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Best practices for tissue collection of bats for -omics analyses and primary cell culture

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Dear Dr. Lyndsay Troyer, Staff Editor,

It is my pleasure to resubmit our manuscript "Best practices for tissue collection of bats for -*omics* analyses and primary cell culture" for further publication consideration in the *Journal of Visualized Experiments*.

We have taken into consideration the suggestions of the reviewers and have addressed their comments and concerns to the best of our abilities.

We would like to remind the journal, because there are two methods protocols within this manuscript, the filming of the two protocols will take place in two locations. The DNA/RNA dissection protocol will take place at Stony Brook University in the United States while the cell culture protocol will take place Max Planck Institute for Psycholinguistics in Nijmegen, The Netherlands. We have brought this detail to the attention of *JoVE* and the journal expressed willingness to accommodate this scenario. We acknowledge that this may entail a slightly higher cost and have discussed splitting the cost among the two last authors of the manuscript.

None of the material presented in this paper has been previously published or is submitted for consideration elsewhere. Please address all correspondence and concerns to Dr. Laurel Yohe with the contact information listed above.

Thank you for your consideration. Please do not hesitate to contact us if you need any further information.

Warm regards,

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TITLE:

Tissue Collection of Bats for -omics Analyses and Primary Cell Culture

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KEYWORDS:

bats, genomics, transcriptomics, tissue sampling, tissue preservation, dissection, cell culture

SUMMARY:

This is a protocol for the optimal tissue preparation for genomic, transcriptomic, and proteomic analyses of bats caught in the wild. It includes protocols for bat capture and dissection, tissue preservation, and cell culturing of bat tissue.

ABSTRACT:

As high-throughput sequencing technologies advance, standardized methods for high quality tissue acquisition and preservation allow for the extension of these methods to non-model organisms. A series of protocols to optimize tissue collection from bats has been developed for a series of high-throughput sequencing approaches. Outlined here are protocols for the capture of bats, desired demographics to be collected for each bat, and optimized methods to minimize stress on a bat during tissue collection. Specifically outlined are methods for collecting and treating tissue to obtain (i) DNA for high molecular weight genomic analyses, (ii) RNA for tissue-specific transcriptomes, and (iii) proteins for proteomic-level analyses. Lastly, also outlined is a method to avoid lethal sampling by creating viable primary cell cultures from wing clips. A central motivation of these methods is to maximize the amount of potential molecular and morphological data for each bat and suggest optimal ways to preserve tissues so they retain their value as new methods develop in the future. This standardization has become particularly important as initiatives to sequence chromosome-level, error-free genomes of species across the world have emerged, in which multiple scientific parties are spearheading the sequencing of different taxonomic groups. The protocols outlined herein define the ideal tissue collection and tissue preservation methods for Bat1K, the consortium that is sequencing the genomes of every species of bat.

INTRODUCTION:

High-throughput sequencing (HTS) methods have rapidly advanced in efficiency and decreased in cost, and it is now possible to scale these approaches to hundreds or thousands of samples. Insights obtained by the application of these technologies have enormous impacts across multiple scientific disciplines, from biomedicine to evolution and ecology[1–3]. Yet, many HTS applications rely critically on high quality nucleic acids from a living source. This limitation is likely to become increasingly problematic with the development of third generation sequencing based on long-read molecules[4]. For these reasons, there is a need to focus efforts on establishing best practices for collecting fresh tissue samples from wild organisms outside of the laboratory, which maximizes the utility of material and reduces the number of individuals that need to be collected.

Bat1K is an international consortium of scientists with an ongoing initiative to sequence the genome of every species of bat to chromosome-level assembly[5]. Bats represent 20% of mammalian diversity and have exceptional adaptations that have implications for understanding aging, disease ecology, sensory biology, and metabolism[5,6]. Many bats are also threatened or endangered due to human exploitation[7] or are rapidly declining due to pathogens[8,9], and genome-level sequencing is of great importance for conservation of these species. Although Bat1K currently aims to sequence the genomes of all bat species, the standardization of collection of tissue samples for high-quality genomic sequencing remains a key challenge across the community of organismal biologists. In addition to genomic data, functional understanding of the diversity of bat adaptations requires tissue-specific transcriptome and protein analyses, often requiring separate collection protocols. Moreover, as

with all taxonomic groups, while optimal tissue collection and preservation is essential for obtaining the highest quality data for -omics analyses, communicating best practices is often difficult because of rapidly changing technologies and multiple research teams working independently.

The need to adopt best practices for bat -omics research is especially urgent, given that many bat species are rare or threatened. Unlike other small mammals such as rodents and shrews, bats are long-lived, attributable to exceptional DNA repair mechanisms[10] and slow reproduction[11], with most species giving birth to just one or (in a few cases) two young per year. For these reasons, bat populations can be slow to recover from disturbance, and collecting many individuals from the wild is neither advisable nor feasible. In other words, protocols must be optimized to obtain the maximum amount of data for a single specimen, thereby reducing the need to unnecessarily replicate sampling efforts.

Here, this protocol focuses specifically on standardized methods for the collection and sampling of bat tissue for genomic and transcriptomic sequencing and protein analyses. Its top priority is to ensure that bat tissues are collected ethically and responsibly, ranging from the permitting process for the collection, exportation of tissues to the minimization of stress to the animal, and long-term storage conditions. Elaborate dissections have been developed with the aim of future-proofing the usefulness of different materials collected. This manuscript provides a step-by-step guide to collect bats in a humane way that is intended to minimize impact on populations and maximize scientific value. While the focus of this protocol is specifically for use in bats, many of the steps are relevant to other vertebrate taxa, especially mammals.

Tissue collection overview

The procedure for tissue collection, including the temperature for storage and the choice of preserving agent, will be determined by the nature of any downstream analyses planned. However, it is strongly recommended that, when possible, tissue is collected under a range of methods to maximize its future utility even if no specific analysis is planned. In general, tissue is collected and preserved for subsequent analyses of either nucleic acids (DNA and RNA), or protein. For each of these applications, tissue can be optimally preserved by directly flash freezing in liquid nitrogen (LN2). However, immediate immersion in LN2 is not always possible in the field. As technology advances, resources such as specialized vials to store DNA and RNA at ambient temperatures are becoming more readily available. While we have not validated all such materials in this protocol, we encourage other researchers to comparatively analyze the performance of new materials relative to what we present here. We do provide methods to ideally preserve tissue for different applications in situations where LN2 cannot be accessed, e.g., when LN2 transport is not possible due to site access via small plane in the Amazon. In addition, we provide a method for collecting tissue from which live cells can be grown and propagated. Below we outline key considerations for collecting material for each of these respective purposes and an overview of collection methods is given in **Table 1**.

Tissue for DNA

For all harvested tissue collected, the storage media will determine if it can be used for either standard or high molecular weight (HMW) DNA extraction. HMW is required for long-read sequencing and currently required to generate chromosome level genome assemblies, or a "platinum standard" genome. Low molecular weight (LMW) DNA can be extracted from flash frozen, AllProtect (henceforth referred to as "tissue stabilizing solution"), or even RNAlater (henceforth "RNA stabilizing solution")-preserved samples (although flash frozen samples remain optimal). DNA isolated by standard laboratory methods (e.g., silica gel membrane spin columns, phenol-chloroform), may still yield DNA fragments of up to ~20 kilobases (kb). Therefore, provided there is sufficient yield, this form of isolated DNA may be used for single insert size library preparation, in which the insert size is often ~500 base-pairs (bp), and short sequence reads of ~100 bp are generated[12]. This DNA is particularly useful for "resequencing" projects or studies in which full-length chromosomal data is not required. HMW DNA (10–150 kb) is more challenging and can only be reliably obtained using tissue that has been rapidly flash frozen in LN2 following harvest and maintained at a maximum of -80 °C until extraction.

Low molecular weight or fragmented DNA is often sufficient for targeted approaches, including gene amplification via PCR and short-read sequencing[13]. PCR-based investigations using LMW DNA that target only one or a few genes have been highly informative in understanding adaptation and the molecular evolution of bat sensory biology[6,14], physiology[15], phylogenetics[5,16], and conservation[17,18]. Successful targeted sequence recapture of low molecular weight and fragmented DNA has also been demonstrated for numerous vertebrate groups, including bats[19]. These methods are often cost-effective and minimally invasive to the bat, as fecal samples and non-lethal tissue sampling via buccal swabs or wing biopsy punches are also common ways to obtain DNA for low molecular weight analyses[20,21].

