber should be square and parallel so that the coverslips fit closely. The top edges of the chamber are smeared thickly with stopcock grease (16 parts vaseline, 8 parts gum rubber and 1 part paraffin melted together) so that after the coverslip is applied, the chamber is completely sealed with the exception of the open end, and so that the triple nosepiece can be swung through the immersion oil without altering the position of the coverslip.

Pipettes

The making of pipettes is illustrated by Chambers (1918). Make them from glass tubing of 4 mm. outside and 2 mm. inside diameters. Draw out one end to 1 mm. diameter for about 4 cm. and seal the extremity. Leave the shank about 10 cm. long, firepolish the open end and plug loosely with cotton. Make a scratch half way along the constriction with a file or glass knife to assist in breaking off the capillary tip to be formed at a later stage. Pack the partially finished pipettes in glass tubes 1.5 by 30 cm., plug these with cotton and sterilize by dry heat. Make a quantity in advance. Either pyrex or soft glass may be used for pipettes. The novice will find that the fine capillaries are more easily made from pyrex, but soft glass will save time after the operator becomes adept.

The capillary tips are made one at a time, as used in the manipulator. Dust and cotton particles collect on the points of finished pipettes during storage and interfere with the making of droplets. The capillary ends are made in a microburner. This is a piece of pyrex tubing 4 mm. in diameter drawn out to a capillary point, bent at a right angle and mounted on a board so that the flame is about 5 cm. above the bench. Illuminating gas is

used for the flame and fed past a screw clamp on a rubber tube to the burner. The flame should be adjusted to burn about 2 mm. high. It is well to practise making the capillary pipettes before any isolations are attempted. The operator is usually advised to make the capillary end in the microflame, and afterwards to bend the capillary portion to a right angle above the flame, so that when the pipette is placed horizontally in the mechanical holder the capillary tip will project vertically underneath and towards the coverslip. We combine the operations of drawing out and bending. This reduces the length of the vertical portion of the pipette, thereby giving more vertical freedom in the moisture chamber. Proceed as follows: By means of forceps sterilized in the flame remove one of the unfinished pipettes from the tubes in which they were sterilized. Grasp the plugged end of the shank horizontally in the left hand. Grip the 1 mm. portion of the pipette in the sterile forceps and heat it in the microflame until the glass softens. When the glass begins to give, jerk the forceps sharply upwards. Clip off the bent capillary portion with the sterile forceps about 5 mm. from the bend. Clamp the pipette in the holder.

Made in this fashion a pipette will have an abrupt bend and after it is in place in the moisture chamber it can be easily centered, approximately, in the low power field. The bend must be square, as otherwise there will be difficulty in ejecting the drops on, and removing them from, the coverslip film. Clipping with forceps almost invariably gives a clean, square opening on the capillary end of the pipette. The bore of the capillaries should be about 5 micra. The first practice pipettes may be measured by means of an ocular micrometer and the heating and pulling timed when subsequent pipettes are made, so that the open capillary end has approximately the recommended size. After the first few satisfactory pipettes have been made, the operator can judge from the "tug" at the time of drawing out whether the pipette is satisfactory. Many of the capillaries taper when the drawing and bending operations are combined. The operator can clip the capillary longer or shorter than 5 mm. where he judges the inside diameter to be about 5 micra. Brief practice

will perfect the knack, although the "feel" of the operation must be modified for hard and soft glasses and for different gas mixtures —as for example, for coal gas in comparison with gasoline gas. Care must be taken to maintain sterile the 1 mm. portion of the pipette and the adjacent end of the shank, which otherwise may contaminate the tubes containing suspensions of organisms or culture media.

It is frequently necessary to use pressure for discharging the pipettes and suction for filling them. This is best done by mouth. A rubber tube connected to the shank of the pipette for this purpose, should be at least one meter long to avoid disturbing the adjustment of the pipette when the operator moves his head. It will be found convenient to connect the rubber tube to the mouth by means of a glass tube bent over the ear. This keeps the tube in readiness when it is not actually being used for sucking or blowing, and also prevents the rubber tube from becoming filled with saliva, which runs back into the mouth through the glass ear lug.

