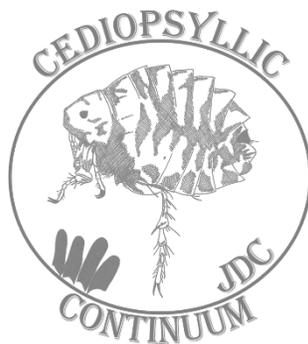


How to Mount Your Flea

A GUIDE TO THE PRESERVATION, PREPARATION, CLEARING,
AND MOUNTING OF SIPHONAPTERA

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How to Mount Your Flea

“The flea is a remarkable insect. From a much simplified larva... it transforms through a pupal stage into a incomparably specialized imago. No part of the external anatomy of an adult flea could possibly be mistaken for that of any other insect. The head, the mouth parts, the thorax, the legs, the abdomen, the external genitalia all present features that are not elsewhere duplicated among the hexapods.”

Robert E. Snodgrass, 1946

Author's Note:

All steps and materials used in this guide were chosen to provide the easiest, and quickest method for producing cleared and mounted flea specimens. This was done to aid technicians within the public health fields that need to produce accurate identification of species within a timely manner for disease testing or surveillance purposes. Any methods or steps can be exchanged for any that suit the user best.

I also want to encourage all that are starting out for the first time to practice with as many mounting mediums as possible to find the one that works best for you and your abilities.

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Materials and Tools Needed

Equipment

Starting with the basic hand tools you will want to acquire: 1) minute spatula; 2) microscopy probe or single barb microscopy tool; 3) bent microscopy probe (optional, works well for manipulating fleas); 4) bent teaser (optional); 5) small paint brush; 6) wooden applicator sticks; 7) cotton swabs (Q-tips®, etc.); 8) small microfiber towel or Kimwipes (Kimtech®; Kimberly-Clark Professional); 9) 1.5mL screw-cap vials; 10) box of slides; 11) round cover slips No. 1 or smaller (0 or 00) ranging from 12 to 17 mm, 12) slide labels (sometimes referred to as “slide end labels”); 13) pen with archival ink (MICRON®, etc.); 14) reliable pair of forceps, 15) Preferred or reliable dissecting microscope.

Hand tools (pictures)



Figure 1. The basic hand tools you'll need

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Figure 2. Microscopy tools; from left to right: 1) probe or single barbed tool; 2) bent probe; 3) minute spatula

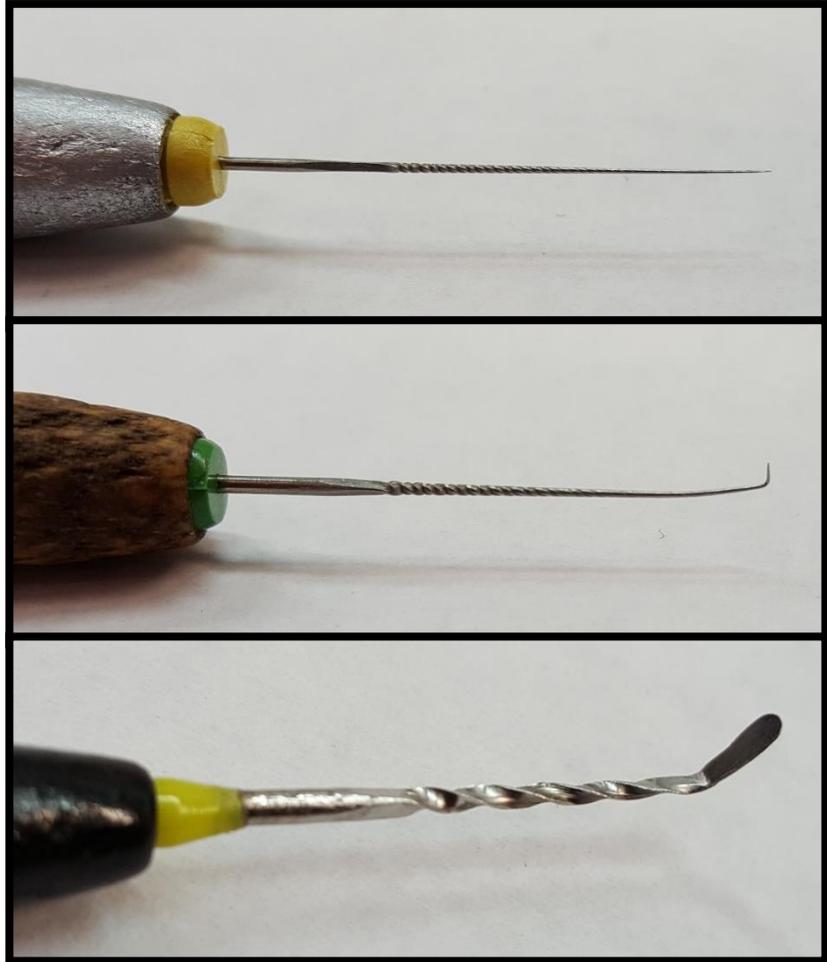


Figure 3. Closeup of microscopy tools; from top to bottom: 1) probe; 2) Bent probe; 3) minute spatula



Figure 4. Handmade probe/incision tool

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Figure 5. X-ACTO® knife with #11 blades (Z series), and #16 blades



Figure 6. From top to bottom: 1) Pixnor® size 11 forceps (my preferred size for handling fleas); 2) Featherweight forceps (Bioquip Product, Inc.; work well for later steps when fleas might become brittle)

Slide Materials (pictures)



Figure 7. Materials needed to create a slide



Figure 8. Box of slides



Figure 9. slide stand; included with box of slides

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Figure 10. Round coverslips



Figure 11. Slide labels (slide end labels)



Figure 12. Slide ringer (Bioquip Products, Inc.)



Figure 13. Appropriate wipes for your slides; from top to bottom: 1) Kimwipes (Kimtech®); 2) small microfiber towel

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Chemicals

The chemicals you will want to acquire are: 1) distilled water; 2) 95-100% ethanol; 3) 10% Potassium hydroxide (KOH) solution (can be diluted from a stock solution, discussed later); 4) Cellosolve (Bioquip Products, Inc.); 5) PVA mounting medium (Bioquip Products, Inc.); 6) Barber's Relaxing/Cleaning Fluid (Bioquip Product, Inc.); 7) Clear finger nail polish.

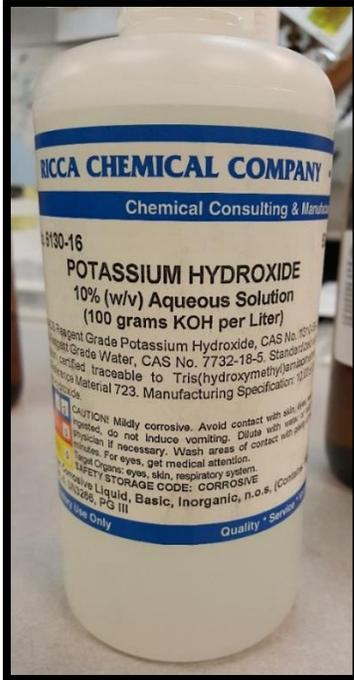


Figure 14. 10% KOH solution



Figure 15. Clear finger nail polish

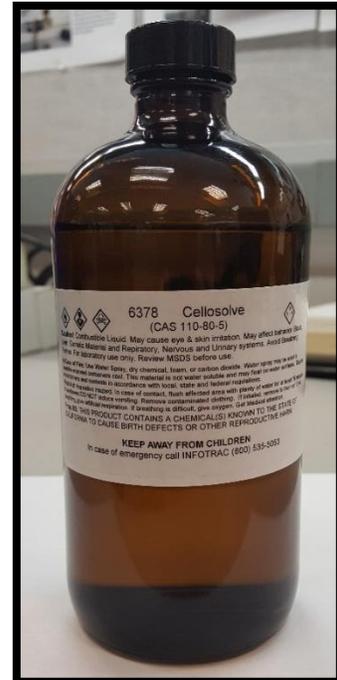


Figure 16. Cellosolve (Bioquip Products, Inc.)

