Single-spore isolation using a hand-made glass needle

Teik-Khiang Goh

Fungal Diversity Research Project, Department of Ecology and Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong; email: tkgoh@hkucc.hku.hk


A method to isolate fungi from single spores using a hand-made glass needle is described and illustrated. Glass needles are made by joining glass Pasteur pipettes by heating with an alcohol-burner and pulling them apart while molten. Procedures for using glass needles in the manipulation of single fungal spores are detailed. Examples of successful spore isolations are given. The usefulness and limitations of this technique are discussed.

Key words: fungi, microbiological techniques, pure cultures, skills, spores, tools.

Introduction

The method used to isolate a particular fungus from a natural substratum, and to obtain a pure culture, depends somewhat on circumstances. Certain species may produce abundant fruiting bodies, whereas others may produce few spores. If fungal growth is luxuriant, with numerous visible aerial fruiting structures, then the method of isolation is usually straightforward. Isolation can be achieved by using fine forceps and a good hand-lens or dissecting microscope, to pick off a few spores or a single spore-head and transfer the spores to a suitable culture medium. A pure culture will often result from this transfer. It is seldom, however, that a fungus found growing on the natural substratum is entirely free of other organisms, and there is always a danger that direct transfer of spores will carry contaminants. In the case of slow-growing fungi, it is likely that the contaminant will overgrow the fungus. If this species is placed in a culture collection, this could cause serious problems of misidentification, as the fungal species in culture would not be the one it was supposed to be.

Another problem commonly encountered in the isolation of fungi from natural substrata by direct spore transfer is bacterial contamination. Fungal colonies growing on antibiotic-incorporated medium may still be contaminated
with bacteria. Another complication is that certain bacteria strongly inhibit the growth of fungi (Smith, 1969). If such bacteria are present, the isolation of fungi would be unsuccessful.

A method which is of particular valuable in the purification of a sterile fungus, or one which produces spores very sparingly, is hyphal tip culture. This technique, however, can be used only when the initial rate of growth of the fungus to be purified is greater than that of the contaminant. Other than employing hyphal tip techniques, single-spore isolation is certainly the best way to obtain pure fungal cultures. Many microbiologists regard a culture as pure only when it has been obtained by germination of a single spore (Kirsop and Doyle, 1991). In certain studies, single-spore cultures are essential. For example, investigations on the genetics of Neurospora species, and of yeasts, necessitate not only making numerous single-spore isolations, but also in serial order of all the spores in a single ascus. In order to prove the connection between an ascomycete and its conidial state it has usually been necessary to make cultures derived from both single ascospores and single conidia. In industrial work, biochemical studies, and the maintenance of culture collections, the value of single-spore cultures cannot be overstressed.

There are a number of methods available to obtain single-spore cultures. Various methods have been reviewed by Hilderbrand (1938) and Smith (1969). Most of these methods are basically various modifications of the spore-dilution technique. In addition to the dilution techniques, however, two other methods are worth noting. The most elegant method of isolating single spores is by means of micro-manipulators (El-Badry, 1963). These are special microscopes with which it is possible to pick up any particular spore and transfer it to a fresh culture medium, all under the high power of the microscope. Such microscopes are virtually essential in fungal genetics, and in any work involving quantities of single-spore isolates. Matsushima (1975) has used one of these micro-manipulators (Keisuke Matsushima, pers. comm.) to arrange single spores of various fungi for his wonderful photographic illustrations in his mycological lectorum.

Another very ingenious method of single-spore isolation is described by Hansen (1926). This method, although based on the spore-dilution principle, works well with hyphomycetes that have large coloured conidia, such as Alternaria, Corynespora, and Helminthosporium, but is difficult to use with species of Aspergillus and Penicillium. A dilute conidial suspension is made in melted agar medium. This is sucked up into a number of fine glass capillary tubes, of bore slightly wider than the diameter of the conidia, and the medium is allowed to solidify therein. Microscopic examination of the capillaries should
Figs. 1, 2. Preparation stages of the technique. 1. Materials required for needle-making and single-spore isolation, viz. 95% ethanol, 3% water agar plates, 2% potato dextrose agar plates (or any other suitable nutrient medium), alcohol burner, lighter, No. 7 scalpel, beaker for sharps, Pasteur pipettes. 2. The first step of needle-making: prior to heating, the entire front portion of a Pasteur pipette is being broken off in a beaker with fingers protected by a piece of folded paper towel.
show short lengths containing single conidia. These are broken off, sterilized externally with alcohol, and planted in fresh medium. The conidia germinate and the germ-tubes emerged from the ends of the capillary fragments and grow into typical colonies.