Figure 2, plate 1, shows the appearance of the end of the pipette and the opening in it when the microscope is focused on the end of the pipette in place immediately beneath the film on the coverslip. In figure 3, plate 1, and figure 4, plate 2, there can be seen the shadow of the horizontal 1 mm. portion of the pipette.

The Chambers' pipette holder is usually provided with a sleeve for accommodating pipettes made from small tubing. This is discarded when using the larger and more convenient 4 mm. tubing.

Preparation of coverslips

It is generally admitted that the vital point in the pipette method is the treatment given the coverslip on which the isolations proper are made in the moisture chamber. The necessity for painstaking care with this detail cannot be over-emphasized. Success or failure of the whole procedure depends upon the manner in which the grease film is applied to the coverglass.

To begin with, the coverslips must be freed of dirt, lint and grease which if present would interfere with the examinations.

They must subsequently be treated for the following three reasons. Firstly, the microdrops without treatment of the coverslips will spread beyond the confines of the high power field. Secondly, drops made on a dry cover are shallow and the organisms may suffer on account of the more intimate exposure to the atmosphere of the chamber. Thirdly, a grease film on the cover allows a field of minute drops of condensed water to be maintained around the experimental drop, thereby protecting it from drying and serving as an index of the moisture conditions in the chamber. Hence the grease film applied to the cover must fulfil this threefold object. The film must be of a thickness which furnishes deep, hemispherical drops which remain clear and transparent. There must be sufficient grease to prevent spreading, but not so much that streaks become visible under the oil immersion lens. Many using the method prepare a number of coverslips in advance. We prefer to treat them one at a time as required for the reason that one satisfactory film is adequate for an isolation and preferable to a number of ready-made ones which may later be found unsatisfactory on account of some error in technic.

A grease film which meets the requirements is prepared as follows: Prepare in advance grease-free silk for rubbing the films. Silk is preferable to cotton or linen which may leave fibers on the coverslip. Use a yard remnant of crêpe silk cut into six or eight pieces. Render grease-free by boiling in 10 per cent sodium carbonate solution and rinsing in distilled water. Hang to dry where dust is not likely to blow. Store in a clean tin box.

Use a coverslip 22 by 50 mm. of No. 1 thickness for the oil immersion lens. Boil in acid-dichromate cleaning solution. Wash with distilled water, holding in forceps. Dry with grease-free silk. With a dry finger rub over one side of the coverslip just sufficient Chesebrough's Blue Seal vaseline (antiseptic-free) to give the surface the thinnest possible continuous coating. Rub the vaseline-coated side with the silk, using a circular motion, and keeping several thicknesses of the material between the glass and the fingers. Remove most of the grease in this way. Examine the film by oblique reflected light. It should show a blue continuous haze finely crossmarked by the silk. Grip a corner of the cover-

slip in the forceps and pass it through the blue flame of a bunsen burner, *film side down.* A single flaming of about one second suffices. This sterilizes the coverslip and further distributes the grease. The film side must be down during the flaming. This point, mentioned by Schouten (1905) is essential for success but is not specified by other workers. Adjust the burner flame so that it is not quite hot enough for the glass to crack after the passage.

Saturate the filter paper lining of the moisture chamber with warm water. After the coverslip has cooled, attach it securely to the chamber by means of the stopcock grease on the edges of the walls. Mount the chamber in the mechanical stage of the microscope, turn on the 100-watt light, and focus on the film with the 16 mm. lens. Within a minute or two a fine dew should condense on the film from the moisture in the chamber, which appears microscopically as a field of minute discrete droplets each with a regular margin. If this film does not form within a few minutes, run a drop or two of hot, but not boiling, water on the floor of the chamber using a 1 cc. pipette. The film should then form immediately from the vapor. Examine under the 1.8 mm. lens using immersion oil. The droplets should be clear and should not be broken up by grease streaks. After the first ten minutes the droplets should retain their size, neither running together nor evaporating. If the film has been properly made this appearance can be retained for an hour or longer, with no attention other than occasional introduction of a few drops of warm water on the floor of the chamber as the film begins to dry up near the open end. If it is found difficult to maintain the film on account of low humidity in the laboratory atmosphere, the proper conditions can be secured by making a small pool on the floor of the chamber and encouraging evaporation from it onto the film by the heat of an ordinary microscope lamp placed on the foot of the microscope under the stage. The operator should practise making films until these requirements are met. If the proper appearance is not reached shortly after the film is made, and maintained for an hour or longer, it is useless to proceed with the isolations since drops cannot be applied to and removed

from the coverslip satisfactorily, and there will not be sufficient moisture in the chamber to enable the organisms to survive exposure on the cover.