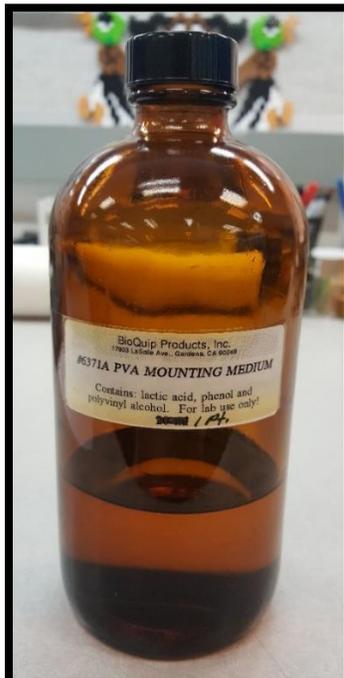


Figure 17. PVA mounting medium (Bioquip Products, Inc.)

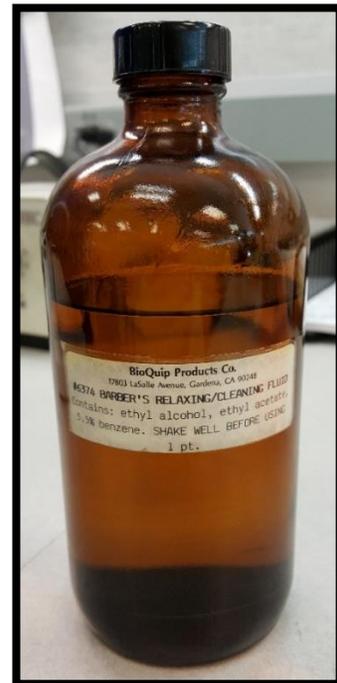


Figure 18. Barber's Relaxing/Cleaning Fluid (Bioquip Products, Inc.)

Preservation

When preserving specimens for later projects (clearing and mounting) the most common method is to use a 70-80% ethanol solution. Using a solution within this given range will keep your specimens soft, pliable, and easier to handle during the mounting procedure. You want to avoid using isopropyl, methyl, or higher concentrations of ethanol, all of which can make the specimens brittle and possibly unusable. Issues might arise if you are looking to keep specimens for later DNA, disease, or other testing. A 70-80% ethanol solution will preserve DNA for roughly one to two years. If you plan to test specimens beyond two years, it is best to use a 95-100% solution of ethanol. At 95-100%, ethanol will keep DNA preserved for up to ten years. Since it is most likely that you will not clear and mount every flea within a given collection/sample it is most practical to remove the number of fleas that you wish to mount on slides (plus a few extra in case you make a mistake or two) and preserve them in 70-80% ethanol until you are ready to begin working on them. For the other fleas that are most likely going to be preserved for future testing it would be best to place them in 95-100% ethanol and store them in a dark cool location to help reduce the speed at which the ethanol will evaporate (high percentage ethanol tends to evaporate very quickly). It is best to store samples in either a glass or polypropylene screw cap jar (avoid using "snap caps" as they can easily come off and provide less of a seal). The polypropylene jars are not as translucent as glass but provide a better storage device for specimens suspended in alcohol.

Making an ethanol solution

If you do not already have 70-80% ethanol on hand you can easily make some. This task can be achieved quite simply by diluting any stock ethanol you may have (95 or 100%). As an example, let's say you want to make 100mL of 70% ethanol, the first step would be to retrieve 70mL of 100% ethanol, and add 30mL of distilled water to it, this will make a 100mL solution of 70% ethanol. If you need a more visual display for this, or prefer calculations you may use the equation $(M1)(V1) = (M2)(V2)$. In our example, M1 equals the percentage of ethanol you wish to achieve, V1 equals the volume of ethanol you wish to achieve, M2 equals the concentration of the stock ethanol used, and V2 equals the volume of stock ethanol you are starting with. For the above example the equation would look as follows:

$$(70\%)(100\text{mL}) = (100\%)(V2)$$

$$7000\text{mL} = (100)(V2)$$

$$7000\text{mL}/100 = V2$$

$$V2 = \underline{70\text{mL of 100\% ethanol}}$$

(Note: the difference remaining to get to 100 mL is the amount of distilled water that must be added to the 70 mL of 100% ethanol)

This equation will work for any percentage of stock ethanol you start with or any percentage of dilute ethanol you wish to achieve.

Clearing Your Flea

Unlike mounting other small arthropods, fleas will need to undergo a clearing process to remove consumed blood and other soft tissues from within the flea to allow key internal structures (spermatheca, and male genitalia) to be clearly visible, as well as making external features sharper. This is probably the most technical and time-consuming part of the mounting procedure, but do not let this cause you any alarm or aversions towards the process, all you really need to master this procedure is practice, and one thing you will notice while working with fleas, if you hadn't already, is that you shouldn't be in short supply of specimens to practice with. To make the process easier I have broken the clearing procedure into four main steps and one optional step.

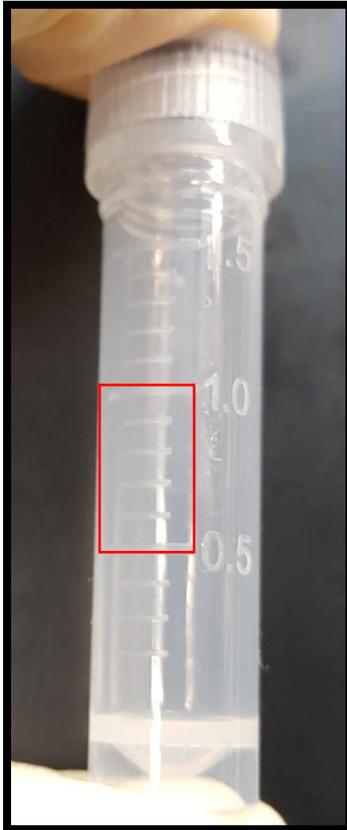


Figure 19. 1.5mL screw cap vial; 0.5 - 1.0 range is highlighted

of blood within the flea's body (usually appearing as a red to black stain within the flea), which blocks or disrupts your view of key morphological features. One of the best ways to clear internal structures and matter from the flea is to use a 10% potassium hydroxide (KOH) solution. If you already have 10% KOH on hand add some to a 1.5mL screw cap vial (around 0.5-0.7mL). If you only have a pure KOH solution on hand you can easily make a 10% solution by taking a 1.5mL screw cap vial and adding 0.75mL of distilled water (roughly half way between the 0.7mL and 0.8mL mark on the vial; if measurement marks are present). After adding the distilled water to the vial, add about 3 to 4 drops of KOH to the vial (adding anymore KOH doesn't help the clearing process and can damage or destroy delicate structures). After adding the KOH, you want to replace the cap, make sure it is on secure, and then invert the vial 8 to 10 times to mix the solution thoroughly.

