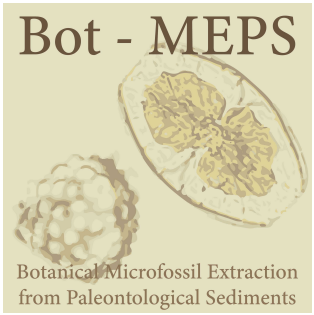

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Botanical Microfossil Extraction from Paleontological Sediments - 'Bot-MEPS' Protocol

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ABSTRACT

Palaeobotanical microfossil analyses are often used to reconstruct palaeoecological histories and past environmental change. To do so, many researchers use lake sediment, for which chronological ordering of stratigraphical layers is possible. Lakes accumulate microfossils including pollen, starch grains, diatoms and phytoliths. Diatom and phytolith assemblages allow inference of vegetation, pH, salinity, water chemistry, and environmental temperature, among others. As an example, we employ this method to study the correlation between palaeobotanical microfossils and desert lake history from Nevada. The preliminary results hint at a complex climatic and environmental variance. Here we propose a protocol split into six sections to isolate diatoms and phytoliths. By following the protocol, one can prepare samples for microfossil identification and interpretation. Researchers can develop an ecological history by analyzing microfossils from different locations and times. A case study is included from a Miocene diatomite deposit paleolake from Northern Nevada. The method resulted in the extraction and isolation of Miocene microfossils dated to approximately 10.3 Mya including phytoliths and diatoms. Another application of this method includes the development of environmental interpretations and hypotheses regarding the future of modern vegetation.

PROTOCOL CITATION

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GUIDELINES

Cross-contamination should be avoided at each step. Disposable glassware should be used when available, and equipment should be cleaned with acetone between each sample.

Make sure to balance samples in the centrifuge: if there aren't enough samples to fill the centrifuge, use blanks or spread the samples out evenly. Plastic tubes should be centrifuged up to a maximum of 3000 rpm, and glass tubes should be centrifuged up to a maximum of 1700 rpm. Remove centrifuge buckets and inserts daily to wash with a brush and warm soapy water. Wipe down the interior of the centrifuge with a moist paper towel.

MATERIALS TEXT

Reagents/Chemicals	Glassware	Equipment	Others
Zinc Bromide (ZnBr2)	Pasteur pipets and pipet bulbs	Balance (up to 0.0)	Chisels (5mm blade)
Hydrogen Peroxide (H2O2)	Falcon tubes (15ml)	Centrifuge	Scoopulas
Distilled Water (dH2O)	Lab coat, nitrile gloves, safety goggles	Hotplate and stirrers	Aluminum foil
Hydrochloric Acid (HCl)	100mL, 1000mL beakers	Specific Gravity Hydrometer	Plastic bags
Acetone	2.0 mL centrifuge tubes	Mortar and pestle	Permanent markers
Immersion Oil (non-drying, Type B, Cargille)	Glass stir rods	Thermometer	Clear nail polish
	1L glass solution bottles		
	25mL, 100mL graduated cylinders		
	Clear glass slides and coverslips		
	Powder glass funnel		

Example workspace; left to right: Pasteur pipet box, hot/stir plate, mortar and pestle, sharpie, prepared slides, 100mL beaker (inside 600mL beaker), 600mL beaker, glass stir rods, scoopula, pipet bulbs, 15 mL Falcon tubes and rack, centrifuge tubes and rack, microscope slides and cover slips, parafilm, centrifuge

SAFETY WARNINGS

Follow standard laboratory procedures, including wearing appropriate personal protective equipment (lab coat, safety goggles, gloves) and familiarizing yourself with safety and first aid equipment. Use chemicals in the fumehood with the intake fan on.

Familiarize yourself with SDS (Safety Data Sheet) and follow the storage requirements of each chemical.

BEFORE STARTING

Create an organized sample inventory and a database of all samples. Include relevant information to identify samples

(country, site, feature, level, etc.). Assign unique label codes to each sample (e.g. SITE CODE/YEAR/ID NUMBER). Digitally prepare and print a suitable form for recording observations during the laboratory preparation of samples.