Plates 1 and 2 illustrate satisfactory films. Any film presenting an appearance intermediate between these two will serve for isolations. The film in plate 1 contained more grease than that in plate 2; the film of plate 2 received a slightly longer flaming. The film of plate 1 was prepared by one of the authors, who works uniformly close to one end of this allowable extreme of greasing and flaming; the other author, whose practice approaches the opposite extreme, prepared the film of plate 2. The operator should be able to tell, after making and examining a few films, what the proper residual amount of grease before flaming should be, and the time of passage through the flame for optimum results. The errors which must be guarded against are illustrated in plate 3. The film of figure 7, plate 3, contained too much grease and the pass through the flame was too rapid. The result is a film so greasy that the streaks persist when the pipette drops are made. Too little grease and prolonged flaming produced the film in figure 8, plate 3. In this case, although the drops are clear, they coalesce and spread beyond the fields of the high power objectives and their shallowness has an unfavorable effect on the organisms. Rubbing the film with a dirty cloth laden with grease from previous work produced the film in figure 9, plate 3. Here pipette drops cannot be examined on account of the debris on the coverslip.

When drops are placed with the micropipette on properly prepared films such as those of plate 1 or plate 2, the outlines of the condensed dew drops originally on the site will disappear and leave a clear drop from the pipette with which the dew drops coalesce. They do not remain outlined in grease after removal. This is brought out in figure 5, plate 2. The field of the oil immersion lens contains about a dozen dew drops with a proper film.

Nutrient media

A quantity of liquid medium adapted to the organism in question should be dispensed in culture tubes, 18 by 150 mm. This

menstruum, used for the suspension from which isolations are made and also to receive the single cells, should be clear and free from sediment or precipitate. If necessary, the medium should be filtered through a Berkefeld candle.

THE ISOLATION

Stock suspension

Before commencing an isolation there should be on hand supplies of sterile, partially finished pipettes, and clear, sterile liquid medium in tubes, and the operator should be able to make the capillaries and coverslip films with facility. As a stock suspension from which to isolate single cells there should be prepared a young culture of the organism on agar or in broth. In the case of spore-formers an old spore slant may be used. Taking a needle in the case of an agar growth and a pipette in the case of broth growth, transfer sufficient of the material to a tube of the specially cleared broth and shake to distribute the cells uniformly. This should give the medium a turbidity which is just distinctly perceptible.

Pipetting of drops

Have the moisture chamber in place on the mechanical stage with a good coverslip film. Adjust each of the three fine movements of the micromanipulator to a central position to permit travel in both directions in each of the three planes. Make a capillary pipette. Fill beyond the bend with the suspension of organisms, using suction if necessary. Turn on the microscope light. Place the pipette in the micromanipulator with the vertical capillary end approximately in the optical axis of the microscope. Make the coarse horizontal adjustments by sliding the pipette along in the holder before clamping tight and by turning the pillar in its socket to move the pipette to and fro. Raise the pillar until the tip of the pipette is within 1 mm. of the coverslip. Using only the coarse adjustments bring the tip of the pipette near the center of the field of the 16 mm. objective. For this purpose focus the objective below the cover and look for the shadow of the pipette in the microscope as it is moved gently in the horizontal directions.

When the pipette becomes visible in the low power field, focus on its tip and center and raise it near the coverslip using the fine adjustments of the manipulator and following the movements through the microscope. The three adjustments must be used in turn since raising the pipette may also move it laterally. After centering the pipette in the low power field, center it in the field of the high dry lens. Finally center under the 1.8 mm. lens using immersion oil. The use of the intermediate power may be omitted, although in this case it may be found necessary to move the pipette a short distance with the fine adjustments to bring it in the field of the 1.8 mm. lens.

Retaining the 1.8 mm. objective, focus on the coverslip film. The tip of the pipette should be visible immediately below. Raise the pipette until it just touches the glass, lower immediately and at the same time blow gently. A drop should be left on the coverslip smaller in diameter than the field of the oil immersion objective. The size of the drops can be regulated by varying the length of time of blowing and of the pipette's contact with the cover. The moisture chamber is moved a short distance for each fresh drop by means of the mechanical stage.