The Four Steps to Clearing a Flea:

- 1) Potassium hydroxide soak (24 hours)
- 2) Spreading and hardening the flea
- 3) Evacuating liquified internal contents
- 4) Cellosolve soak (One hour)

Barber's Relaxing/Cleansing Fluid Soak (Optional Step)

This is an optional step best used for specimens that are desiccated, dirty, have been frozen and thawed out, or have been stored in improper alcohol. To accomplish the soak, fill up a 1.5mL screw cap vial between 0.50 to 1.00mL, and place the flea or group of fleas into the vial. Allow the specimen(s) to soak for 30 minutes to one hour. This soak will help clean off the exoskeleton of the flea and will help loosen the extremities (legs, etc.). If you are dealing with a fresh specimen that has been suspended in the proper alcohol solution you can skip this step and go straight to the potassium hydroxide soak.

Step One: Potassium hydroxide (KOH) Soak

The first step in the clearing process includes making a small incision into the flea's exoskeleton and suspending the flea in solution for a given period of time. One thing you might notice while looking at a flea under a microscope is the presence



Figure 20. Inverting the 10% KOH solution

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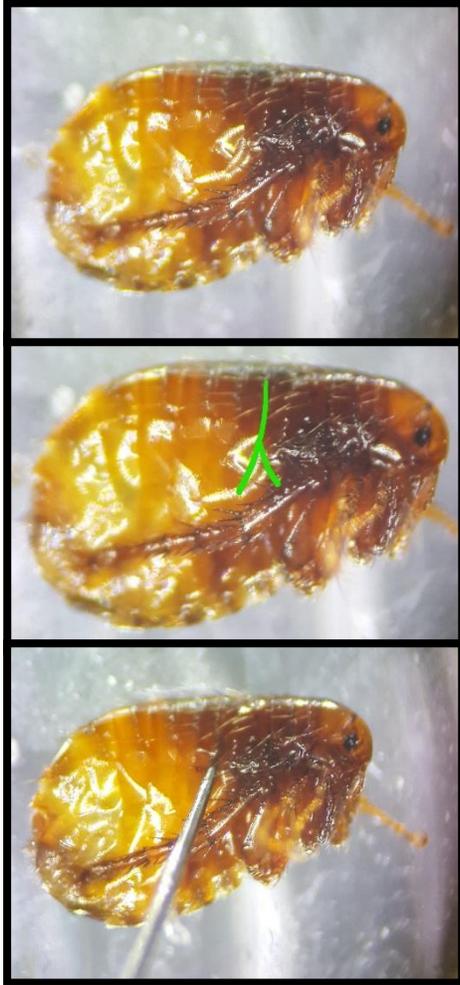


Figure 21. Incision for KOH soak

After the 10% KOH solution is prepared, begin prepping the flea for the KOH soak. Start by placing the flea in a wax bottom petri dish (dissection dish, Pryex[®]) filled with alcohol. The wax bottom helps when making the incision into the flea. Then puncture the flea on its right side between the first and second abdominal segments (it is acceptable to puncture through the flea into the wax). When mentioning the “right side” of the flea, I’m talking from the flea’s prospective (refer to Figure 24 on page 9 for a positioning aid). A good visual marker for the correct placement of the incision is the inverted ‘Y’ shaped “seam” that is created where tergal plate (upper abdominal plate) two converges with stergal plate (lower abdominal plate) two (location is highlighted in Figure 21 on the left). This area is right next to the seam created by tergal plates one and two, the incision point. Also note that fleas lack a stergal plate one. Remember this incision should be made on the flea’s right side. The small incision is necessary to allow the KOH to flood into the flea’s body cavity. The best tool to use for making the incision is a microscopy probe (single barb microscopy tool; a thin twisted piece of metal that resembles a bristle, think porcupine bristle/needle), or you can easily make a homemade tool by taping a double or triple zero mounting pin to

the end of a wooden applicator stick. Sometimes you’ll find it can take quite a bit of pressure to penetrate through the flea’s exoskeleton considering their size. A good way to tell if you have penetrated through the exoskeleton of the flea is to try and lift the pin or probe up with the flea still attached. If the pin stays stuck into the flea, after lifting it up, you have most likely penetrated the exoskeleton.

Once you have made the hole/incision into the flea’s exoskeleton, place the flea into the prepared vial and allow the flea to soak in the KOH solution for a 24-hour period. Try and make sure the flea is fully suspended in the solution, it doesn’t have to be sitting at the bottom of the vial, but you do not want the flea floating at the top of the solution where the KOH doesn’t have the opportunity to enter the flea’s body cavity. If you notice the flea wanting to stay at, or float back to the surface, try inverting the vial, allowing the flea to float to the top of the solution (bottom of the vial), and quickly turn the vial back upright (refer to Figure 22 on the right). This inversion can help catch the flea under the solution of KOH, allowing the flea to stay at the bottom of the solution or hopefully only move part way up the solution and stop before breaking the surface.



Figure 22. Submerging the flea in KOH. Top: inverting the vial. Middle: flea at bottom of solution. Bottom: Flea rising but not breaking the surface.

Step Two: Evacuating Liquified Internal Contents

This step can be the most difficult to perform and is most likely the step that you are going to make mistakes on (this is the main reason I suggested having some back up fleas in your mounting sample). After the KOH soak you want to move the flea to a petri dish filled with distilled water. If you notice that the flea will not sink into the water, you can try pulling the flea down into the water with a pair of forceps or try adding a few drops of 70% ethanol to help break the water's surface tension. Once the flea has sunk to the bottom of the petri dish you want to turn it onto its left side (right side up) and make a minute incision between the thorax and the abdomen (refer to Figure 23 on the right). You can either use an X-ACTO® knife or microscopy probe to make the incision. I find that the X-ACTO® knife works well on large bodied fleas (*C. felis*, *Pulex irritans*, etc.), but when working with smaller species (*E. gallinacea*, etc.) or a small male flea, the probe works best for making the incision. This incision is made on the same side of the flea as the incision made earlier for the KOH and is simply over one segment towards the head. The right side of the flea will be facing the bottom of the slide once mounting is complete, hiding the incisions. As well, the section between the abdomen and thorax of the flea is not used much in the identification of flea species so little to no key morphological features should be disturbed. Placing the incision further up the flea's body, away from the distal end of the abdomen, also helps prevent evacuating any internal structures or organs that you want to keep inside the flea (female's spermatheca, and male's genitalia). If you find it tricky making the incision into the flea, I find that positioning the flea with its back towards you can make the task easier. You can easily achieve this position by placing the flea's head to your left-hand side and placing the legs of the flea towards the arm of the microscope (refer to Figure 24 below).