However, the quality depends heavily on the type of media in which the sample is stored[22]. After systematic and quantitative comparisons of buccal swabs and biopsy punches, wing biopsy punches have been shown to yield consistently higher levels of DNA and were less stressful to the bat during collection[22]. These comparisons also showed that the best results were obtained when the wing punch was preserved in indicator silica (i.e., a type of desiccant made of silica gel beads that changes color when moisture is observed) rather than in other popular storage media such as ethanol or DMSO[22]; although, other storage media including tissue stabilization solution were not examined. Wing punches can also be used to grow fibroblast cells in culture, such as in Kacprzyk et al.[23] and as described below (see section 6). For these methods, the wing or uropatagium should be extended gently, and a clean biopsy punch, typically 3 mm in diameter, should be used to obtain the sample. This approach appears to cause no lasting damage, with scars healing over within weeks in most cases[24].

HMW DNA (10–150 kb) is more challenging and is currently only reliably obtained using tissue that has been rapidly flash frozen in LN2 following harvest and maintained at a maximum of -80 °C until extraction. HMW DNA (10–150 kb) is crucial for long-read DNA sequencing and therefore for *de novo* genome assembly. Indeed, while most commercial kits can be used to isolate some standard HMW DNA, the resulting molecule sizes often do not meet the requirements of third generation sequencing technologies [e.g., those launched by companies

such as Pacific Biosciences (PacBio), Oxford Nanopore Technologies, and 10X Genomics, or through assembly methods offered by Bionano Genomics or Dovetail Genomics]. As such, there is a new demand for "ultra HMW" DNA (>150 kb). When obtaining ultra HMW DNA from bats, fresh samples of liver, brain, or muscle are all suitable, but these must be immediately flash frozen in LN2 without any storage buffer or cryoprotectant. A full description of these steps is beyond the scope of this paper but are available elsewhere[25].

Tissue for RNA

RNA is a single-stranded molecule that is less stable than DNA. Although there are many forms of RNA, -omics analyses tend to focus on mRNA (messenger RNA) and small RNAs (e.g., microRNAs). Following transcription, the mRNA is spliced to form a mature transcript that contains no introns and represents the coding portion of genes/genomes. Coding genes account for a tiny fraction of the genome size (1%–2%), making targeting mRNA a cost-effective means of obtaining sequence data for genes. MicroRNAs are a class of RNAs that regulate the process of translation of mRNA into proteins and are thus important regulatory effectors. RNA transcripts can be sequenced individually, or more commonly for -omics analyses[26–30], as part of a transcriptome; that is, the total of all RNA transcripts present in a given sample.

Sequencing can be performed following several methods (i.e., via short-read RNA-seq or long-read whole isoform Seq), allowing analysis of both RNA abundance and isoform usage. As the quantity and diversity of mRNA transcripts varies among cells and tissues, RNA sequencing makes it possible to study and compare gene expression and regulation across samples. Interest in sequencing small RNAs and whole isoform sequencing is growing, as these methods are becoming increasingly more biologically informative. Preparation of tissue samples to sequence different classes of RNA can be performed in the same way as presented in this manuscript, with only the subsequent extraction methods differing[31,32]. Finally, because transcriptomes offer a high coverage subset of the protein-coding genome, the assembled dataset may be useful in genome assembly and annotation, making collection of RNA-seq data across a range of different tissues an important component of the Bat1K initiative.

In contrast to DNA, RNA is chemically unstable and also targeted by RNase enzymes, which are ubiquitously present in tissue lysates as a defensive strategy against RNA-based viruses. For these reasons, the RNA fraction in cells and tissues begins to degrade shortly after the point of sampling and/or euthanasia. Preserving the RNA therefore requires steps to prevent its degradation. This typically involves preserving freshly collected tissue at 4 °C in a stabilizing agent such as RNA stabilizing solution to inactivate the RNases naturally present in tissues, followed by freezing for longer term storage. As a preferred alternative, tissue can be flash frozen in LN2; although as noted above, transporting LN2 into the field and maintaining levels to prevent the tissue thawing can be logistically challenging.

Tissue for protein

Protein composition and relative abundance vary among cells and tissues in a similar way to what was discussed for RNA; however, proteins are on average more stable than RNA. Protein identification using proteomics typically matches a fraction of, and not the whole, protein

sequence, but it can supply information on expression across tissues and characterize pathogens present. As many protein sequences are conserved across mammals, bat samples for proteomics can easily be contaminated with conserved human proteins, requiring sterile protocols (e.g., gloves, forceps) during collection. While flash-freezing in LN2 is the best way to prevent the degradation of proteins, use of dry ice, -20 ˚C freezers, and even ice are suitable if there are no other means. As temperatures increase, the risk of differential protein breakdown also rises. Stabilizing agents such as tissue stabilization solution are effective in preserving the protein fraction of tissues at room temperature and are suitable for short-term preservation (up to one week) when flash-freezing is not viable.

The enzymatic profile of a given tissue directly influences the preservation of protein therein. Tissues with low enzymatic activity such as muscle can preserve protein profiles even at the higher temperatures in a household freezer. By contrast, liver tissue is enzymatically reactive, and its proteins have higher probabilities of degrading during preparation. The growing number of protocols for obtaining human proteomic profiles from formalin-fixed paraffin-embedded (FFPE) samples suggests that paraformaldehyde fixing of tissues holds promise for low-cost protein preservation when freezing upon collection is not feasible[33,34]. Although highly dependent on preservation time and condition, proteins have been identified via immunohistochemistry from formalin-fixed, ethanol-preserved bat specimens[35]. This approach is not scalable to proteomic-level sampling but highlights the potential for formalin-fixed bat tissues to yield protein profiles when flash-freezing is unavailable and other stabilizing agents are too costly.

Tissue for cell culture

Sampling tissue and flash-freezing offers a finite amount of material to be used, and once the material is used, it is no longer available. Alternatively, cell cultures provide live cells that can be immediately used or preserved for future studies. Cultures also facilitate expansion of cells to increase yield when tissue samples are small. It is particularly useful in cases where tissue collection is limited, such as experiments with rare species in which non-lethal sampling is essential and therefore has wide implications for conservation. Described is a protocol in which cell culture is possible via non-lethal sampling of wing membrane tissue, but culturing is possible with multiple tissue types[36,37]. The protocol provided here selects for adherent cells. The combination of source tissue and growth media used makes this protocol suitable to select and grow fibroblasts, but if desired, alternate protocols can be used to select for other cell types. In the context of the Bat1K project, it is predicted that for rare and threatened species, non-lethal sampling of wing membranes and expansion of samples via culturing is essential to generate the volume of DNA needed for the multiple technologies employed[5].

Bat capture

All people handling bats should be trained by a bat-competent researcher and vaccinated against rabies with a series of pre-exposure injections. If bitten, a further series of post-exposure injections is still necessary. Standard methods for capturing bats include mist nets (**Figure 1**) and harp traps (**Figure 2**). Mist nets are most commonly used and ideal for areas with low to moderate activity, as they require the most care for minimizing bat distress. Small bats

are particularly vulnerable and can die from stress if not tended to quickly. Frequent net inspections minimize bat injury and mortality as well as damage to the mist net. This detail is important because proper tissue collection requires the tissue to be fresh, and improper attention to the mist nets in bats can lead to unnecessary mortality or premature mortality before the researcher can properly process samples. Because several bats can rest in a harp trap with minimal distress, this approach is ideal for areas with high bat activity, such as near a cave or large roost. Detailed instructions for proper bat capture and data processing for collection of morphological and demographic information are available in the supplemental methods.

PROTOCOL:

All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Stony Brook University (protocol #2013-2034).

1. Euthanasia

1.1. Moisten a cotton ball with an isoflurane anesthetic or another available anesthetic.

1.2. Place the cotton ball into a resealable, airtight plastic bag. The bag should be large enough to hold a bag in a cloth bat bag comfortably.

1.3. After several minutes of allowing the bag to permeate with anesthetic, place a bat bag containing the bat into the plastic bag.