The first few drops may contain numerous organisms indicating that the suspension is too heavy. In this case prepare a lighter suspension and repeat the process with a new pipette. If the first few drops do not appear to contain any cells, they may have settled into the lower portion of the capillary. Blow out a large drop to bring this denser suspension into the tip. Continue making smaller drops. When a suspension of the proper density has been used, one or two organisms will be visible in the next few drops made. Continue making microdrops until one is obtained which appears to contain only one cell. The appearance of clear drops with the microscope in focus on the film and the shadow of the horizontal arm of the pipette in the field is brought out in figure 3, plate 1, and figure 4, plate 2.

Examination of drops

All examinations are made under the oil immersion lens. Because the pipette drops are hemispherical rather than flat, the

focusing must be changed continually during an examination so that each drop can be searched thoroughly from top to bottom. A cell may be invisible in the lower part of a drop when the lens is focused sharply on the lower side of the coverglass. Figure 6, plate 2, shows two drops, one free from cells and the other containing a single rod. The microscope is focused below the film on one end of the rod, which is floating obliquely.

Removal of organisms

When a drop has been obtained, definitely containing one cell, turn off the microscope light, lower the pipette, remove from the holder and discard.

Make a new pipette, and fill the tip from one of the tubes of sterile medium. Do not fill beyond the bend. Place in the manipulator, center as before and bring into the field of the 1.8 mm. objective immediately below the drop containing the single selected organism. Make a second confirmatory examination of the drop. Raise the pipette until the tip just enters the drop. The drop should be drawn promptly into the pipette by capillarity. The organism may frequently be seen to be carried into the opening of the pipette. If the drop does not run into the pipette at once, apply gentle suction. When the drop has been removed, focus on the film and examine to see whether the organism was taken by the pipette. The site of the drop should be clear. Occasionally a tiny residual drop is left behind. In either case it is possible to decide immediately whether the organism was removed by the pipette. Move the chamber to the right to minimize the danger of touching any part of it with the pipette. Remove the pipette. Transfer the organism to a tube of sterile nutrient medium. To do this, slant the tube of medium and place the pipette in its mouth, tip down, so that only the sterile 1 mm. portion enters the culture tube. Blow out the contents of the pipette. Wash out several times with the medium. Press the tip of the pipette against the tube so that the end breaks off at the filemark and drops into the medium. Wash into the liquid any fragments of glass, to which the organism may be adhering, by tilting the tube. Discard the pipette and repeat the process using a new pipette filled from the stock suspension.

Precautions

The necessity of maintaining the moisture films on the coverslip throughout the operations has already been emphasized. The film should be inspected from time to time and warm water dropped into the chamber whenever signs of drying are evident. A vegetative cell once stranded on the coverslip due to the drying of the drop in which it was contained had best be abandoned.

Attention must be paid also to maintaining sterile the 1 mm. portion of the pipette during handling and care must be taken that drops on the film or the sides of the chamber are not brushed when pipettes are being placed in, and removed from the chamber.

Exposure of the organisms to the microscope light should be reduced to the time during which examinations are being made. When a pipette has been filled with a suspension and no drops have been secured containing one satisfactory organism after several minutes of trial, the pipette should be removed and discarded and a new sample of the suspension taken in a fresh pipette.

Many workers maintain a suspension of organisms on the coverglass together with a pool of the nutrient medium so that dilutions can be made in the chamber on the coverslip for filling pipettes. We prefer to use only test tube suspensions, to be diluted if too dense and reinoculated if too light to contain organisms in the ratio of one to the microdrop. In this way the exposure on the coverslip is reduced to a minimum.

It is interesting to note that the percentage of successful transfers is generally higher on a dark, rainy day than in bright, dry weather. Since conditions on a dull day favor the survival of the cells, it is advisable to duplicate them, in so far as is possible, in the laboratory.

It may be thought advantageous to make a series of single cell drops on the coverslip from a single pipette, marking the positions by means of the scale on the mechanical stage. This also increases the exposure of selected organisms on the coverslip. The percentage of successful growths will be found higher if the directions are followed without modification. In this way the

selected organisms are taken from the bulk of the suspension, and after isolation are inoculated into the new medium with a minimum of delay.