Figure 23. Incision for evacuating liquified internal contents

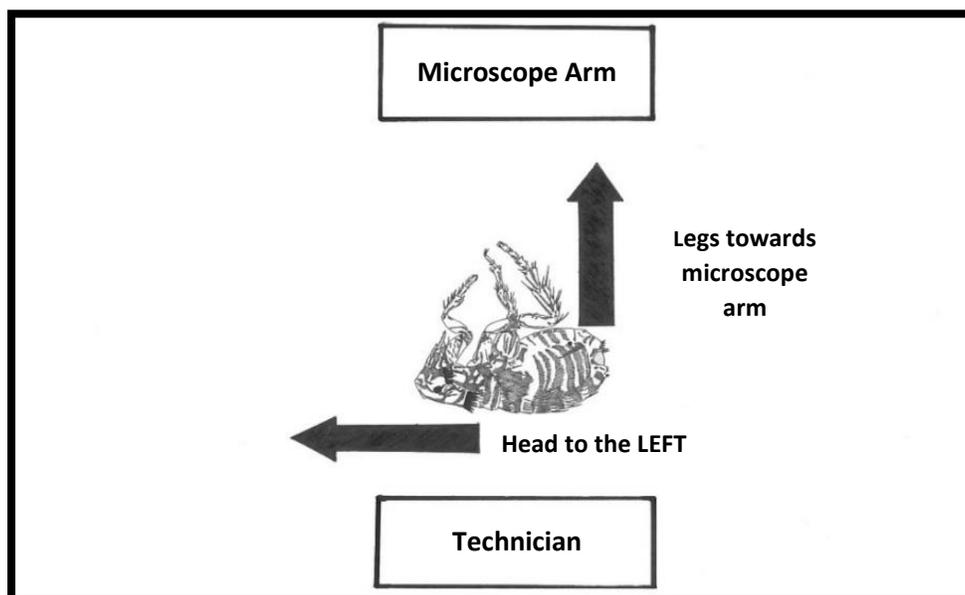


Figure 24. Flea positioning for incision

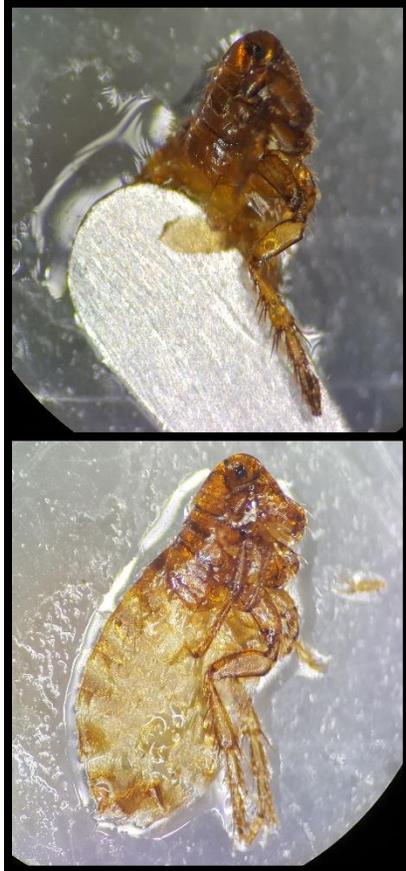


Figure 25. Applying pressure to the flea's abdomen with a minute spatula

the exoskeleton of the flea. Due to the thicker contents in the flea, you will need to apply more pressure to evacuate them from the flea's exoskeleton, and this means taking even greater care to avoid damaging the seta and other surface structures that are key for identification while pushing the spatula down onto the flea's abdomen. What I found works best, is to start by applying pressure near the incision which helps start the excretion of the internal contents (refer to Figure 26 to the right). By initially applying pressure near the incision you will avoid pushing out too much matter at once, which can cause the incision to rip open damaging the exoskeleton further, and possibly causing any structures you wanted to keep inside the flea to evacuate. After applying pressure near the incision, you want to locate the spermatheca or male's genitalia on the flea and start applying pressure to the abdomen in the area just in front of these organs to help avoid pushing them out of the body completely or too far out of place. Once most of the contents from in front of the key organ has been evacuated, you can take your time and push the contents out that are located beside and behind these organs. Once enough contents have been removed, you are ready for step three, spreading and hardening the flea.

Once the incision is made, start applying pressure to the flea's abdomen with a minute spatula, hand-made versions are acceptable, until the contents start to excrete from the incision (refer to Figure 25 on the left). If the KOH soak goes well the liquified internal contents should come out of the flea rather easily and will look like a plume of orange to copper liquid evacuating the incision, or out of the "seams" formed from the individual plates of exoskeleton making up the flea's body. In some instances, you may notice that the flea doesn't need to be flattened and most of the internal contents have been cleared out already.

Not all the flea's liquified internal contents may evacuate on the first squeeze, or application of pressure. If this does occur, simply let the flea sit in the distilled water for a few minutes and start squeezing other fleas. Once water has filled into the flea's body cavity (through osmosis) you can squeeze the flea again. This process of squeezing and re-squeezing can be repeated as many times as necessary. Be careful while reapplying pressure to avoid damaging the exoskeleton and/or seta of the flea.

If you didn't allow for the full 24-hour soaking period or your KOH solution was too dilute, the internal contents of the flea might not be completely liquified, and more "jelly" like. If this is the case, you want to take your time when applying pressure to the flea's abdomen. If done too fast and/or hard you can evacuate all the contents of the flea including the key organs that you want to keep (female's spermatheca or the male's genitalia), or possibly destroy



Figure 26. Applying pressure near the incision to evacuate thick gel-like internal matter; Notice the widening of the incision, "jelly" like matter.

Step Three: Spreading and hardening Your Flea

After the KOH soak is complete and you have cleared any remaining liquified internal contents, it is time to spread the flea's legs, and harden the specimen. You may notice that some, if not most, of the fleas that you work with will have their legs tucked in, which blocks some key morphological features found on the legs. To perform the spreading, start by acquiring a slide and securing it under a dissection microscope. Next, retrieve one of the fleas that have been cleared and place it on the slide. After the flea is on the slide, take a small piece of paper towel, tissue, or any absorbents material on hand and dab it onto the water surrounding the flea to soak it up. Once the water is removed apply a small drop of Barber's Relaxing and Cleansing Fluid (Bioquip Products, Inc.) onto the flea. Once again take the small piece of absorbent material and soak up the fluid surrounding the flea leaving just enough to coat the flea's exoskeleton and keep the flea adhered to the slide. Note, you will want to keep the flea moist and not allow it to dry throughout the spreading process by reapplying small drops of the fluid while you are working on spreading the legs. To draw the legs out away from the flea's body several microscopy tools can be used from a bent microscopy probe, minute spatula, hand-made probe, etc. I Found that using the combination of a bent microscopy probe in my dominant hand, and a minute spatula in my off-hand worked best, by using the bent probe to spread/straighten the legs, and lightly using the spatula to keep the flea secured in place, if it starts to move.

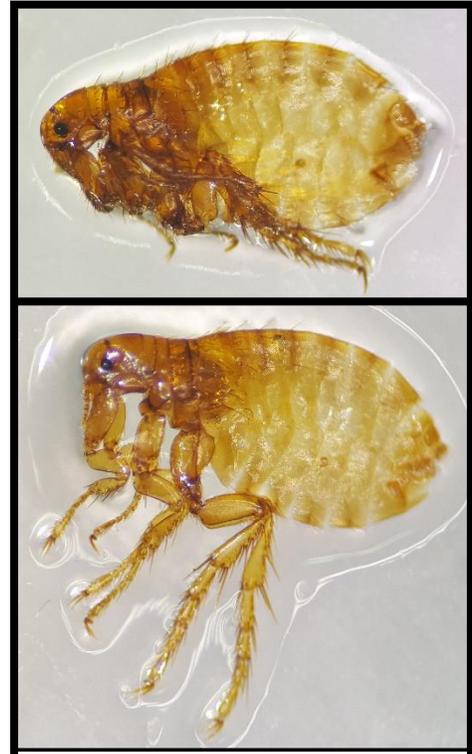


Figure 27. From top to bottom: 1) flea before spreading; 2) flea after spreading

Once the legs are spread out it is time to harden the specimen. Start by absorbing any of the remaining fluid from the slide and place a dry cover slip over the flea. When the dry cover slip is in place acquire a small amount of 95-100% ethanol and apply a small drop of the alcohol to the *low* side of the cover slip. Once the alcohol is pulled under the cover slip, make sure the alcohol completely coats the flea. If the first drop is not adequate to completely coat the flea, keep applying drops one at a time until the alcohol surrounds the flea. The ethanol should harden the flea within seconds, but to make sure the flea is hardened keep it under the slip for 5 minutes and reapply alcohol as it evaporates. when the ethanol soak is complete carefully remove the cover slip from the slide, and to avoid damaging the hardened flea wash it off the slide with your 95-100% ethanol.