1.4. Wait several minutes until the bat is euthanized. Bats ~20 g or less in weight require approximately 5–10 min. Bats larger than this may require up to 20 min. Note that the length of time might also depend on the permeability of the bat bag. It is recommended to use the thinnest possible cloth material to maximize diffusion of the anesthetic.

1.5. Check for breath and heartbeat to ensure the bat has been euthanized before dissection begins.

2. Dissection preparation

2.1. Keep all instrumentation clean between dissections, using the following protocol.

2.2. Wash instruments with water and dish soap. Wipe them down with 10% bleach, away from the dissection area, as bleach will break down nucleic acids.

2.3. Wipe down with 70% ethanol.

2.4. Wipe down with RNAse decontamination reagent.

2.5. Repeat this section after each dissection.

3. Preparing the vials for tissue samples

3.1. For RNA:

3.1.1. Prepare all vials before starting any dissections to avoid delays.

3.1.2. Set aside the number of vials needed for each specimen. Label each tube with the tissue type that will be collected, as well as standard specimen identification information. Fill the vials 50% full of RNA stabilizing solution, and ideally chill to 4 °C.

3.1.3. Take care not to fill the vials completely with RNA stabilizing solution, otherwise the tube may explode when placing it in LN2. When taking tissue samples, remember that large lumps of tissue will not be fully permeated by the RNA stabilizing solution. Therefore, cut the tissue into smaller pieces of no larger than 0.5 cm in one dimension, and maintain a ratio of at least 10:1 volume for RNA stabilizing solution:tissue.

3.1.4. At minimum, dissected tissues need to be placed in RNA stabilizing solution, even if access to LN2 or cold storages in unavailable.

3.2. For DNA:

NOTE: The nuclear DNA content is the same for almost all tissues; therefore, sampling can be flexible. However, it should be noted that higher densities of mitochondria in muscle tissue may lead to a loss of reads for the nuclear genome[38].

3.2.1. For low molecular weight DNA, take one or more replicates of wing membrane for storage in silica, and/or muscle for storage in RNA stabilization solution or tissue stabilization solution.

3.2.2. For ultra HMW DNA, flash-freeze in LN2 without any storage reagent, then transfer to long-term storage at -80 °C or colder. From the cranial dissection, brain tissue is the most suitable tissue type for retrieving ultra HMW DNA[39], whereas from postcranial dissections, liver or muscle are more suitable[40].

3.2.3. For the cranial dissection, use 5 mL vials (as opposed to the standard 2 mL vials) of RNA stabilizing solution for some tissue. All vials should be cryogenic. Keep the vials on ice while dissecting.

4. Cranial dissections for RNA

4.1. Immediately after euthanasia, decapitate the specimen with a large pair of scissors or bone cutters (**Figure 3**).

4.2. Remove the eyes with forceps using strong enough force to detach the optic nerve. Place the eyes in 2 mL vial of RNA stabilizing solution.

4.3. Using your tool of choice, skin the skull from hair, fascia, and skull muscles, including the skin on the nose. Take care not to break the front end of the nose.

4.4. Using scissors, make a sagittal cut on the ventral portion (**Figure 3**) of the skull starting at the neck, taking care not to damage the brain.

4.5. Using forceps, gently pull back both sides of the skull until it has cracked open and the brain is exposed.

4.6. On the caudal end of the skull, the cochleae should now be laterally visible on each side of the head. They are small, spherical bones on the left and right side, just rostral to the neck and posterior to the masseter muscles. Using forceps, gently pull the cochleae and put them in one 2 mL vial of RNA stabilizing solution.

4.7. Gently scrape the brain (which will be very soft) with forceps. The olfactory bulb will become visible, sitting at the ventral portion of the interior of the skull. Try to keep the olfactory bulb attached.

4.8. If the olfactory bulb has not already been removed, gently scrape away the tissue, and the cribriform plate will become apparent. This is a critical bone to keep the researcher oriented. It can be identified as the most anterior region of the skull with multiple foramen and the point at which two grooves where the olfactory bulb rests.

4.9. If possible, keep the brain shape intact and immediately place on dry ice to keep shape or in a 5 mL vial of RNA stabilizing solution. If immunohistochemistry is to be done on the brain, place in 4% paraformaldehyde, if available.

4.10. Make two incisions where the top and bottom jaw join, and remove the mandible.

4.11. Once the mandible has been removed, remove the rostrum (upper jaw) from the remaining part of the skull. Ensure that the jaw includes the cribriform plate.

4.12. Place the nose in RNA stabilizing solution and store at 4 °C overnight. Because this is a dense tissue, it requires time to allow RNA stabilizing solution to permeate the entire tissue.

4.13. Place the nose vial in LN2 following the overnight soak in RNA stabilizing solution.

4.14. From the lower mandible, cut the tongue with scissors and place in a 2 mL vial of RNA for later.

4.15. This protocol forfeits most of the skull. Teeth, particularly those from the mandible, can be recovered and may be useful for species diagnostics. Bone tissue can be stored in 1x phosphate-buffered saline (PBS).

5. Postcranial dissections for RNA

5.1. Use a scalpel to pierce through the abdominal cavity, making a longitudinal incision up to the ribs (**Figure 3**). This forfeits the skeletal frame. If bone is to be preserved, carefully dissect from skin in muscle and store in 1x PBS after soft tissue dissection is complete.

5.2. Strip the skin to reveal the pectoral muscle, take at least two samples of muscle, one for RNA stabilizing solution and one to remain frozen for HMW DNA, placing immediately in a vial to put into LN2.

5.3. Cut through the sternum and pull away the ribs to collect samples from the lung.

5.4. Collect the heart, which can be taken whole but should be sectioned in halves, so the RNA stabilizing solution soaks thoroughly.

5.5. Take samples from the liver. Take at least two samples of liver, one to remain frozen for HMW DNA, placing immediately in an empty vial to put into LN2. The liver is very enzymatic, and it is important to make the samples small enough for the RNA stabilizing solution to fully soak in.

5.6. The hepatic duct, which functions to drain bile from the liver, connects the liver to pancreas and small intestine. This vessel is easily identifiable by probing the inferior/posterior portion of the liver and tracing the vessel in a posterior manner. The duct is often a greenish color, as well. Follow the hepatic duct to find the pancreas and gallbladder and collect these separately. Place in respective vials of RNA stabilizing solution.

5.7. Collect the stomach and, next to it, at its base and appearing as a different shade of purple, is the feather-like spleen. The pancreas should also be visible here as a white structure (**Figure 3**). Place in respective vials of RNA stabilizing solution.

5.8. Collect small samples of the small and large intestine. Place in respective vials of RNA stabilizing solution. Intestines may also be screened for endoparasite. If a parasitologist is in the field, an inspection for parasites can be performed. If this is to be done at a later time, the entire intestine can be taken in a 5 mL cryogenic vial.

5.9. Take one of the kidneys and follow their ducts to the bladder. Place in respective vials of RNA stabilizing solution.

5.10. Use the other kidney as a guide to find the testes (if male) or uterus and through it the ovaries (if female). Collect one or both gonads, if possible.

5.11. Keep samples of various parts of the skin of the wing in separate vials (muscular vs. non-muscular part).

6. **Tissue culture collection and preparation**

6.1. Prepare growth medium for the cells by making up Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 50 µg/ mL gentamycin. Aliquots of growth media should be made fresh every day of the protocol.

6.2. After collection, wing biopsy punches must be directly placed in 1 mL of cold growth medium in a well-sealed 1.5 mL centrifuge tube. Wrap the tube in parafilm to seal.

6.3. Tubes should then be transported in a polystyrene box with cooling elements to keep samples at 4 °C. Transport should be expedited; although, healthy cells can be made from punches that have been stored in this manner for up to 6 days but less optimally[23].

6.4. Once transported to tissue culture facility, the following protocol can be used to generate cell cultures. Standard sterile techniques should be used for the rest of the protocol[41].

7. Day 1 tissue culture: dissociation of tissue

7.1. Transfer the contents of the tube (growth medium containing the wing membrane biopsy) into a 15 mL conical centrifuge tube.