The motility and morphology of the organisms can be studied in the microscope, which is of advantage when isolations are made from a culture of doubtful purity. The lighting is adequate for the observer to distinguish between rods and diplococci, and between rods of different sizes, with the facility of any hanging drop preparation. Spores are plainly visible on account of their high refractivity. In general, any cell which does not show some movement, with the exception of the largest non-motile rods, is stuck to the coverglass and useless for isolation. Spores and the smaller non-motile rods are in continual Brownian movement. The larger rods can be seen to quiver under the Brownian influence. Motile rods display a gyrating and undulating movement in the microdrops precisely as in other hanging drop preparations. They may frequently be observed cruising around the edge of the single cell drop. These factors must be taken into consideration in searching a drop for organisms and in making a rough selection of the more viable cells for transferring singly. An organism which is in rapid movement when first released on the coverslip, but which soon restricts or ceases its activity will not be likely to grow. It may be inferred that the cell was injured mechanically during the operation or has succumbed to the light or to the intimate atmospheric contact of the microdrop.

It should be possible to make ten or more isolations without changing coverslips during a period of two hours. We get from one to five successful growths from ten transfers of single cells from the same suspension.

The portion of the coverslip near the closed end of the chamber is usually the most satisfactory area for the isolations. When the coverslip has been in place for an hour or two, the part at the closed end usually becomes saturated with large dew drops and there is increasing difficulty in maintaining the film at the open end. If it is desired to make further isolations after this has occurred, the coverslip may be turned end for end on the chamber. A new film will then form on the unused portion of the glass.

DISCUSSION

The chief advantage of the pipette method of isolating single cells with a procedure such as the one we have described is the certainty with which the examinations can be made. By working under the oil immersion objective the operator may be sure that when growth results from a transfer, one and only one organism was its source. Exponents of the various agar-block methods criticize the pipette technic as offering too great an opportunity for contamination. Experience with the method will readily show, however, that this objection is without foundation. During the isolation of more than 100 strains we have not encountered a single contamination. Since this is the universal experience of those using the pipette method, it can be said that possible contamination is not a factor in the procedure. It would seem that many of the agar-block methods offer better chances for the appearance of adventitious organisms in cultures.

While it is not possible to follow the development of the isolated cell into a colony when the pipette technic is used, it must also be remembered that when solid films (nearly all of which contain interfering granules) are examined for organisms under a magnification of 440 times or less, there is always the possibility that nearby dormant cells which have escaped notice may afterwards grow and merge with the selected colony without the knowledge of the operator.

Finally, the micromanipulation requires no more time than do many of the methods based on different principles for which celerity is claimed.

Alternative methods

For the sake of completeness we include references to the better known procedures, not requiring the use of pipettes. A "harpoon" system for transferring from colonies of known ancestry has been used extensively by Ørskov (1922a, 1922b, 1924), and it is described in various alternative forms by Hort (1919–20), Levinthal (1927), and Stearn and Stearn (1927). A combination of micromanipulation and agar-film sytem has been devised by

Dickinson (1926a, 1926b). Dickinson places the organisms in the moisture film on a thin layer of agar and moves them about in a capillary bead of liquid formed between the film and a micro-needle. This ingenious device permits examinations to be made under the oil immersion lens.

A departure from the usual solid medium method is the one used by Topley, Barnard and Wilson (1921). This also permits examination under high power. The organisms are placed on a solid medium under quartz covers. Isolated organisms are covered with a bubble of mercury and the unshielded remainder are subjected to ultra-violet raying to destroy all other cells. This method, however, does not appear to effect a saving of time in comparison with micromanipulation, and care is necessary to make sure that all organisms but the one required are killed.

Dark field illumination has been used for making isolations, but in this case the presence of the isolation chamber complicates the problem of lateral lighting. Péterfi and Wámoscher (1926), and Péterfi (1926–27), who have adapted the dark field microscope for micromanipulation, recommend a chamber 4.5 mm. in height for high magnifications. In this restricted space the manipulation of the pipette becomes difficult. A 10 mm. chamber can be used for large organisms, such as yeast, which can be handled under the lower magnifications, as is done by Hahn, Schutz and Wámoscher (1926).