Figure 28. Fleas soaking in cellosolve

When you have completed spreading and hardening your fleas, you may move to the next step in the process, the cellosolve soak, or you can keep the flea(s) stored in a vial filled with 95-100% ethanol until you are ready to move on to the next step.

Step Four: Cellosolve Soak

The cellosolve soak helps remove water and other substances, such as the KOH crystals, from within the flea. For this soak, begin by prepping a 1.5 mL screw cap vial with a few drops of cellosolve from a 2mL disposable pipet. Cellosolve can be harsh on your tools so disposable or older tools would be best to use. After the vial is prepped, place the flea(s) into the

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vial and allow it to soak for one hour. During this soak, just as with the KOH soak, try and make sure that the flea stays as fully suspended in the solution as possible. Soaking fleas any longer than one hour sees minimal gains and can make the specimen brittle and difficult to work with in later steps. The same vial and solution can be used multiple times, to soak multiple specimens at the same time, throughout the day. Specimens that are still soaked/covered with cellosolve stay somewhat pliable, so avoid washing or soaking the flea in other solutions after the cellosolve soak is complete. This added pliability will aid with handling the specimen, and any adjustments that might need to be made to the flea once it is in the mounting medium.



Figure 29. Allowing 1st PVA drop to fall into the bottle

Mounting Your Flea

The mounting process can be broken down into two smaller steps: 1) Preparing the slide (applying mounting medium); 2) Mounting the flea.

Preparing the Slide

While your fleas are soaking in Cellosolve you can take the time to start gathering all the tools that you'll need for mounting the flea including: slides, round or square coverslips (preferably round) No. 1 or smaller (0 or 00) ranging from 12 to 17 mm, PVA mounting medium (Boiquip Products, Inc.), slide labels, wooden applicator sticks, cotton swabs, minute spatula, microscopy probe and/or barb, forceps, slide ringer, and a bottle of nail polish along with a small paint brush (used for sealing the slide). Once your tools are together, start cleaning slides for the mounting procedure by wiping them off with either a Kimwipe, or small microfiber towel to remove any dust and other particles that might have accumulated on the slides. After you have cleaned off all the slides that you are going to be using, start prepping slides with PVA (if the cellosolve soak is almost finished). First, take a wooden

applicator stick and dip it into the PVA bottle, making sure that around a fourth to a third of the stick is submerged into the PVA. Wait a few seconds before pulling the stick out of the medium, and once you have pulled the stick out of the medium, allow at least one drop to fall off the stick back into the bottle. I found that this first drop tends to fall off the applicator stick well before you get the stick lined up on the slide just where you want to drop the PVA, causing you to either miss the slide or place the drop off-center on the slide, wasting mounting medium and causing you to either clean or replace the slide. Once you feel that you have dropped an adequate amount of PVA on the slide (I found that four to five drops worked best for the slides and covers that I was using), place the slide under a microscope and check the PVA droplet for air bubbles, especially in the center of the droplet. If air bubbles are present, simply set the slide aside and start to prepare other slides with PVA. Allowing the slide to sit for a minute or two will allow any air bubbles to dissipate, leaving you with a clean and clear droplet of PVA. If you would like to get more practice working under the microscope, you can try to either pop the bubbles or move them to the side of the droplet, so they do not obstruct view of the flea once the cover slip has been placed on the slide (refer to Figure 30 on the right). Moving or popping the bubbles can be a tedious process, so take your time and try not to disturb the droplet too much.

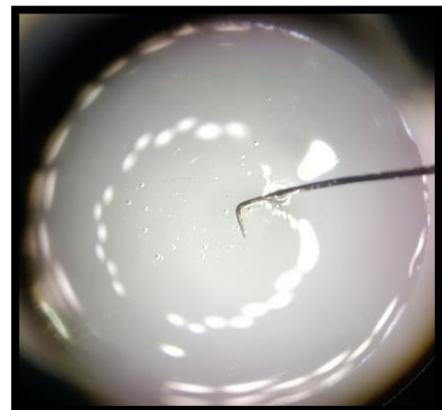


Figure 30. Removing bubbles from the PVA

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Mounting the Flea

Once you are done prepping the PVA droplet on the slide you are ready to place the cleared flea into the mounting medium. Start, by removing the flea from the cellosolve by grabbing the flea with a pair of forceps (Featherweight forceps; Bioquip Products, Inc.) in a comfortable manner that will allow you to place it into the medium in the mounting position. The mounting position can be achieved by placing the flea's head to your righthand side and the legs facing away from you towards the microscope's arm, placing the flea's "back" towards yourself (refer to Figure 31 to the right). Keeping this positioning fresh in your mind will help you avoid placing or mounting the flea on the slide improperly, a common mistake made when first starting out.

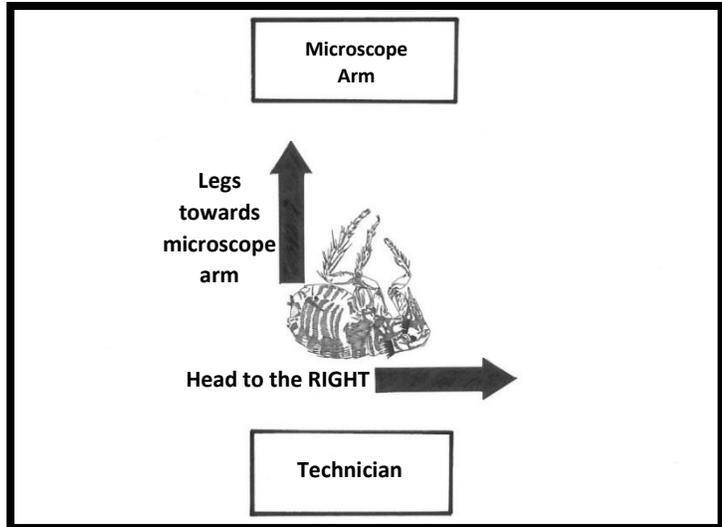


Figure 31. Diagram of the mounting position



Figure 32. Positioning the flea in PVA

Place, and submerge the flea into the droplet slowly to avoid disturbing the droplet's shape too much. Once submerged, you can tweak the position of the flea, making sure it is sitting flat against the slide and as parallel to the length of the slide as possible (this will give you the best viewing of the flea's anatomy through the microscope). Due to their importance in identification, remember to readjust the legs if they do happen to get moved out of place. Do not try to force the legs to move as they might break off at this point in the process. I found a bent microscopy probe works well for moving and manipulating the flea while it is in the PVA.

You may notice when the flea is submerged that bubbles will form in its body cavity, if this does occur you want to carefully try to push the bubbles out of the flea using the incision made earlier to evacuate the liquified internal contents (take caution during this to avoid evacuating the female's spermatheca or male's genitalia). When you are satisfied with the position of the flea you can place the cover slip over the droplet and flea. As with many of the steps, take your time when placing the cover slip and try to drop or place it down evenly to avoid pushing the specimen out of position or pushing and/or creating new air bubbles over the specimen. Once the cover slip is on, the medium on the slide needs time to settle and air dry.