7.2. Carefully remove the growth medium. Gently wash the biopsy two times with 500 µL of sterile PBS.

7.3. Add 500 µL of collagenase IV (1 mg/ mL) to the tube. This will cause digestion of the tissue into individual cells.

7.4. Incubate overnight (maximum 16 h) at 37 °C without agitation.

8. Day 2 tissue culture: plating cells

8.1. Make up fresh growth medium and pre-warm it to 37 °C.

8.2. Prepare a 6 well tissue culture plate by adding 1 mL of fresh, pre-warmed growth medium in each well of the plate to be used (1 well per 3 mm wing biopsy). Store this plate in a 37 °C and 5% CO_2 incubator until needed (step 8.7).

8.3. Remove the 15 mL tube containing the cells from the incubator and quench the digestion reaction by adding 1 mL of fresh growth medium (pre-warmed to 37 °C).

8.4. Resuspend the cells by carefully triturating the solution with a P1000 pipette tip to achieve a single cell suspension.

8.5. Gently spin down the cells in a table top centrifuge for 3 min at 300 x *g*.

8.6. Discard the supernatant by gently removing 80%–90% of the liquid with a P1000 pipette.

8.7. Resuspend the pellet in 500 µL of pre-warmed growth medium, gently triturate the suspension to ensure that the pellet or large fragments of the pellet are no longer visible and that cells are sufficiently suspended. The media may look cloudy due to the presence of cells.

NOTE: At this stage, a viable cell count can be performed in order to assess the quality of the cells suspension and yield of cells derived from the wing clip (see step 10.11 for further details).

8.8. Gently pipette the entire volume of cell suspension into a single well of a 6 well plate.

NOTE: Perform the above step immediately and do not allow cells to settle after step 8.7. Use a pipette to gently distribute the cell suspension across the surface of the well in a dropwise fashion. Do not pipet the entire solution into the center of the well, as this would result in the cells clumping in the middle of the well.

8.9. Gently rock the plate from side-to-side and front-to-back 2x–3x to help cells distribute over the well surface in a single layer.

8.10. Check the plated cells under the microscope, as they should be single cells that appear balled up and floating but very dense.

8.11. Carefully place the plate into an incubator pre-set to 37 °C and 5% CO_2.

8.12. After ~24 h, observe the cells under the microscope to determine health of the culture. Cells should now be attached to the plate surface and appear flattened (**Figure 4A**). Different cell types may be visible in the culture when looking through the microscope.

8.13. There will likely be some floating cells that have not attached. A proportion of these cells are dead. If there is a high proportion of floating cells visible in the well, the media should be refreshed. In this case, carefully aspirate ~50% of the medium from the well and gently add 1 mL of pre-warmed growth medium to the side of the well so as not to disturb the cells.

8.14. Maintain the cells in an incubator pre-set to 37 °C and 5% CO_2. Cells should be observed under the microscope regularly to determine the need for media refreshment or splitting.

NOTE: When cells are newly plated, they will divide quickly and so should be checked every day. After some time in culture, growth will slow, and they can be checked every 48–72 h.

9. Refreshing media

9.1. Check the cells under the microscope regularly to observe their growth and quality of the culture. Monitor the color of the media as an indicator of its quality. Cells should remain in the same media for a maximum of 3 days or until passaging is necessary, whichever comes first.

9.1.1. If cells are growing quickly and exhausting the media, this will also be visible to the eye, as the color of the media will turn from red to yellow.

9.2. Whenever necessary carefully aspirate ~50% of the medium from the well and gently add 1 mL of pre-warmed growth medium to the side of the well so as not to disturb the cells.

9.3. Return the cells to the 37 °C and 5% CO_2 incubator.

10. Passaging cells

10.1. Passaging cells refers to taking an existing culture and splitting it into new wells. Passaging should take place when the cells are ~80% confluent [i.e., when they occupy ~80% of the surface of the well and only ~20% of the plastic is still visible as gaps (**Figure 4B**)].

10.2. Carefully aspirate ~90% of the growth medium.

10.3. Wash the cells very gently by adding 1 mL of sterile PBS to the wall of the well so as not to disturb the cells. Gently rock the plate back-and-forth and side-to-side 2x–3x. Carefully aspirate all the PBS from the plate.

10.4. Repeat step 10.3 to wash the cells again.

10.5. Gently add 250 µL of trypsin-EDTA to the well and incubate for 1 min at RT.

10.6. Quench the reaction by adding 1.5 mL of fresh pre-warmed growth medium.

10.7. Pipette up and down ~5x to wash the cells from the surface of the plate and ensure the cells are in suspension.

NOTE: The solution should become cloudy due to the presence of the cells.

10.8. Place the cell suspension in a 15 mL tube and spin down the cells in a table-top centrifuge for 3 min at 300 x *g*.

10.9. Discard the supernatant by gently removing 80%–90% of the liquid with a P1000 pipette.

10.10. Resuspend the pellet in 1.5 mL of pre-warmed growth medium and gently triturate the suspension. Ensure that the pellet or large fragments of the pellet are no longer visible, and cells are in a single cell suspension.

10.11. Count the cells using an automated cell counter as described here, or manually by using an hemocytometer as described in detail in the reference video[42]. Mix a 10 µL aliquot of the suspended cells 1:1 with Trypan blue to detect viable cells.

10.12. Incubate for 1 min, and pipette 10 µL of the solution onto the counting slide. Insert the counting slide into an automated cell counter to count the cells using the appropriate settings. The yield should be around 1–2 million cells from a confluent single well of a 6 well plate, although this may vary depending on size of the cells and species. If cells are not in a single cell suspension, the cell count will not be accurate.

10.13. These cell cultures will generally tolerate a 1:2 split [i.e., one confluent well of cells can be split into two new wells (total)]. To do this, gently triturate the cells again, then take the cell suspension in two halves (~730 µL each) and place them into each of two new wells containing 750 µL of pre-warmed growth medium in dropwise fashion.

NOTE: After 2–3 days the wells should be confluent and again ready for splitting. At this point, it is recommended to preserve one well of cells by the viably freezing protocol (section 11). Further stocks of cells can be frozen during further passages as desirable.

NOTE: After ~6 passages, the cells tend to enter senescence and will no longer divide. This is an indication that the cells can no longer be split or expanded to increase cell numbers. This is also an indication that the cells will not be viable for much longer.

11. Freezing viable living cells

11.1. Prepare freezing media by combining DMEM with 20% FBS, 10% DMSO, 1% P/S, and 50 µg/ mL gentamycin.

11.2. Freezing is performed by taking cells pelleted during the passaging process. The pellet should be prepared from an 80%–90% confluent well of a 6 well plate (representing ~1–2 million cells), as per steps 8.1–8.9.

11.3. Resuspend the pellet in 1.5 mL of freezing medium.

11.4. Place ~750 µL of cell suspension in each of two separate cryovials.

11.5. Place the vials in a cryogenic freezing container, which results in the cells freezing slowly to maintain cell vitality. Immediately place them in -80 °C.

11.6. After 24–48 h, transfer the cryovials of cells to LN2 for long-term storage. Stored in this way, cells can be revived and will be viable for years.

12. Thawing frozen cells

12.1. Prepare a 6 well plate containing 750 µL of pre-warmed growth medium in each well that will be used (1 well per vial of cells being thawed). Maintain plate in the 37 °C and 5% CO_2 incubator until needed.

12.2. Take one vial of cells from LN2 and place it in a warm water bath (37 °C) to rapidly thaw cells. This should take 2–3 min.

NOTE*:* Do not leave the vials thawing for longer than 3–5 min, as the DMSO in the freezing media is toxic to the cells once thawed.

12.3. As soon as the solution in the vial is thawed, gently pipette up and down to homogenize the solution and immediately place the entire solution (~750 µL) in one well of a 6 well plate (pre-prepared in step 12.1).

12.4. Gently rock the plate from side to side and front to back 2–3 times to help cells distribute over the well surface in a single layer.