Single-spore isolation by means of micro-manipulators or capillary tubes, as described above, are useful but have limitations. Unfortunately, micro-manipulators are expensive and such outlays are usually not justified. On the other hand, a great deal of skill is required before one can suck up spores into the fine capillary tubes and break the tubes into short fragments containing single spores. In this paper, a further method of single-spore isolation is introduced. This technique does not require the expensive use of a micro-manipulator, and requires less skill than the capillary method.

For more than 17 years, I have been using hand-made glass needles for isolation of fungal spores, since the technique was taught by my former Professor, Dr. Wen-Hsui Hsieh, in the Department of Plant Pathology, National Chung Hsing University, Taiwan. In fact, this isolation method can be traced back to Dr. W.C. Snyder's laboratory at The University of California, Berkeley, where the skill was employed for the studies of *Fusarium* genetics. To the best of my knowledge, however, this technique has not previously been formally described and illustrated. The objective of this paper is therefore to present the procedure of the technique in stepwise detail, so that others can use this excellent technique for single-spore isolation.

**Materials and Methods**

Single-spore isolation using a hand-made glass needle requires: (a) the making of the glass needles, and (b) the use of the needle for isolation of fungi. Equipment and materials that are required (Fig. 1) include glass Pasteur pipettes, an alcohol burner, a lighter or a box of matches, a bottle of 95% alcohol, a piece of cloth or paper towel, a No. 7 scalpel with a surgical blade, 3% water agar plates, potato dextrose agar plates/slants or other suitable culture media, a container for disposal of sharps, a bench with clear work area, and a dissecting microscope.

**The making of the glass needles**

Glass needles are produced by joining a pair of Pasteur pipettes by heating in the flame of an alcohol-burner and then pulling the pipettes apart while the glass is still molten (Figs. 2-6). The use of a Bunsen burner instead of the alcohol burner is not recommended because the flame of the former is too intense and the correct timing of heating the pipettes is hard to control. The
Figs. 3, 4. The joining of the glass rods. 3. The two pipettes are held horizontally, and their tips are heated in the flame of an alcohol-burner. Note that the portion of the flame for heating the glass is the apex. 4. The tips of the two glass rods have become red-hot and are joining together in the flame.
Figs. 5, 6. The pulling of the glass rods to make the needles. 5. The pipettes with the glowing bridge have been removed from the flame for brief cooling just before the pulling action. 6. The pipettes are pulled horizontally outside the flame, with an acceleration in movement. Arrows show direction of pulling.
Figs. 7, 8. Treatment of bad needles. 7. A bad needle is broken off into the beaker with fingers protected by a piece of folded paper towel. 8. Two pipettes with the bad needle part broken off into the beaker, with the conical portion of the glass rods ready for the next trial of flaming.
flexibility of the glass needle would not be perfect if the duration of heating is not appropriate. Practically, alcohol-burners are very convenient and safe to use, and heating time can be controlled to produce good quality needles.

The following are step-wise directions for making the glass needles:

1. **Minimize airflow in the work area.** The best place for the making of the glass needles is inside an enclosed compartment, such as the lamina flow (with the airflow turned off), fume cupboard, or any other enclosed cabinets. During the joining and pulling of the pipettes, it is essential to keep the flame of the alcohol-burner vertical. If the flame is moving due to air currents, more time will be taken in heating the two glass tips till they glow. The glass would become fragile and the needles would not be flexible. It is therefore essential to minimize airflow in order to prevent flame movement. If you are using an open bench in the laboratory, then turn off all air-conditioners or electric fans in the laboratory so that air movement is minimal. If feasible, ask your fellow workers in the laboratory not to walk behind you while you are heating the pipettes, or wait till they have gone home.

2. **Preparation of glass rods.** Take two unused glass Pasteur pipettes, one in each hand. Before heating the pipettes, break off the entire narrow tube (i.e. the front portion) of the pipettes up to the conical part of the remaining tube, as shown in Fig. 2. Use a paper towel or cloth to protect fingers. The crushed glass should be disposed into a suitable container for sharps.