Figure 33. Placing the cover slip on the slide



Figure 34. Slides settling and air drying in slide book

Air Drying and Settling the Slide

When you are done placing the cover slip on the slide, carefully move the slide to a dark, dry, and undisturbed area (old slide books work great) where the slide can settle and dry. Allow the slide to settle and air dry for around 48 to 120 hours (two to five days). During this period most of the air bubbles formed in the mounting medium from the mounting process should dissipate as the medium starts to harden over (same process as letting the PVA droplet settle and dissipate the air bubbles). Be cautious while handling slides after this settling period as the glue will still be soft.

Removing Excess Mounting Medium

You may notice that you are using too much mounting medium and it is gushing out from underneath the cover slips, a slide ringer can be used to assist in removing the excess medium. A slide ringer is a device that will spin the slide in a 360-degree range, this will allow for the removal of any excess medium, and aid in the application of any sealants later. A manual slide ringer (one that you physically spin) is more than sufficient to do the job. To start the process, secure the slide to the slide ringer, making sure to place the coverslip over the circle in the center of the slide ringer. To remove excess medium after 48 to 72 hours of settling and air drying, use a cotton swab to remove the excess. To do this simply place the cotton end of the swab against the surface where the coverslip and slide contact at a 45-degree angle (refer to top image of Figure 35 on the right), and slowly began to spin the slide ringer. To remove the excess medium after 96 to 120 hours of settling and air drying, or any time the medium is too stiff for a cotton swab to wipe clean, use an X-ACTO® knife with a #16 blade, or an old blade no longer used for incisions, and place the beveled edge (cutting edge) down against the slide and position the point of the blade as close to the surface were the coverslip and slide contact (refer to Figure 35 on the right). Once the blade is in position, start dragging the blade slowly around the edge of the coverslip. When you feel you have gotten a handle on the angle and distance at which to hold the blade, start slowly and steadily spinning the slide ringer. Make sure to take the time to wipe the blade clean as you work around the coverslip, as built up glue tends to fall off the blade and back onto the slide or cover slip. Take your time during this process to avoid damaging the slide and coverslip with the blade, or accidentally removing the coverslip while removing the excess medium.

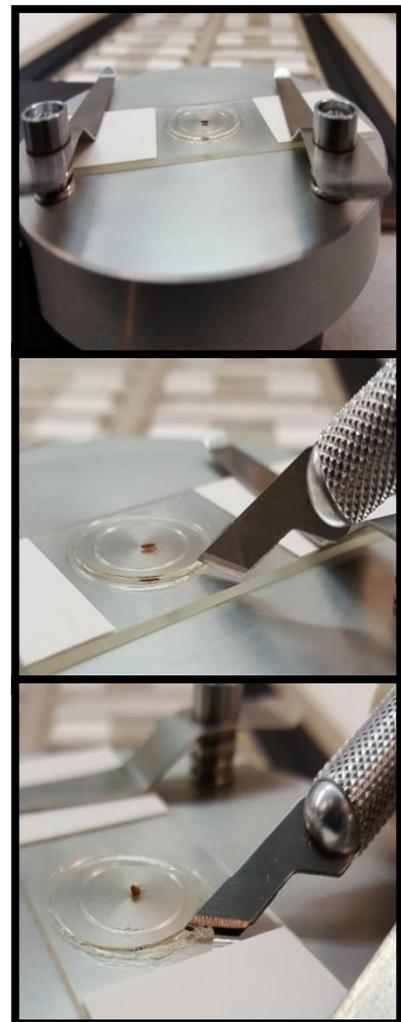


Figure 35. Removing excess medium from the slide with an X-ACTO® knife

How to Mount Your Flea

When you have finished removing any excess medium with the blade, you may notice that small pieces of excess glue are left behind on the slide or cover slip. You can easily remove this excess glue with a cotton swab. Leave the slide on the slide ringer, take a cotton swab, and simply follow the same path that you took with your blade (refer to top image of Figure 36 on the right). If you have any excess glue on the coverslip be careful while wiping it clean with your cotton swab, as the glue will still be soft, and the coverslip will be moveable (bottom image of Figure 36). You can remove any glue that builds up on the cotton swab by wiping it on to the top of your latex/nitrile gloves or wiping it on to a piece of paper. Once you feel that you have removed all the excess medium, you are ready to seal the slide.

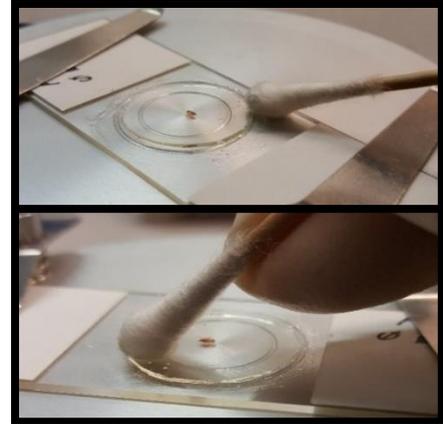


Figure 36. Cleaning off slide with cotton swab following the X-ACTO® knife

Sealing the slide

After removing any excess mounting medium keep the slide on the slide ringer and acquire some nail polish. Retrieve either the brush that comes attached to the cap of the nail polish or your small paint brush. The brush attached to the cap of the nail polish is best to use, because it requires less maintenance and care to keep in a usable condition. Start by dipping the brush into the nail polish, and lightly begin to apply polish by following the same path used while removing the excess medium from the slide. This is easily achieved by keeping the brush in contact with the surface where the slide and coverslip meet, and slowly spinning the slide ringer (refer to top image of Figure 37 on the left).

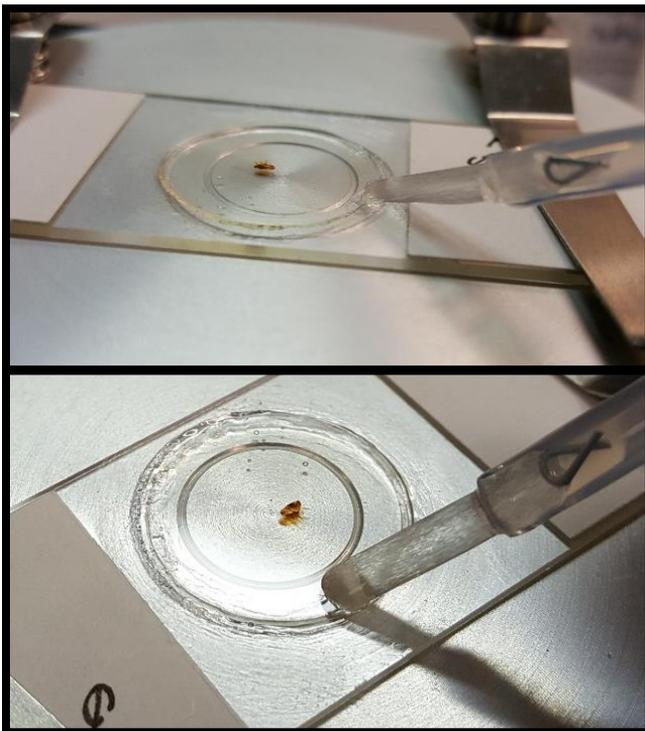


Figure 37. Sealing the slide with nail polish

After making a full pass around the coverslip, add a thin layer to the outer most edge on the top of the coverslip to complete the seal (refer to lower image of Figure 37 on the left). You may also choose to follow your application of nail polish with a cotton swab wipe to remove any excess nail polish that may have been applied.