12.5. Place the cells in the incubator at 37 °C and 5% CO_2 incubator for 24–48 h.

12.6. Monitor and passage the cells as described above.

REPRESENTATIVE RESULTS:

DNA

For standard low molecular weight (LMW) analyses, DNA was extracted from three neotropical bat species. Tissue samples were collected in the field from the Dominican Republic and Costa Rica, following the protocols described in this paper. Following dissection, small pieces (<0.5 cm at the thinnest section) of mixed tissues (brain, liver, and intestine) were placed in RNA stabilizing solution, flash frozen, then stored at -80 °C. Extractions were carried out using standard DNA extraction protocols with RNase treatment[43]. Assessment of DNA integrity with an automated electrophoresis tool revealed the following representative sample peaks of fragment sizes: species 1 (two separate extractions) consisted of 22 kb and 20 kb; species 2 consisted of 25 kb; and species 3 consisted of 24 kb (**Figure 5**, **Table 2**).

DNA extracted for third-generation sequencing is required to be collected in high concentrations and minimally fragmented. HMW DNA extracted from flash-frozen brain tissue from an Old World fruit bat (*Eidolon helvum*) collected in Ghana was obtained. HMW DNA was extracted using the Bionano extraction protocol for subsequent analysis on the Irys platform. This DNA was 607,463 Mb in length and had an average molecular N50 of 194.5 Mb.

RNA

A common indicator of success for RNA extractions is the RNA integrity number (RIN) value. It is often not recommended by sequencing facilities to carry out an RNA-seq library preparation or sequencing protocol when extractions have a RIN value less than eight, as values below this threshold begin to demonstrate high signatures of degradation[44,45]. **Figure 6** shows the RIN values for RNA extractions of various tissue types following this protocol. Extraction of tissue collected with the protocols has resulted in high quality RNA, independent of field sampling locality. When LN2 was not immediately available in the Amazon, tissues were placed in RNA stabilizing solution and kept at 4 °C for 1 week until placed into LN2. Even in such cases, RIN values were comparable to those immediately placed in RNA stabilizing solution and directly into LN2. RNA stabilizing solution is an essential stabilizing agent.

Tissue culture

Immediately after plating, cells will be balled up and floating. However, within 24 h, the fibroblasts should flatten and attach to the surface of the plate (**Figure 4A**). At this point, the cells should be at roughly 20%–30% confluence to ensure survival. A value lower than this may result in death of the entire culture. Initially, they must have ample space between each other to allow expansion, as the cells will divide and expand in culture. They should be split when they reach 80%–90% confluence [i.e., they cover >80% of the plate surface (**Figure 4B**)]. In this way, cells can be maintained in culture for a number of passages (usually >6 passages) before undergoing senescence. If cells are maintained in culture beyond this time or not split when they become confluent, they may change morphology and become larger and longer (**Figure 4C**). Cells with this morphology may still divide, but this usually indicates that the cells are or will soon enter senescence and will no longer be able to be maintained or expanded in culture.

FIGURE AND TABLE LEGENDS:

Figure 1: A common set up for mist nets to capture bats in the forest. Keeping one hand covered while disentangling with the opposite hand is a way to safely remove the bat while minimizing stress. This photo was taken by Jon Flanders.

Figure 2: A harp trap is often used to capture outside caves, large roosts, or fly-aways. Bats will accumulate in the lower pouch of the trap with minimal entanglement and easy removal by the investigator. This photo was taken by Stephen Rossiter.

Figure 3: Workflow of dissections and tissue sampling for RNA tissue preparation.

Figure 4: Bat cell cultures derived from wing punches of *Phyllostomus discolor*. (**A**) 24–48 h after plating, cells will become flattened and attach to the plate surface. (**B**) Cells cover 80%–90% of the plate surface and maintain the original morphology. This represents the optimal stage for splitting. (**C**) After >6 passages, cells will change their morphology to become larger and longer, and the cells will enter senescence. In all pictures, bright, balled-up cells represent dead cells.

Figure 5: Representative results of DNA extractions from preservation for standard DNA analyses from three bat species, DNA was extracted twice from Species 1. A single large band indicates minimal fragmentation and similar sized fragments of DNA from each respective extraction.

Figure 6: RNA integrity numbers (RIN) of RNA extractions from 13 different tissue types from neotropical bat species sampled at four different localities. These extractions were prepared following the described protocol and then were used to prepare HiSeq/NextSeq Illumina transcriptome libraries. MOE is the main olfactory epithelium. VNO is the vomeronasal organ. Tissues sampled from species in the Andes, Costa Rica and Dominican Republic were placed directly in LN2 after soaking in RNA stabilizing solution at 4 °C overnight. Tissues sampled from species in the Amazon were placed in LN2 approximately 1 week after placing in RNA stabilizing solution and kept at 4 °C continuously. *N* represents the number of individuals represented for each tissue extraction.

Table 1: Overview of preservation methods and applications. MW = molecular weight, FFPE = formalin-fixed paraffin-embedded tissue. Checkmarks indicate preferred preservation method for each respective intended application. X-marks indicate preservations that should not be used.

Table 2: Representative results for DNA extractions based on preservation methods in this protocol. Values correspond to the gel electrophoresis wells from **Figure 5**.

DISCUSSION:

The protocol discussed in this manuscript describe the best sampling practices for various high-throughput molecular analyses of bats. All successful -omics studies require high quality tissue, but sampling bat tissue, as well as other non-model organisms, often occurs in field conditions that cannot be set to the same standards as those of a controlled laboratory setting. Sampling often occurs in remote locations, with minimal resources, including limited access to electricity and freezers. It is difficult, and often impossible, to ensure completely sterile sampling conditions. Thus, outlined are the protocols identified as the most successful in a variety of sampling localities and field conditions. In order to have standardized sampling methods for the Bat1K initiative and related efforts and projects, it is suggested that bat biologists follow these protocols while planning field expeditions to the extent that is permitted.

Protocol suggestions

While thoracic compression was a common approach for euthanasia in the past, it is suggested to never use thoracic compression to collect bats for –*omics* analyses. It is no longer an acceptable form of euthanasia in the United States[46], and this method may lead to the enzymatic degradation of a range of tissues no matter the species of interest[47]. If an animal will be euthanized, it is suggested that tissue for both genomic and transcriptomic analyses be collected. Both require harvesting fresh tissue that would ideally be flash frozen in LN2, but that

can also be stored in different media to facilitate DNA, RNA, or protein extraction. It is suggested working in parallel with two people at cranial and postcranial dissections to reduce degradation of the RNA in tissues. One person should dissect the cranium while the other works on the post-cranium.

Major limitations of carrying out the dissection protocols include the personnel involved in the dissection and space available for tissue preservation (e.g., in the LN2 tank). Preparation of vials in advance and sufficient numbers of people to assist with labeling tubes and documenting vials and tissues collected will contribute to smooth sample processing. It should be noted that proper documentation of samples is essential to the proper permitting processes. The postcranial dissection protocol, for example, demands more than 20 vials per animal.

If space and time are limited, the following should be prioritized: 1) prioritization for one specimen for species, or at minimum, one specimen per genus; prioritization for a male specimen to allow sequencing of both X and Y chromosome and minimization of the probability of sampling a reproductive female; 2) for HMW DNA: brain, muscle, and liver should be flash frozen without any reagent; 3) for RNA: brain, digestive tissues, spleen, and sensory organs are of high priority. Each tissue sample should be stored in RNA stabilizing solution. Muscle tissue should also be taken as a control for expression analyses of specialized tissue; 4) at the very minimum, tissues should be kept cold; 5) the availability of LN2 may also be of concern. LN2 tanks must be refilled at minimum every two weeks but more frequently in warmer climates or if the tank is frequently opened. LN2 is often commercially available in all countries, as it is frequently used in agriculture and healthcare to transport samples. Usually these services are provided by companies that also sell other gases, such as carbon dioxide or oxygen. Ice cream shops sometimes another option for dry ice or LN2 (or at least provide a recommendation for purchase), and it is suggested to ask local personnel for assistance with this; 6) **Figure 6** outlines successful tissue extractions for some collection expeditions in which conditions were limited.

With more people and more space, more tissue can be collected in different ways. The following are additional protocols published elsewhere that investigators should consider if the proper experience and materials are available: 1) the bat metabolome, or all the excreted low-molecular weight metabolites of cells and tissues, can provide insight into exceptional bat longevity or hibernation and metabolic flight demands. Blood should be collected from an easily accessible blood vessel, such as the ankle veins, into a vial with heparin[48], and fecal samples should be frozen in LN2[49]; 2) immunomics, or the response of the animal to pathogens, can be analyzed by taking femurs and humeri and placing them in tissue culture solution to culture macrophages downstream[50]; 3) feces may be useful for two types of downstream nucleic-acid based analyses, such as determining diet[51] and probing the gut microbiome[52–54]. In both cases, reagents such as tissue stabilizing solution reduce the degradation of rare variants present in the feces; 4) lipidomics, or the phospholipid repertoires of a tissue type, has been revealing in understanding the white-nose syndrome fungal pathogen[55,56]. Preparation of tissue is suggested to be frozen immediately after dissection.