3. **Joining and pulling the glass rods apart.** Hold the broken pipettes horizontally with the conical parts facing each other, and heat the tips in the flame of an alcohol-burner (Fig. 3). The best portion of the flame for heating is the tip where it is the hottest, although it is this portion that moves the most. Within a few seconds, the two pipettes can be joint together when their tips turn molten-red (Fig. 4). Once the tips have fused, and while still heating, try to pull the tips slightly apart in such a way that a bridge of about 5 mm long and 2 mm wide is formed between the pipettes (Fig. 4). Hold the bridge in the flame for a few more seconds till it starts to glow red. Keeping the pipettes horizontal, remove them from the flame (Fig. 5). Wait for about 1 second so that the bridge cools slightly, and then pull the pipettes apart horizontally (Fig. 6), with a quick acceleration. Successful pulling of the pipettes results in two usable needles, or at least one good enough for the isolation purposes. These needles are reusable, as long as they are not damaged.
Figs. 9, 10. Good and bad needles. 9. An ideal glass needle with the handle ca 10 cm long and the flexible needle ca 2 cm long. Note that the needle is straight in respect to the handle and smoothly attenuated towards its end. 10. Illustration of one ideal (a) and five faulty needles, viz. b. too long, c. too short, d. crooked, e, f. stiff and with a hooked or truncate apex.

Good and bad needles

An ideal needle (Figs. 9, 10a) should have the following features, listed in descending order of importance:

1. *The needle must be very flexible.* The flexibility of the needle is essential to the sweeping, dragging, and other manipulation of spores on the agar surface. A stiff needle would be fragile and cut into the agar when broken.
2. **The needle must be smoothly attenuated at its tip.** The tip must have a smooth acute end (e.g. not truncate). A needle with a truncate tip would puncture the agar surface and spores under the dragging motion often would fall into the holes created by the blunt needle and disappear.

3. **The axis of the needle should be straight in respect to the axis of the handle.** A straight needle would facilitate manipulation.

4. **The needle should be about 2 cm long.** A short needle (e.g. 1 cm, Fig. 10c) is not good because the glass handle would be too close to the agar surface during manipulation. A long needle (e.g. 3-5 cm, Fig. 10b) usually does not have the right flexibility due to the length.

5. **The glass rod (i.e. the handle) should be about 10 cm long.** It would be inconvenient during manipulation if the rod is too short (e.g. 6 cm) or too long (e.g. 15 cm). A short handle would be difficult to hold, whereas a long one would be awkward when working under a dissecting microscope.

The timing of the heat-joining and cool-pulling of the glass is critical to produce good needles. If the timing is incorrect the needles may not be useful. If the red-hot bridge between the two pipettes has not been allowed to cool...
Figs. 12-18. Photomicrographs under a dissecting microscope illustrating representative stages of single-spore isolation with a hand-made glass needle. 12. Close-up of an ideal glass needle made before the spore isolation. Note that it is straight and smoothly attenuated towards its end. 13. Lanes scored with a sterile scalpel on the surface of a 3 % water agar plate. 14. A sample containing conidia of *Tetraploa aristata* transferred from the natural substratum to agar surface. It was placed on the left of and at a distance apart from the pre-scored lanes of the agar. 15. Single conidia of *T. aristata* dragged with the glass needle into individual lanes on the agar. 16. The agar plate turned 180° (note the reference alpha (α) sign in Fig. 13) and the lanes containing individual conidia lifted with a sterile scalpel and transferred to appropriate culture media. 17. Close-up of the agar block with a single conidium of *T. aristata*. The block has been transferred from the water agar plate and is now situated on potato dextrose agar plate. 18. The same agar block in Fig. 17. after 24 hr incubation at room temperature. The single conidium has germinated. Bars: 12 = 5 mm, 13-18 = 1 mm.
slightly before pulling, a long thread-like needle will result (Figs. 7, 10b). The acceleration during pulling is essential to create a smoothly attenuated needle with an acute end (Figs. 10a, 12). The overall pulling action takes about half a second. If the timing of pulling is not right, the needles would have one or more of the following undesirable features (Fig. 10b, c, d, e, f): (a) thread-like, with no attenuation; (b) not flexible but stiff (i.e. like a spur instead of an eye-lash); (c) the tip is not acute at its very end, but truncate. This is because the glass loses its plasticity when cooled, due to overdue pulling time. The pulling breaks the glass which results in a truncate tip. The truncate tip might not be noticeable unless the needle is examined under a dissecting microscope. By practice, however, one could still be aware of such faulty needle tips since the breakage of the glass can be detected by a “click” during the pulling action.