Accessory equipment

We believe that the pipette method as we have described it can be applied successfully with any type of microörganism. A satisfactory proportion of growths was obtained with the pipette method by Barber (1920) and by Starin (1924) in the case of the anaerobes.

Organisms which cannot be grown in a clear liquid medium can be suspended in an indifferent menstruum for the purpose of isolation, and in this case it is advisable to reduce the actual time required for an isolation to a minimum. The most direct method of attaining this end is by the use of two micromanipulators so

that an isolated organism expelled in a drop by one pipette can be immediately taken off with the other and removed to a suitable culture medium. We believe that the simpler single pipette system will be found inexpensive and convenient for most work, but if the transfers fail to grow when it is used, the apparatus described by Wright and McCoy (1927) is recommended. This is a special form of the double manipulator for bacteriological work. By the use of this machine the length of time during which the organism is exposed on the coverslip can be reduced to the few seconds required for complete examination of the drops.

When warm stage isolation is indicated, as in the case of the gonococcus, the device of Péterfi (1927) may be utilized. Péterfi has perfected a warm stage for micromanipulation of tissue and for the isolation of bacteria. A thermoelectric regulator keeps the temperature of the moisture chamber at 37 degrees.

If organisms are encountered which repeatedly fail to grow after isolation, despite the use of double manipulators and a warm stage, it may be advantageous to circulate air containing approximately 5 per cent carbon dioxide through the moisture chamber. It is advisable to pass the sterilized gas through a bubbler containing warm sterile water before allowing it to enter the chamber. This suggestion is based on the general favorable effect of carbon dioxide on bacteria and bacterial products which has been reported recently by Valley and Rettger (1927) and Valley (1928).

SUMMARY

1. The pipette method of procuring single cell bacterial cultures is described in a form which can be applied as a routine procedure requiring a minimum of special equipment.

2. Attention is directed to the steps in the technic which are essential for success and certain refinements are offered.

3. Accessory equipment recently devised by various workers and of service in work with difficultly cultivable organisms is reviewed briefly.

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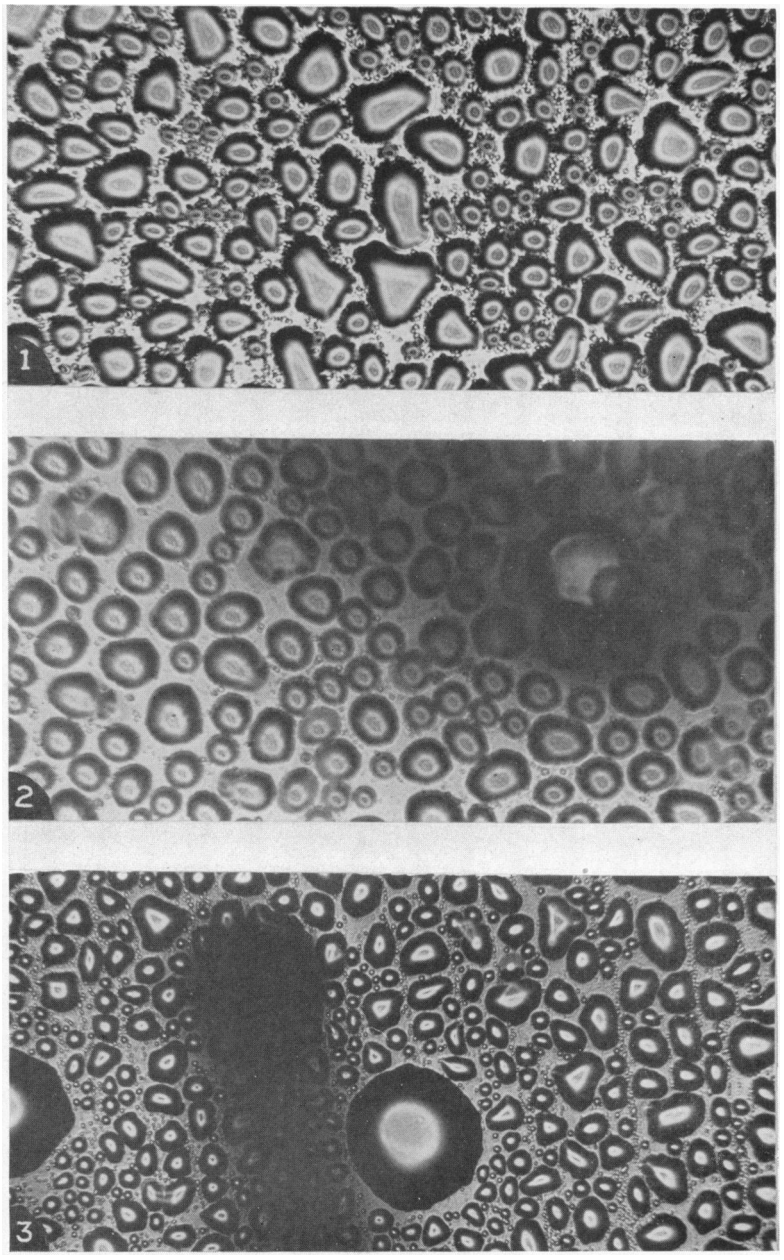
PLATE 1