Tissue archives for Bat1K

Bat1K aims to maintain a tissue bank of each individual bat that is used to generate the genomes. Currently, these tissue banks and the relevant phenotypic and ecological data collected per individual are maintained in the laboratories/museum collections of the Bat1K contributing members. As the project progresses, Bat1K will centralize and maintain tissue collections and relevant databases through networked repositories such as the Global Genome Biodiversity Network <http://www.ggbn.org/ggbn_portal/>.

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The authors have nothing to disclose.

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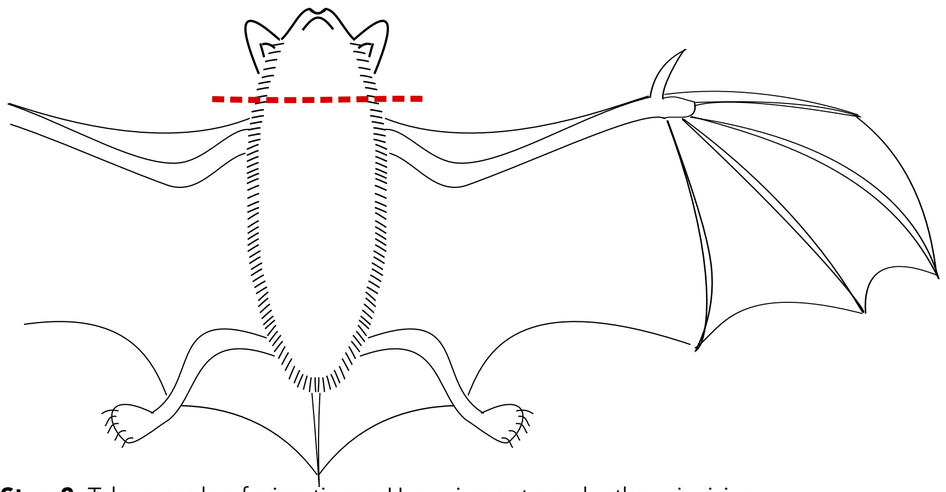
Figure 1

Click here to access/download;Figure;fig1_mistNet.png

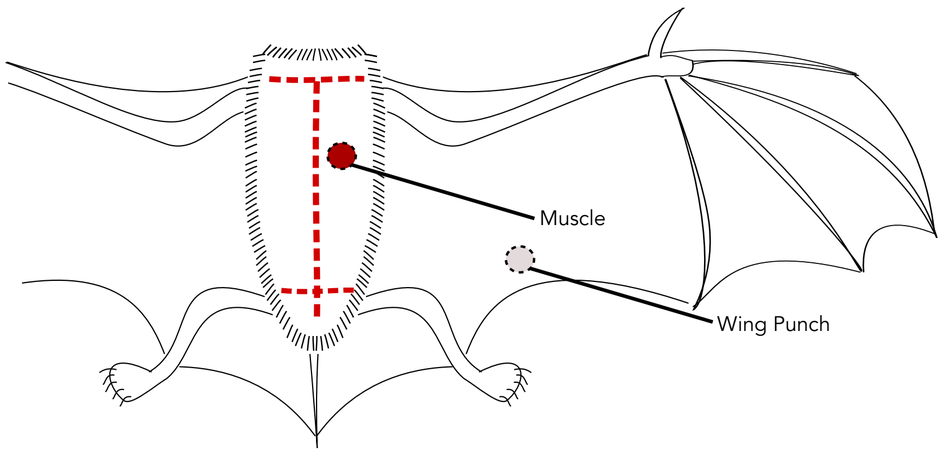
Figure 2

Click here to access/download;Figure;fig2_harpTrap.jpg

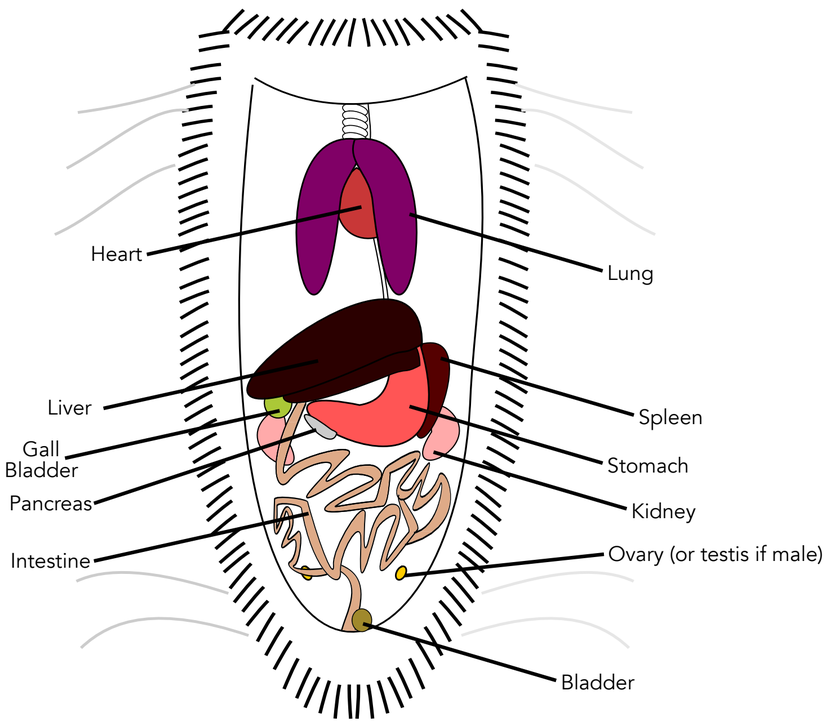
Step 1: Remove head using bone cutters or strong scissors.



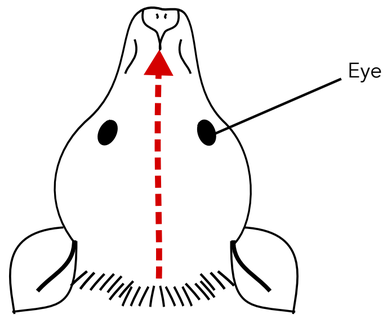
Step 2: Take sample of wing tissue. Use scissors to make three incisions along chest. Cut through pectoral muscle to expose internal organs. Take muscle and wing tissue and put in RNA storage solution.



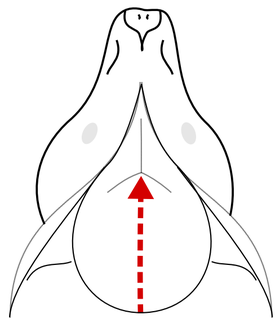
Step 3: Dissect postcranial anatomy, taking at minimum the tissues listed below. All tissues should be placed in RNA storage solution.



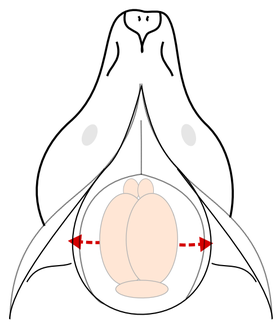
Step 4: Make sagittal cut under skin to remove. Use forceps to remove eyeballs and place in RNA storage solution.



Step 5: Peel skin and muscle away from the posterior end of skull to expose the skull. Using bone cutters, cut skull posterior to anterior until reaching cribriform plate. Take care to not damage brain.

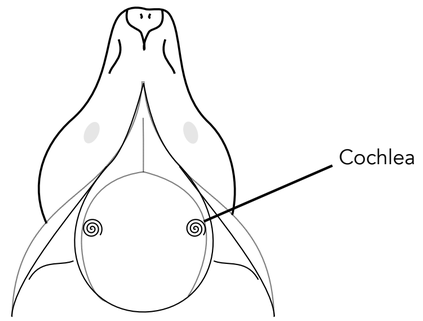


Step 6: Using forceps, gently pull away the pieces of the skull in a distal fashion to expose the brain.