Sediment Preparation

1 Determine how thick the sampling region should be in relation to the thickness of the geological layers. If the source specimen is densely layered, then a thinner sampling region will result in a higher accuracy of placing microfossils within correct historical timelines.

E.g.: varve sediments (such as the examples included here) will include years of deposition in just a few centimeters. In contrast, metamorphic samples might include single-event deposition throughout meters of stratigraphy.

0.2 Using a chisel, outline a section to be used for the study. This should be done in a dust collector or a fumehood with a protective surface (e.g. aluminum foil), laid down to protect the researchers and sample.

0.3 Prepare sediment block by cleaning (see further instructions in *steps 1.1-1.3*) the exposed sections. Stratigraphical layers should be visible for best sampling strategy as per next steps.

Note

Here, our samples have layers/laminations. These layers can be separated to achieve finer stratigraphical resolution.

0.4 Use a flat tool to separate the sample from the rock. A flat chisel can separate portions until the whole sub-sample is isolated.

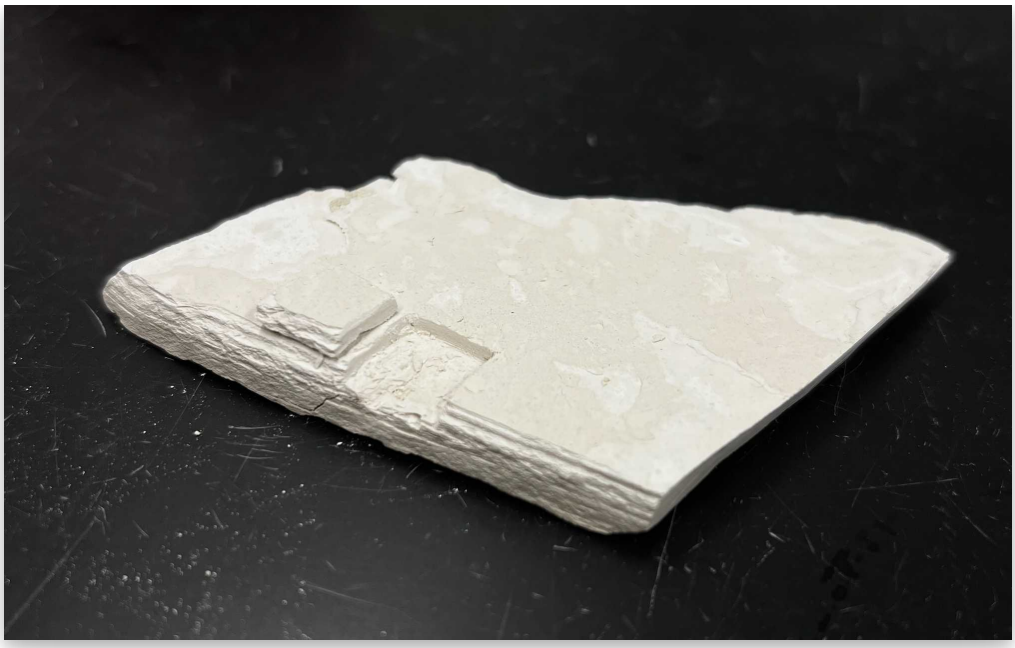

Image 1: example sample of first chiseled sub-sample (composed of several layers) removed. Removed layer is approximately 0.5cm in depth.

0.5 Mass the sample.

Note

Make sure to maintain equal mass across sub-samples throughout separation. Depending on the material sampled the mass will vary (e.g. diatomite achieved good microfossil density at 0.5g). For optimal results maintain an accuracy of ±0.05g.

0.6 Repeat *Steps 1.2-1.4* for each sample while cleaning the tools and using new aluminum foil to prevent cross-contamination.

0.7 Transfer each sample into a new plastic Ziploc bag, and then put that bag, sealing, into a 2nd new plastic Ziploc bag.

1 Place the bag into a mortar and use a pestle to crush the samples (as finely as possible).

Note

This works best if some air is kept in the inside bag to allow the sample to move during grinding.