If the making of an ideal glass needle is not successful, try to do it again. After a short period (e.g. 30 min.) of practice it should be possible to master the production of perfect needles. Faulty needles should not be reheated for joining again because the glass has become fragile after the first heating, and hence it would no longer result in a flexible needle. Faulty needles should be broken off manually into a container for sharps before the conical portion of the pipettes can be heated again for the next effort in needle-making (Figs. 7, 8).

**Isolation of single spores using the glass needle**

Once an ideal glass needle (Fig. 12) has been made, it can be a very useful tool to carry out single-spore isolations of various fungi. The fungal spores that are of appropriate size can be dragged on the surface of a water agar plate by means of water tension at the tip of the glass needle. Practically 3 % water agar is of appropriate hardness, with surface water tension just right for this manipulation. Water agar plates which are too watery and soft (i.e. contain 1.5-2 % agar powder) are not ideal because excessive surface water and the soft agar texture physically do not facilitate the dragging of spores on the agar surface. The details of the isolation process are described as below:

1. At the centre of a clean 3 % water agar plate, score under a dissecting microscope with a sterile scalpel (flamed with the alcohol burner) a vertical line followed by a series of horizontal lines as shown in Fig. 13.
2. From either the natural substratum or a sporulating colony in culture, transfer with a scalpel a minute sample of the mycelium with spores onto the surface of the water agar on the left* of, and at a distance apart from, the pre-scored lanes of the agar (Fig. 14).

* If you are left-handed, then it should be on the right, respectively.
3. Sterilize the hand-made glass needle by dipping it into 95% alcohol. The alcohol on the needle tip can be removed by means of gently touching the tip a few times on a clean area of the agar surface. Do not autoclave nor flame the needle for the purpose of sterilization or removing excessive alcohol because the heat will distort or damage the needle tip.

4. Under the dissecting microscope, single spores of an appropriate size (ca 5-30 \( \mu \text{m} \) wide) can be dragged with the glass needle due to the water tension of the agar surface and the needle tip. Manipulation involves repeated picking-up and dropping-off of the spore using the needle tip, along a distance of clean agar surface towards the pre-scored lanes. The spore is actually rolled and cleaned by the dragging movement on the agar surface. Any dirt or bacterial cells on the surface of the spore should be removed by repeating rolling (i.e. the pick-and-drop manipulation). The spore is eventually dragged into one of the pre-scored lanes. More single spores can then be dragged and cleaned in this manner until sufficient spores have been isolated by this manipulation, each in one of the pre-scored lanes as shown in Fig. 15.

5. The agar plate can then be rotated 180° in such a way that the lanes on the left of the vertical line (i.e. those with the isolated spores) become on the right for the ease of cutting and lifting agar blocks (Fig. 16) with the sterile scalpel in your right hand. The lifting of the agar blocks should be carried out under the dissecting microscope.

6. Individual agar blocks lifted by the scalpel, each contains a cleaned single spore (Fig. 17), can now be transferred directly to appropriate culture media (either as Petri plates or slants in tubes) and incubated for spore germination and mycelial growth (Fig. 18). The ability of the spores to germinate and grow will depend on the fungal species isolated.

Safety Precautions

To use the hand-made glass needles in the isolation of fungal spores, a few safety precautions need to be addressed in order to avoid any risk of punctures, scalds, and burns. These are noted as below:

1. To avoid punctures and cuts by the glass needles: While making needles, failure in producing a perfect needle is common. When you manually break off the faulty needles, care should be taken. Always break the needles with paper towels or a piece of cloth for protection and always dispose the glass into the specific container for sharps (Figs. 2, 7, 8).

2. To avoid scalds, burns or fire: Never break the needle immediately after you find it to be faulty, because the glass may still be hot, especially the conical portion of the rod. The heat may scald your fingers, burn the paper towel, or
light a piece of paper in the rubbish bin. Wait for at least 30 seconds for the glass to cool before the faulty needle is broken manually. Similarly, make sure the needle is cool before it is put on the bench, because the hot glass would scald the bench surface, especially if covered by plastic or paper sheets.

3. How to keep the needles when not in use? When not in use the needles should be kept appropriately to avoid accidental cuts or punctures. To protect the delicate tips of needles, keep them in an upright position (Fig. 11). An easy way is to hold the needles in a test tube rack. They can also be held in holes or slots made in polystyrene blocks or synthetic sponges. The holder with the needles should be stored in a safe area, e.g. on top of a shelf.