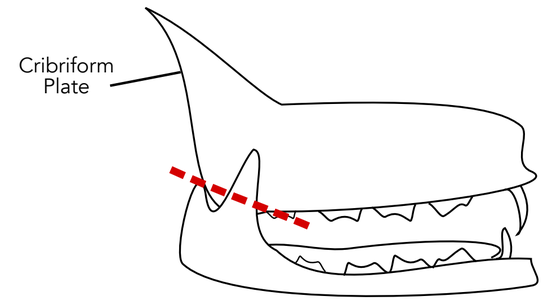


Step 7: To maintain the integrity of the brain, gently scrape the brain away from the skull with forceps. Place the brain on dry ice, in RNA storage solution, or in 4% paraformaldehyde, depending on the intended purpose.

Step 8: Once the brain is removed, the cochleas should be readily exposed. Gently pull them away from skull and place in RNA storage solution.

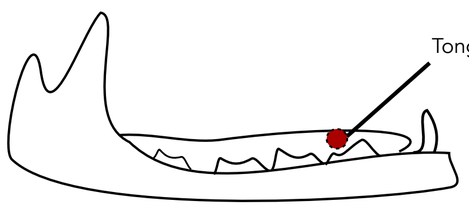


Step 9: Peel off the remainin of the skin. Using bone cutters, detach the mandible from the maxilla at the temporomandibular joints.



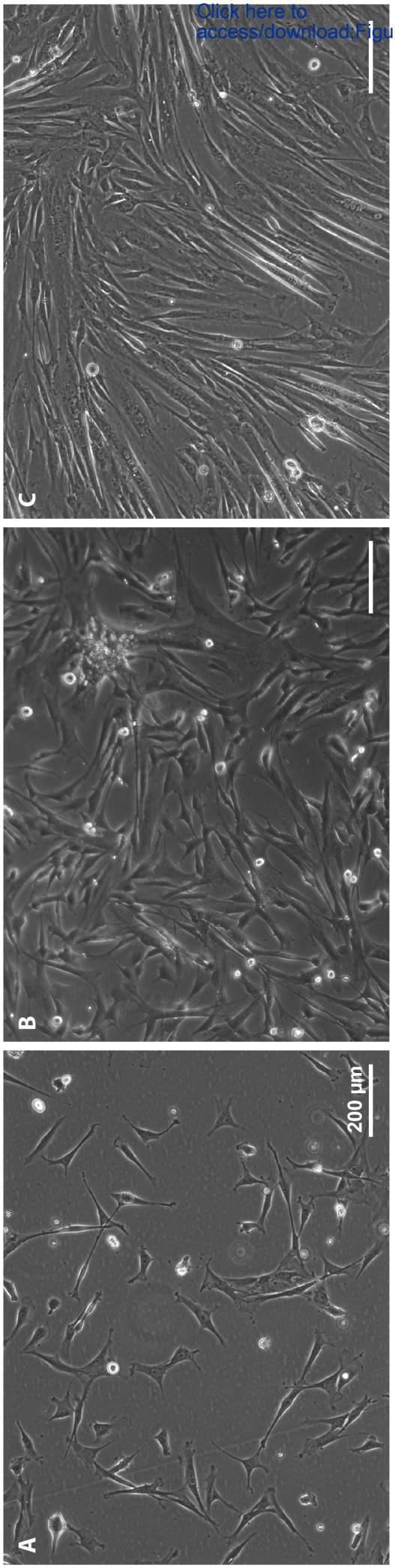
Step 10: Place entire maxilla, including the cribriform plate into a 5 mL vial of RNA storage solution. Soak overnight at 4 °C before flash freezing to allow RNA storage solution to penetrate.

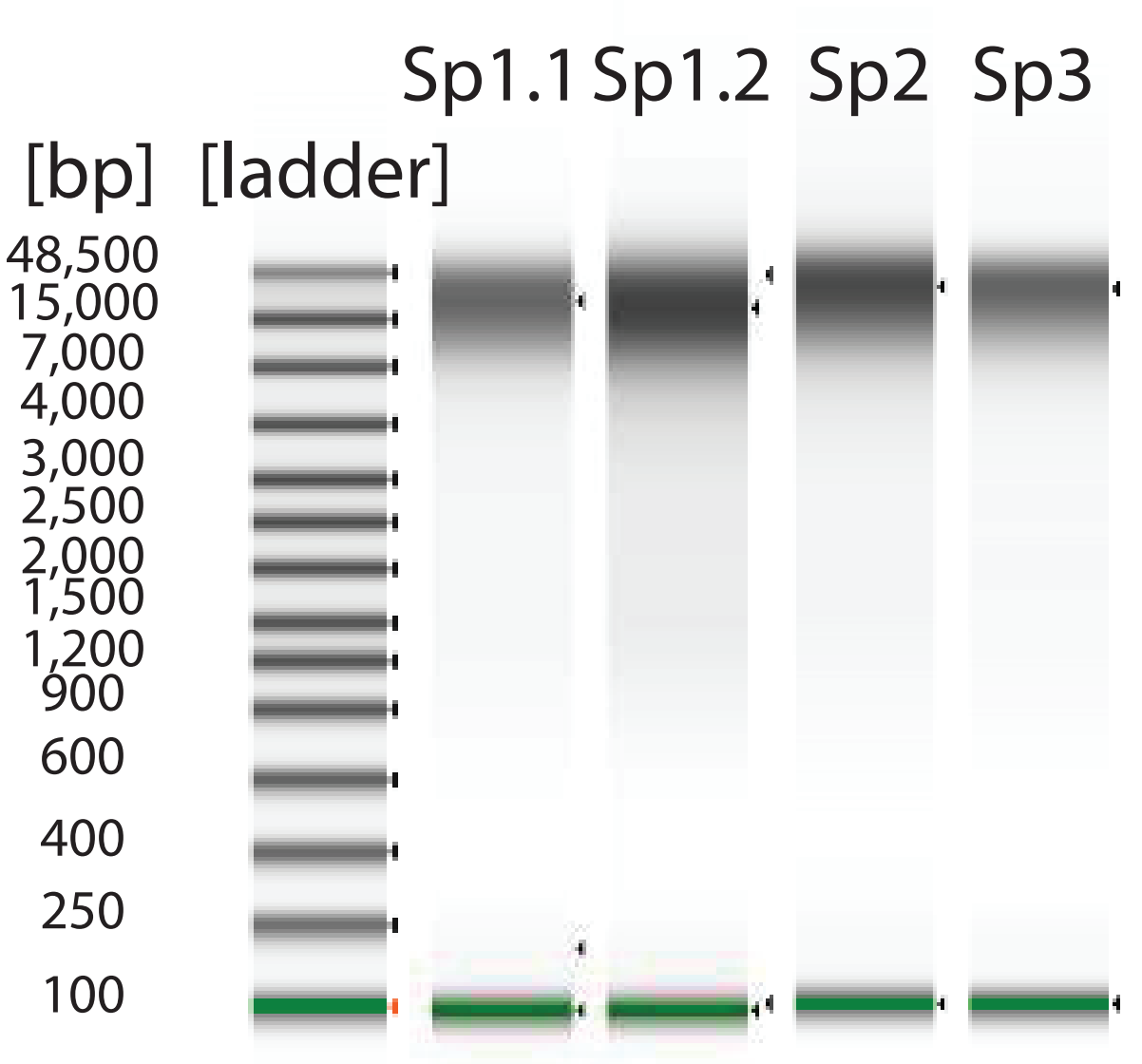
Step 11: Cut the tongue from the mandbile and place tissue in RNA storage solution.



Step 12: Once dissection is complete, record all vials collected and place all 2 mL vials in liquid nitrogen.