SATISFACTORY FILM, MAXIMUM AMOUNT OF VASELINE

FIG. 1. Film of dewdrops.
FIG. 2. End of pipette just below film.
FIG. 3. Microdrop of sterile broth and shadow of receding pipette.

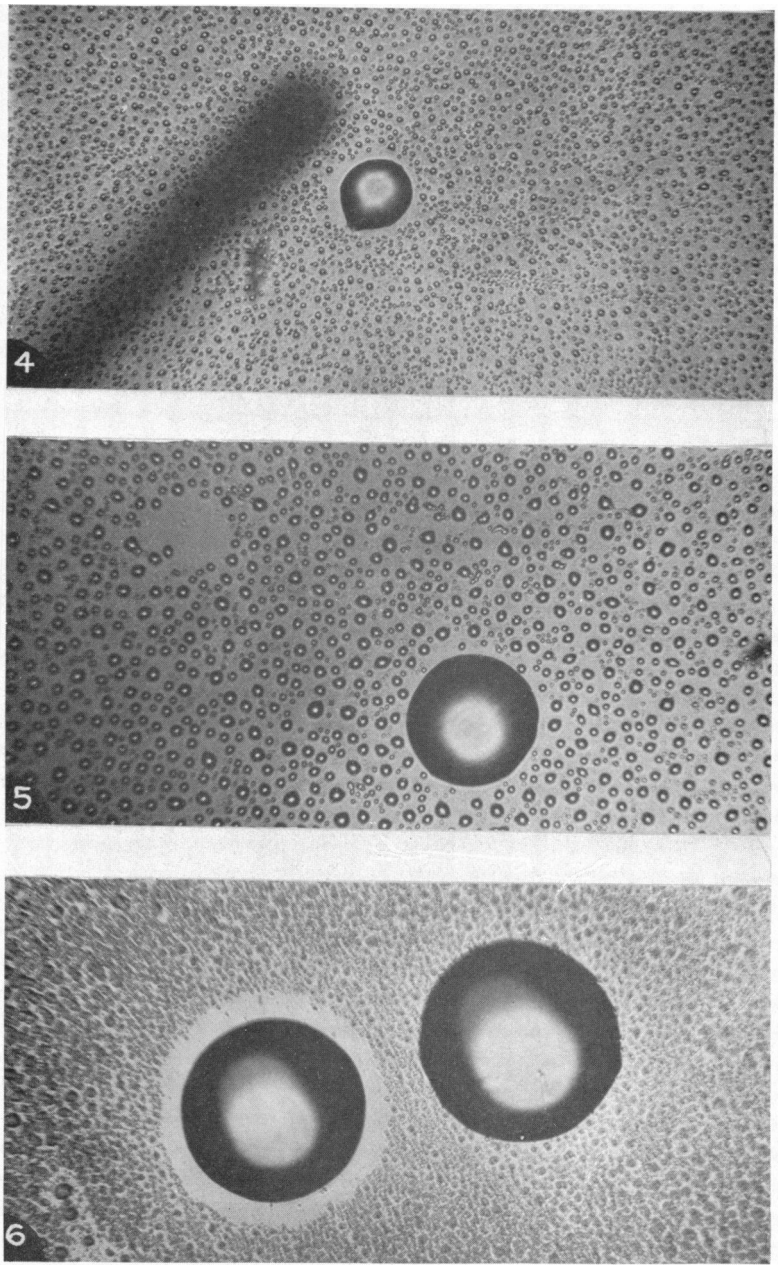


(Gee and Hunt: Single Cell Technic.)