The nail polish will act as a sealant to prevent any further moisture loss or gain, and stop the entry of oxygen, or foreign invaders (fungi, etc.) into the medium and specimen. Once you have finished applying the nail polish, place the slide into an incubator (drying oven) overnight for around 24 hours, within a temperature range of 37-43 °C. After this heat treatment the glue should hardened and any bubbles that were still in the medium should have dissipated. At this point the slide is ready for use under a microscope, or to be stored in a vertical microscope slide case/holder.

Labeling Your Slide

When your slide is done hardening off, you are ready for the final step, labeling the slide. There are many different conventions for completing this task, and the place you work for or even you yourself might have a specific format for labeling in use. The most common labeling convention for slide mounted ectoparasites will be discussed. This convention is simple and follows some of the same rules for a pinned arthropod. A minimum of two labels will be used when making a slide (refer to Figure 38 below).



Figure 38. Slide after being sealed, and harden off

The right label will contain the locality/collection data. Locality/collection data includes city, political subdivision (county, providence, or department), state or country (when possible use standard grid coordinates to aid in placing the physical location), date of collection: written out as "Day Month Year" where a three-letter month abbreviation is used (example: 15 SEP 2017), name of collector (always located at the bottom of the label) written out as "coll. J. Smith" where the collector's first name is abbreviated to the first initial, and "coll." is used to abbreviate "collected by".

The left label will contain the determination of the flea species. This label should only be filled out once the specimen has been examined under a compound microscope, by a person who is trained or knowledgeable in Siphonaptera (or the order you are working with). The determination label contains the name of the specimen (genus, species, and subspecies), the name or initials of the person who described the species, and the name of the person that determined/identified the specimen followed by the year it was identified, written as "det. J. Smith 2015"; the abbreviation "det." stands for "determined by". If needed or desired, an additional third label, for collection methods, can be added to the bottom of the slide directly behind or in line with the locality label.



Figure 39. Slide with complete labels



Figure 40. Fleas soaking in Cellosolve

Cellosolve Quick Clearing Method (used for identification purposes only!)

A cellosolve soak can be used as a quick or fast way to clear fleas for identification purposes. This method is best suited for situations when you are looking to identify a given flea rather than preserving it. The cellosolve will help lighten up the exoskeleton and some of the internal organs and fluids which helps make seta/hairs more visible as well as the female's spermatheca and male's genitalia. Cellosolve does appear to harden the insides of the flea as it removes water from the body cavity, sometimes making features difficult to see clearly, especially when compared to a fully cleared and mounted specimen. Due to this clarity issue I suggest using this method on fleas that you are not quite positive on the identification of, but have a good idea of what genus and/or species it might be. For this quick method you can simply skip the first two steps of the clearing process (KOH soak, and clearing liquified internal contents) and go straight to the cellosolve soak. You may still make a small incision/hole in-between the flea's first and second abdominal segments to allow the solution to enter the fleas body cavity. After making the incision (or

not), place a few drops of cellosolve into a 1.5mL screw cap vial, and place the flea into the solution. Allow the flea to soak for a minimum of 30 minutes, but you may allow the flea to soak overnight if timing or scheduling require you to do so. I have left specimens in cellosolve for periods of 30 minutes, 1 hour, 24 hours, and 48 hours (picture results on the following pages). There is no further clarity gained from leaving the specimen in the cellosolve soak past 30 minutes to one hour. If you do choose to leave specimens in cellosolve past 30 minutes to one hour, you will want to handle them with care for the remaining steps after the soak, as the specimen might become brittle the longer it is left in the solution. Once the soak is complete, remove the flea from the cellosolve and place it on a slide. The flea is now ready to be placed under a compound microscope and identified. For this technique you do not want to place or secure a cover slip on to the slide. Due to the flea's internal matter not being removed, which flattens the fleas body, the cover slip will not sit properly on the slide and large or numerous air pockets will form in the mounting medium making identification and later use of the slide difficult. Once you are done identifying the specimen you may dispose of it properly.

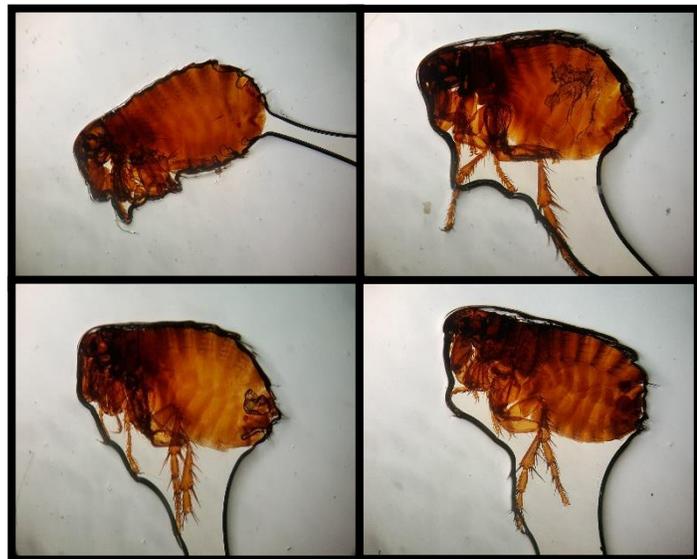


Figure 41. Specimens that were mounted after the quick clear method; Notice the air pockets that formed around the flea's body

The next four pages will cover the photographic results from soaking fleas for 30 minutes, one hour, 24 hours, and 48 hours.

30 Minute Cellosolve Soak Results



Figure 42. Overall picture of female flea after a 30 min. cellosolve soak



Figure 43. View of female's spermatheca after a 30 min. cellosolve soak

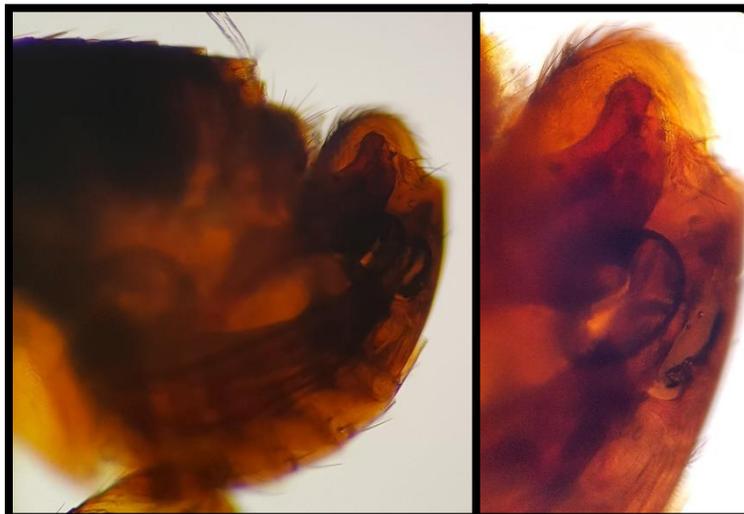


Figure 44. View of male's genitalia and claspers after a 30 min. cellosolve soak

One-hour Cellosolve Soak Results



Figure 45. Overall picture of female flea after a one hour cellosolve soak



Figure 46. View of female's spermatheca after a one hour cellosolve soak

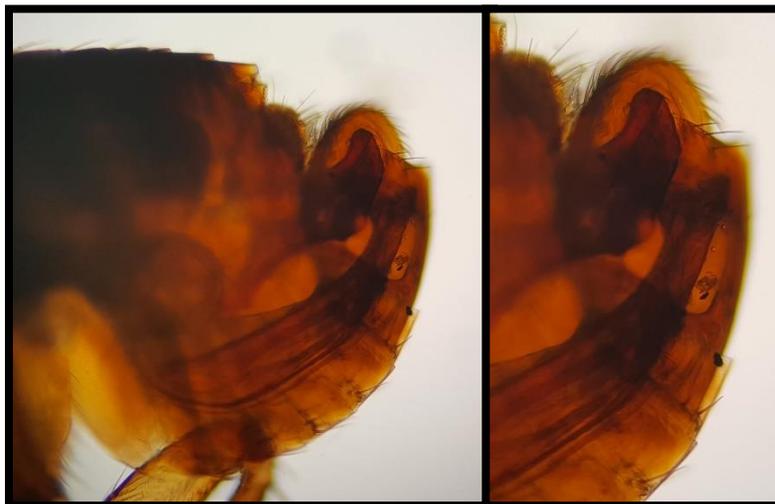


Figure 47. View of male's genitalia and claspers after a one hour cellosolve soak