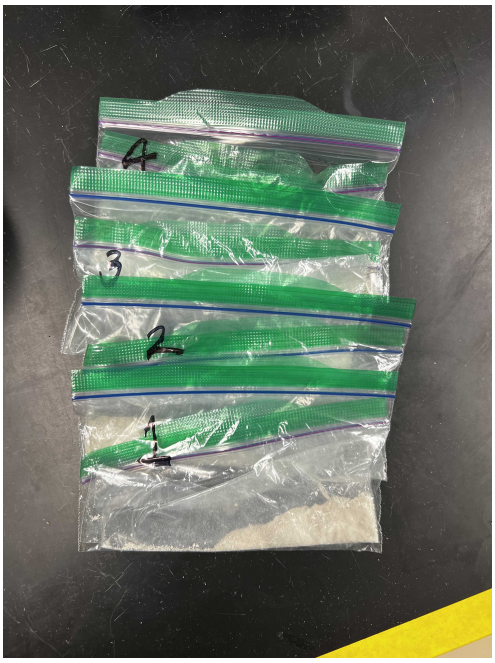

Image 2: sections separated into double bags with sample codes.

1.1 Pour the ground powder through a funnel into sealable falcon test tubes for storage.

1.2 Transfer sample to a centrifuge tube, filling to 1/3 of the height to allow volume for chemicals.

1.3

Safety information

In this protocol approximately 0.5 mL of sediment were processed per sample in 2.0 mL centrifuge tubes. If more sediment is desired, larger centrifuge tubes will be necessary. This is applicable for sediments with a lower concentration of microfossils.

Carbonate Removal (HCl)

2 If the sample contains carbonates, they will have to be removed for quality viewing of the microfossils.

To remove carbonates, start by pouring 50 mL of 30% Hydrochloric Acid **Contributed by users** into a small beaker.

Note

Complete these steps in a covered fumehood, wearing proper personal protective equipment: gloves, lab coat, safety goggles, etc.

3 Carefully pipet a small amount of Hydrochloric Acid **Contributed by users** into each centrifuge tube, and stir with a clean pipet to further expose carbonates to acid.

Note

If carbonates are present, there can be a vigorous exothermic reaction. Use an ice bath if necessary to control the reaction. Stir gently to avoid a violent reaction.

Safety information

Keep an ice bath in fumehood to cool samples immediately in case of a violent reaction (by placing samples in ice water).

4 Pipet more Hydrochloric Acid **Contributed by users** to fill the tube, leaving enough room to close the top.

Note

Again, add the acid slowly to avoid bubbling over.

4.1 Stir again with a clean pipet tip.

5 Wait until the bubbling stops and the reaction ceases.

5.1 If a reaction is present but slow (low rate of bubbling), heat the samples on a hotplate to a maximum temperature of 70 °C until the bubbling ceases.

5.2 Allow the samples to cool and stir.

6 Centrifuge at 1700 rpm for 00:10:00 to pellet the sediment. 10m

7 Carefully pipet the supernatant into a 1000 mL waste beaker half-filled with water.

8 Fill the centrifuge tube with dH2O **Fisher Scientific Catalog #BP2470-1** , and repeat *steps 7* and *8* to rinse the sample. Be sure to remix the sample with HCl to unpellet before centrifuging again.

8.1 Repeat until the water is clear, approximately two times.

9 Dispose of waste Hydrochloric Acid **Contributed by users** by flushing down the sink while cold water pours from the tap. Leave the water running for 00:05:00 . 5m

10 Dry the samples by repeating *step 9* with Acetone **Contributed by users** . Pipet the supernatant into a 100 mL waste beaker.

10.1 After decanting the waste Acetone **Contributed by users** , place samples and the 100 mL beaker of decanted acetone in the fumehood to evaporate for 1-2 days. After 3 days, wash out now empty beaker.

Organics Removal (H_2O_2)

11 If the sample contains organics, they will have to be removed for quality viewing of the microfossils.

To remove organic material, start by filling the centrifuge tubes halfway with Hydrogen peroxide 30% **Merck Millipore Catalog #822287.1000** (H_2O_2).

Note

Complete these steps in a covered fumehood, wearing proper personal protective equipment: gloves, lab coat, safety goggles, etc.