PLATE 2

SATISFACTORY FILM, MINIMUM AMOUNT OF VASELINE

FIG. 4. Microdrop of sterile broth and shadow of receding pipette.
FIG. 5. Left, site after removal of microdrop; right, sterile microdrop.
FIG. 6. Left, sterile microdrop; right, microdrop containing a single bacillus.



(Gee and Hunt: Single Cell Technic.)

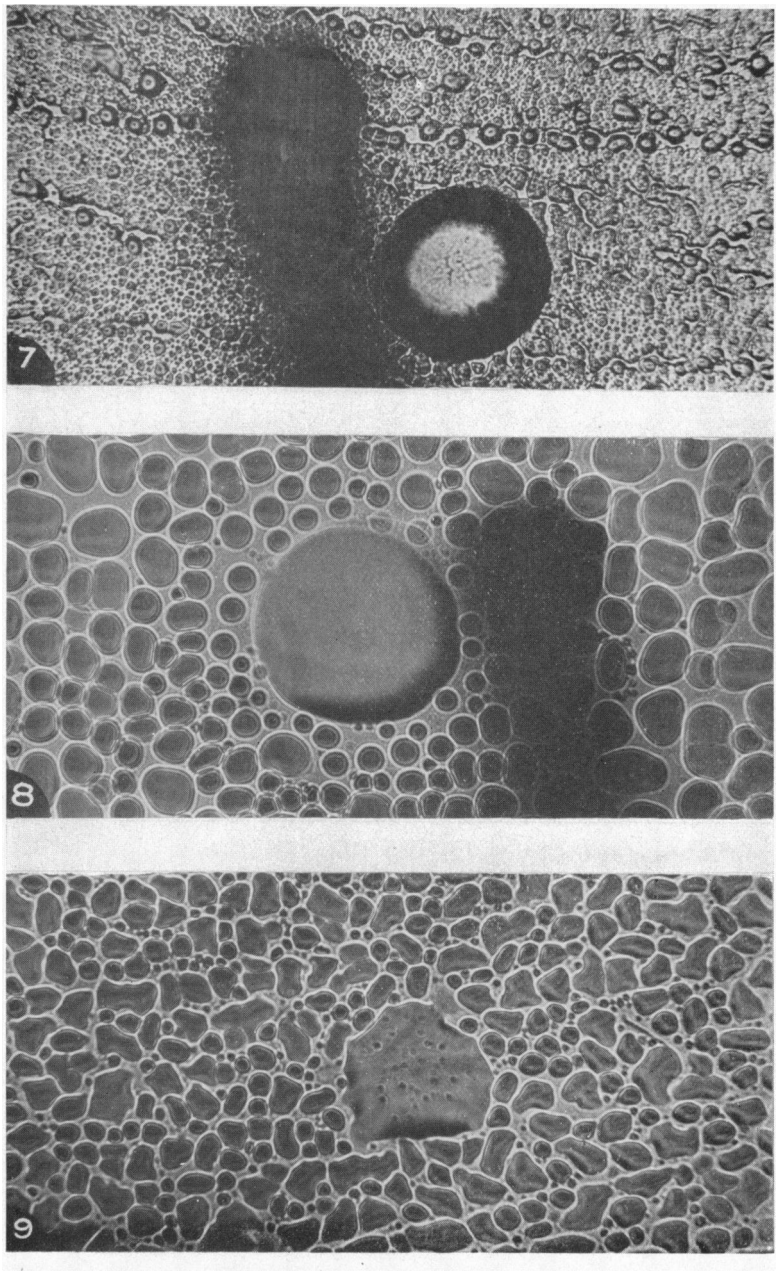
PLATE 3

FILMS USELESS FOR ISOLATION PURPOSES

FIG. 7. Excess of vaseline, distortion of dewdrops, streaks in microdrop.

FIG. 8. Insufficient vaseline, spreading of dewdrops, clear but shallow microdrop.

FIG. 9. Film prepared with dirty silk.



(Gee and Hunt: Single Cell Technic.)