24 Hour Cellosolve Soak Results



Figure 48. Overall picture of female flea after a 24 hour cellosolve soak

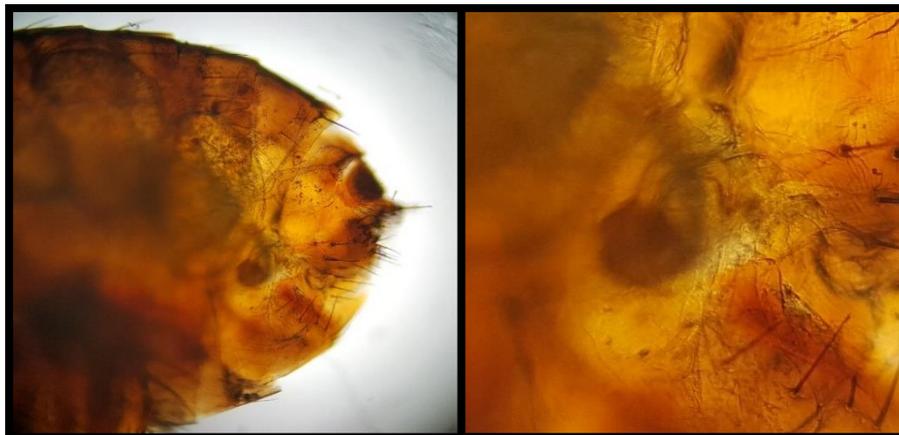


Figure 49. View of female's spermatheca after a 24 hour cellosolve soak

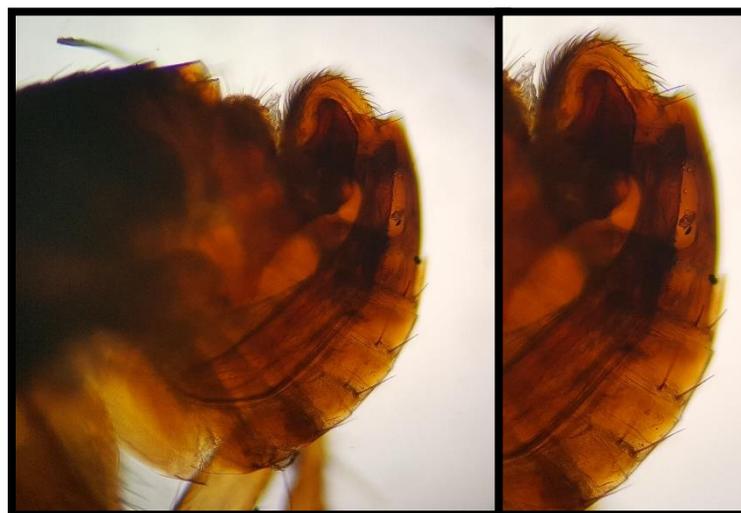


Figure 50. View of male's genitalia and claspers after a 24 hour cellosolve soak

48 Hour Cellosolve Soak Results



Figure 51. Overall picture of female flea after a 48 hour cellosolve soak



Figure 52. View of female's spermatheca after a 48 hour cellosolve soak

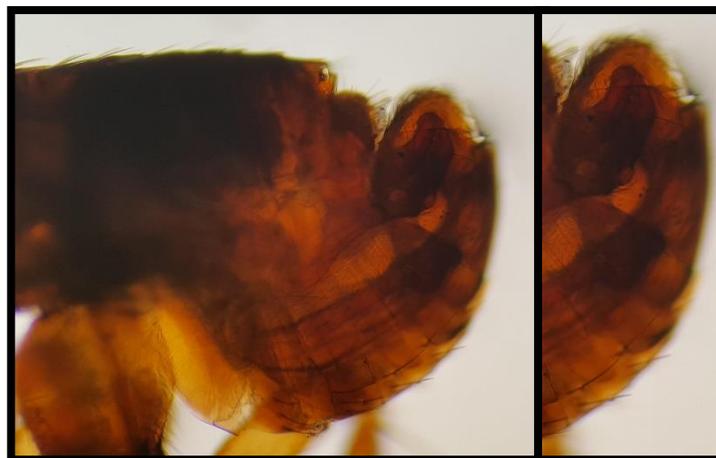


Figure 53. View of male's genitalia and claspers after a 48 hour cellosolve soak

Common Mistakes

I created this section to highlight just a few of the mistakes that might occur while you are working with your fleas. The biggest key or route to success when mounting Siphonaptera or anything for that matter is to practice. A lot of the techniques that you will use when mounting or clearing fleas will be performed under the microscope. Working in such a manner can feel alien at first, especially when handling or trying to make incisions into a very small arthropod that is laterally flattened like a flounder or halibut. So, do not be afraid of making mistakes, or become discouraged if your first attempts don't live up to your expectations. Remember the old saying "practice makes perfect".



Figure 54. Ruptured flea after applying too much pressure while evacuating internal contents; try to be careful when handling or manipulating the fleas, their tough for their size, but still easily broken.



Figure 55. Specimen is mounted backwards. Always remember the mounting position when placing your flea in the mounting medium.



Figure 56. Spermatheca was lost while evacuating liquified internal contents. always keep an eye on the key organs that you are trying to keep.



Figure 57. Using too little mounting medium. Remember excess medium can be removed, but too little can ruin the slide.



Figure 58. Expanded air bubbles ruining the view of the specimen and key morphological features. Remember to allow your slides to air dry and settle to reduce or eliminate air bubbles from the medium.



Figure 59. Legs either damaged and/or not spread out far enough. Remember to always take the time to spread out the legs, this is one step that many technicians skip or over look.



Figure 60. Damaged abdomens after being too aggressive while applying pressure to evacuate liquified internal contents. Remember to be careful and avoid damaging the exoskeleton and seta.

References

- Austin Community College. (n.d.). *Slide Mounting Techniques for very to microscopic animals*. Retrieved from Austincc.edu:
www.austincc.edu/sziser/Biol%201413/animal%20collections/Making%20Slides.pdf
- Brigham Young University The college of Life Sciences. (n.d.). *Flea Mounting Procedures*. Retrieved from BYU The College of Life Sciences Fleas of the World:
<https://fleasoftheworld.byu.edu/Systematics/MountingTechniques.aspx>
- Hubbard, C. A. (1947). *FLEAS OF Western North America*. New York: Hafner Publishing Company, INC.
- Lewis, R. E., Lewis, J. H., & Maser, C. (1988). *The Fleas of the Pacific Northwest*. Corvallis: Oregon State University Press.
- Snodgrass, R. E. (1946). *THE SKELETAL ANATOMY OF FLEAS (SIPHONAPTERA)*. Washington : The Smithsonian Institution.

Please visit www.ocvector.org/fk for more references and to download the *Pictorial Key to Some Common Fleas of Southern California*, a key developed by the author covering common fleas found in Southern California.