As organics are destroyed, the solution's color will change from black to red/brown to orange to a clear yellow/green and emit hazardous fumes.

12 Put centrifuge tubes on a hot plate and warm to 40 °C . If the reaction is vigorous, remove from heat and place in an ice bucket until reaction has ceased.

13 After approximately 00:30:00 to 01:00:00 , increase the temperature to 80 °C . If reaction is none or minimal then increase temperature to 100 °C . Stir carefully with a pipet tip every 5 minutes. Once no reaction is visible, move to *Step 15*. 1h 30m

14 Completely fill the centrifuge tubes with Hydrogen peroxide 30% **Merck Millipore Catalog #822287.1000** .

Note

As organics are destroyed, the solution's color will change from black to red/brown to orange to a clear yellow/green.

14.1 If the reaction has not ceased within 8 hours, leave on hot plate overnight at 80 °C . Top off with hydrogen peroxide in the morning if evaporated.

15 When the samples are a clear yellow or yellow/green, remove from heat and allow to cool.

16 Centrifuge at 1700 rpm, 00:10:00 to pellet. 10m

17 Remove supernatant with pipet, rinse with water, and dry with acetone by repeating *step 11.*

$ZnBr_2$ Solution Preparation

18 After carbonate and organic steps are completed or skipped depending sample content, microfossils have to be separated. This can be done using a specific gravity solution such as $ZnBr_2$ and centrifugation.

To prepare the $ZnBr_2$ solution, start with a magnetic stirrer and slowly add 500 g of solid Zinc Bromide **Fisher Scientific Catalog #B22510.36** ($ZnBr_2$) to 175 mL of dH2O **Fisher Scientific Catalog #BP2470-1** .

Note

This reaction is exothermic and will produce heat.

18.1 Based on literature review and trial and error, we generated a $ZnBr_2$ solution with density of 2.3 g/mL using:

$$Density\ of\ ZnBr_2\ Solution = \frac{Mass_{ZnBr_2} + (Density_{H_2O} \times Volume_{H_2O})}{\frac{Mass_{ZnBr_2}}{Density_{ZnBr_2}} + Volume_{H_2O}}$$

If needing to prepare more or less solution, use the following equation to determine the volume of water and mass of $ZnBr_2$ necessary for a density of 2.3 g/mL. The amount of solution is dependent on sample size, quantity, and sediment type.

18.2 Below is a practical example:

Creating a 2.3 g/mL solution of ZnBr2 using 250 g of ZnBr2 with a 4.22 g/mL density from the manufacturer:

$$2.3\frac{g}{mL} = \frac{250\ g\ ZnBr_2 + \left(0.99802\frac{g}{mL\ H_2O} \times V_{H_2O}\right)}{\frac{250 g_{ZnBr_2}}{4.22 g/mL_{ZnBr_2}} + V_{H_2O}}$$

Multiplying both sides by denominator gives:

$$136.26\ g\ ZnBr_2 + 2.3\ \frac{g}{mL}\ V_{H_2O} = 250\ g\ ZnBr_2 + 0.99802\ \frac{g}{mL}\ V_{H_2O}$$

Rearranging to have like terms on each side:

$$1.30198\ \frac{g}{mL}\ V_{H_2O} = 113.74\ g\ ZnBr_2$$

Dividing both sides by 1.30, leaving:

$$V_{H_2O} = 87.36mL$$

∴ To make a 2.3 g/mL solution of $ZnBr_2$, one should mix 250 g of solid $ZnBr_2$ with 87.36 mL of H_2O.

Safety information

Make sure to check the correct density was achieved by using a hydrometer with the correct specific gravity range.

3h

19 Stir the $ZnBr_2$ solution and cool down for 01:00:00 to 02:00:00 hours, until room temperature or 25 °C , per a thermometer.

20 Check density using a hydrometer.

21 The specific gravity density should be 2.30 ± 0.057 g/mL. If higher, add water slowly to the beaker until density decreases to the accepted range. If lower, add $_2ZnBr_2$ to bring density up to the accepted range.