Results and Discussion

Comparisons with other methods of single-spore isolation

The method described in this paper is somehow a combination of features exhibited by the micro-manipulator as well as the capillary methods. Like the micro-manipulator, this method enables one to pick up any particular spore visible under the dissecting microscope, but the cost is much cheaper than that of the micro-manipulator. Like the capillary method, this glass needle method works particular well with species of dematiaceous hyphomycetes, but the needle is solid instead of a capillary. In contrast to the fracturing of capillary tube into fragments containing single spores, in this method a small agar block with isolated spore is transferred to fresh medium where it germinates and grows directly into the medium.

Usefulness and limitation of the technique

Of the various techniques used for single-spore isolation, I have found the glass needle method very convenient, economical, and useful.

The technique is convenient because isolation of fungi need not be in a sterile environment (e.g. in a lamina flow), but can be done in the laboratory without fear of contamination. This is because single spores are cleaned during the dragging process and are transferred directly to a fresh medium for axenic growth.

The technique is economical because antibiotics are not needed in the water agar or the nutrient medium, since the manipulation renders the isolated spores free of bacterial contamination. Unlike other isolation techniques, the method also saves money on excessive use of sterile pipettes, agar plates, alcohol, and other indispensable items used for sterile conditions and spore dilutions.
The technique is useful because it can be modified for the isolation of various fungi belonging to different taxonomic groups. Using the glass needles, I have successfully isolated various fungi. These include (i) hyphomycetes, from their conidia, e.g. Cercospora and allied genera (Hsieh and Goh, 1990), Helminthosporium and allied genera (Goh, Hyde and Lee, 1998b), and Brachydesmiella (Sivichai et al., 1998); (ii) ascomycetes, from ascospores: e.g. Melanospora zamiae (Goh, Hyde and Hanlin, 1998a); and (iii) chytridiomycetes, from zoosporangia: e.g. Gonapodya (Fuller and Clay, 1993).

The glass needle method works well for fungi with relatively large spores, no matter what the shape (e.g. amerospores as in Melanospora, scolecospores as in Cercospora, and staurospores as in Tetraploa). As in the case of the capillary method (Hansen, 1926), however, the glass needle fails to manipulate smaller spores like those of Aspergillus, Penicillium and Cladosporium.

Axenic cultures obtained from the glass needle technique have been employed in different aspects of mycological studies. These include (i) DNA extractions for molecular studies (Goh et al., 1998b), (ii) physiological studies: verifications of homothallism and teleomorph-anamorph connections (Goh et al., 1998a), and (iii) isolation of individual ascospores from a single ascus* for studies of fungal genetics. Other applications of the glass needle technique include (i) the orientation of ascomata during resin-embedding for transmission electron microscopy (Goh and Hanlin, 1998), (ii) the manipulation of delicate asci during staining and washing for fluorescence microscopy (Goh and Hanlin, 1997), (iii) the purification of fungal structures from the contaminated environment** (Fuller and Clay, 1993). The glass needle can also be used to arrange fungal spores artistically and aesthetically on a microscope slide for

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* To isolate the individual ascospores for genetic studies, an ascus is transferred to the water agar and is then gently squeezed with the needle tip to break the ascus and release the ascospores. This works best for cylindrical asci (e.g. those of Neurospora and other sordariaceous ascomycetes), for which individual ascospores can be squeezed out from the ascus sequentially. The individual ascospores can then be dragged with the glass needle and isolated accordingly.

** To isolate and/or purify a zoosporic fungus from a natural substratum (e.g. Gonapodya on dogwood seeds, Saprolegnia on a dead insect), a small portion of the thallus containing zoosporangia or oospires is transferred to a microscope slide containing a few drops of sterile water. With the glass needle, individual zoosporangia or oospires can be gently agitated in the water drop in order to remove the bacterial cells on the fungal surface. The process can be repeated several times until the fungal structures are almost clean. The washed fungal structures can then be transferred to the surface of a water agar plate to be dragged with the glass needle and eventually isolated.
photographing purpose, as similar to the use of a micro-manipulator (Matsushima, 1975).

Every method of single-spore isolation has its own advantages and limitations. The most difficult part of the technique described in this paper is the production of an ideal glass needle and the manipulation of the fungal spores using the needles. With sufficient practice and patience, the technique should prove to be convenient and useful for manipulating fungal structures. It is, however, not restricted to the isolations of single fungal spores. The basic skill of manipulating fungal structures using the glass needle can be modified so that the technique may be applied to various aspects of mycological or other biological studies.

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