22 Pour the solution into a glass bottle, cap, and label it with the date and density.

Heavy Liquid Flotation Separation (with ZnBr2)

23 To separate paleobotanical microfossils from inorganic sediment, first prepare a new set of labeled centrifuge tubes and a set of pipets with bulbs.

24 Add dried sample to the new centrifuge tubes.

25 Fill the centrifuge tubes with the $ZnBr_2$ solution using a new pipet, leaving room so the tube can be closed.

25.1 Gently mix with a clean pipet.

26 Centrifuge at 1700 rpm, 00:05:00 5m

Note

Heavy materials will sink, whereas microfossils will float forming a ring at the water-air interface.

27 For each sample, use a new pipet to draw off the microfossil surface layer. Pipet into the new labeled test tube. If no material is visible, draw off 0.5-1 mL of liquid anyway from as close to the surface as possible. Focus on the ring close to the tube wall.

Image 3: floating microfossils in a ring at top of the solution.

10m

28 Fill a clear wash bottle with hot non-distilled water (any source including sink will suffice). Add water to dilute the microfossil solution. Stir well with a pipet to mix water and microfossil-$ZnBr_2$ solution. Centrifuge at 1700 rpm, 00:10:00 and decant supernatant with a new pipet into a 'Waste $ZnBr_2$' container. Do not pour down the sink.

29 If no evidence of floated material is present on the surface of the solution, it could be an indication that only a small amount of paleobotanical microfossils are present. In such case, top off the original sample tubes with $ZnBr_2$ and do a second float separation repeating *steps 24-29*.

30 Repeat *step 29* two more times.

31 Acetone wash the paleobotanical microfossils using the same procedure from *step 11.*

Mounting Slides

32 Label a glass side and carefully add a drop of
Immersion Oil (non-drying TYPE B) **Cargille Catalog #CODE 1248** onto a glass slide.

32.1

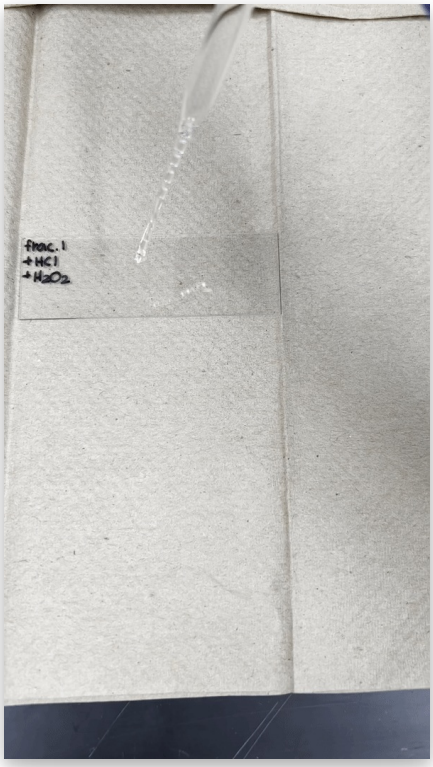

Image 4: example video of slide preparation (*steps 35-38*)

33 Using a clean spatula, add about a quarter of a pinky fingernail of powder from *step 32* to the immersion oil on the slide.

34 Mix the oil and sample with the tip of a new glass pipet.

35 Slowly place the coverslip on top of the oil/microfossil mixture.

Note

This is best done by touching the edge of the coverslip to the slide, then by letting it press on itself. Finally, gently push the coverslip to create a flat layer of microfossils.

36 If the intention is to keep the slide for future use rather than observing right away, seal the coverslip onto the slide with clear nail polish.

Note

This is best done by slowly placing a bead of nail polish along all the edges, letting the nail polish solution seep between the two glass layers by capillary action.

36.1 Air dry for at least 24 hours before handling.

36.2 Repeat *step 37 and 37.1* for better sealing of material, if desired.

Microscopy (Brightfield)

37 Use a compound microscope, binocular or trinocular, for brightfield viewing (light placed below sample, viewing from above).

38 A polarizing lens will help discern shapes and features in phytoliths and diatoms.

39 A magnification of 40x is good for the initial observation. For individual identification of microfossils and microscope photography, a magnification of 100x will be required.

40

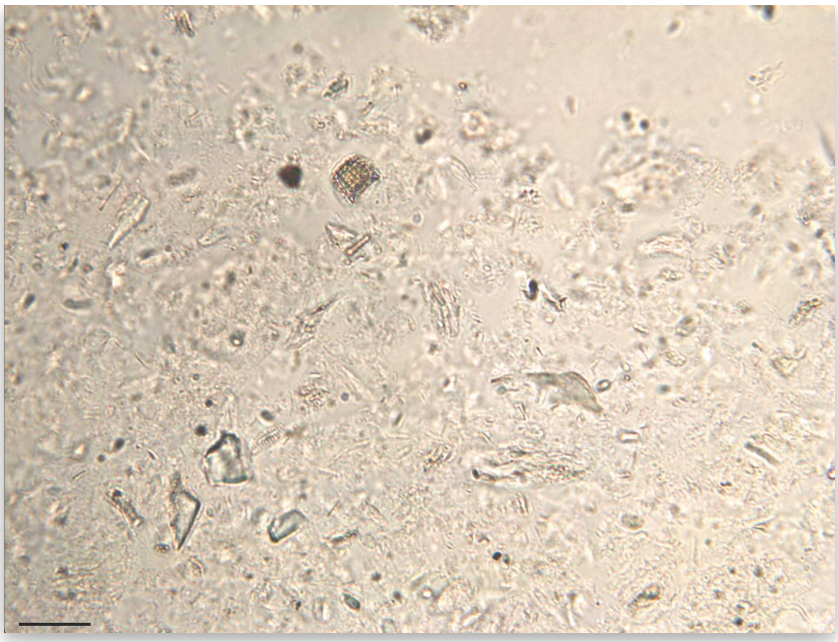

Image 5: Microscope field of view of sample, magnified at 40x. Notice the very dense concentration of palaeobotanical microfossils. Scale bar (in black) is approximately 20 microns.

41

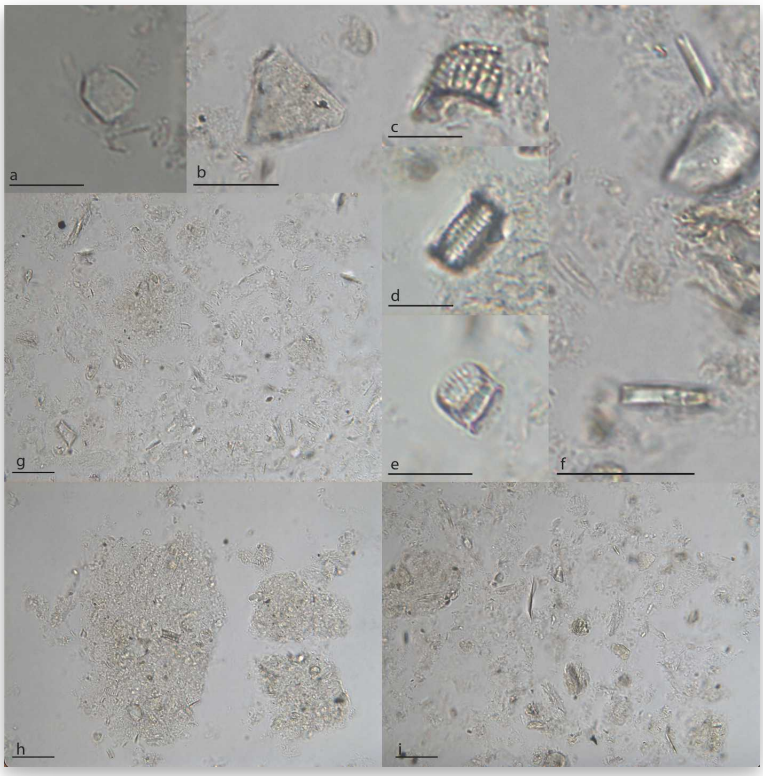

Image 6: Examples of microscope photographs showing: phytoliths (a, b; 100x magnification), diatoms (c, d, e; 100x magnification), sponge spicules (f; 100x magnification), and clusters of palaeobotanical microfossils (g, h, I) at 40x magnification. All scales (in black) are approximately 20 microns.