Figure 4




Sp1.1
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48,500
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Figure 6

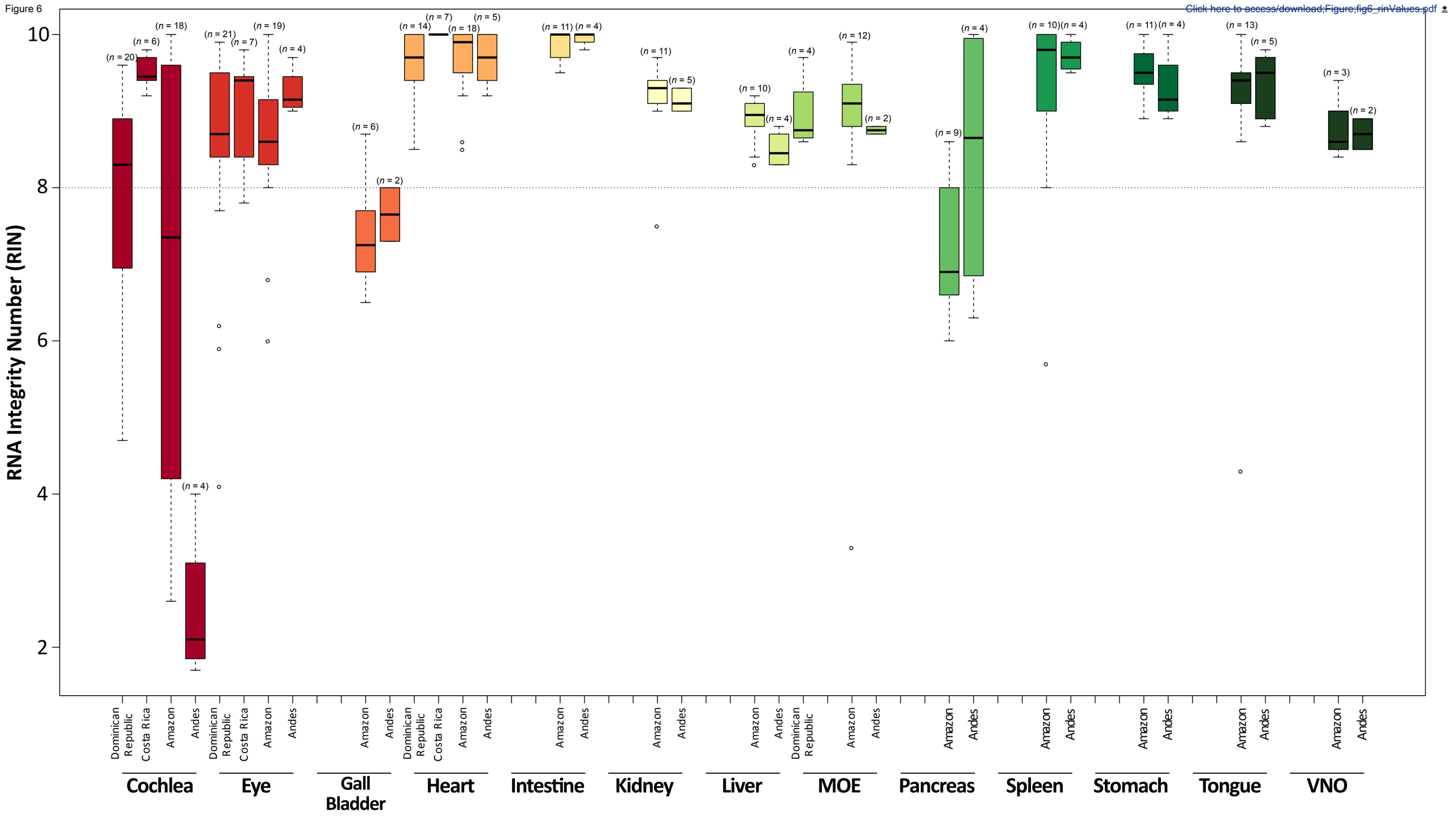

Table 1

Application	Flash frozen (L2)	RNAlater	AllProtect	FFPE	Media
High MW DNA	✓	×	×	×	×
Low MW DNA	✓	✓	✓	×	×
RNA	✓	✓	✓	×	×
Protein	✓	×	✓	✓	×
Cell Culture	×	×	×	×	✓

Species ID	Sample ID	Concentration (ng/µL)	Sample fragment peak size (bp)
Species 1	1.1	31.8	21,885
	1.2	22.8	19,633
Species 2	2	30.4	25,198
Species 3	3	32	24,386

Table of Materials

Click here to access/download;Table of Materials;Jove_batTissue_materialsList_final_PDv.xlsx

Name of Material/ Equipment	Company	Catalog Number
1.5 mL Eppendorf Safelock tubes	Fischer Scientific	10509691
15 mL tubes	Sarstedt	62,554,002
2 mL cryovials	Thomas Scientific	1154P75
3-mm biopsy punch	Medline	MIL3332
6 well plate	Greiner	83,392
Allprotect Tissue Reagent	Qiagen	76405
Cell counting slides for TC10/TC20 cell counter, dual chamber	Bio-Rad	145-0011
collagenase IV	Stemcell Technologies	7909
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D2650-5X5ML
DMEM–Dulbecco's Modified Eagle Medium	Thermo Fisher	12491-015
Dulbecco's PBS	Invitrogen	14190169
Fetal bovine serum (FBS)	Fischer Scientific	10270106
Gentamycin sulfate salt	Sigma Aldrich	G1264-250MG
Nalgene Mr. Frosty	Thermo Scientific	5100-0050
PARAFILM	Sigma	P7793
Penicillin-streptomycin (pen-strep), 100x	Invitrogen	15140130
Phosphate-buffered saline (PBS) 10x, pH 7.4	Thermo Fisher	10010023
RNAlater	Thermo Fisher	AM7021
RNAse away	Genetech	83931-250mL
Silica gel	Fisher Scientific	7631-86-9
TC20 Automated Cell Counter	Bio-Rad	1450102
Trypan blue	Bio-Rad	145-0013
trypsin-EDTA	Sigma Aldrich	T4049

	Comments/Description
1.5 mL sterile tubes	used during cell culture protocol
15 mL sterile tubes	used during cell culture protocol
cryogenic vials	for tissues to be stored in liquid nitrogen
wing biopsy punch	for cell culture
Culture vessle	
	for fecal samples; tissue stabilizing solution
	Chambers for the count cells using the automated cell counter TC20 by Bio-Rad
	For dissociation of primary cells
	Prevents crystalization of water during freezing of the cells.
culture media	
	Balanced salt solution used for washing cells
	Serum-supplement for in vitro cell culture of eukaryotic cells
	Antibiotic for culture media
	Freezing container which provides 1°C/min cooling rate
	Wrapping tubes etc for sealing
	Antibiotic for culture media
	salt buffer used for washes and storage of bone tissue; dilute to 10X using de-ionized water
RNA stabilizing solution	
	breaks down enzymes that lead to RNA degradation
	dessicant agent
Automated cell counter	
	Cell stain used to assess cell viability
	For dissociation of cells during splitting

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Title of Article: Best practices for tissue collection of bats for -omics analyses and primary cell culture

Author(s): Laurel R Yohe, Laura Baas, Kalina TJ Davies, Joshua HT Potter, Stephen J Rossiter, Emma C Teeling, Sonja Vernes, Liliana M Dávalos

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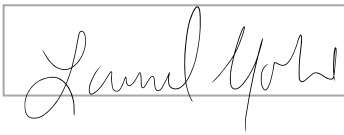
Signature:	 Date: 12/5/2018

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Editorial comments:

General:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.* **Addressed.**
2. *Please ensure manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.* **Addressed.**
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   RNAlater, AllProtect, RNase AWAY are all referenced in the Table of Materials and Reagents. We used generic terms for Eppendorf, Falcon, DNeasy, and Agilent TapeStation. The inclusion of the sequencing technologies is imperative to the purpose of the manuscript and we have left these in the text.

Protocol:

1. *Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.* **Addressed.**
2. *Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a 'Note'. Please move the introductory paragraphs of the protocol to the Introduction, Results, or Discussion (as appropriate) or break into steps.* **Addressed.**
3. *Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.* **Addressed.**
4. There is a ~10-page limit for the Protocol, but there is a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. **Because we are filming two separate protocols in two separate labs, we are requesting for double the page length for the scripted material.**
5. *For each step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps*. **Addressed.**

Specific Protocol steps:

1. *Euthanasia: Please give approximate times here if you are able.*

Added.

Figures:

1. *Figure 3: Please remove commercial language from the figure as well (RNAlater). Please define PFA. Please add a space between '4' and '°C' (Step 10).* **Addressed.**
2. Figure 4: 'µm', not 'uM'. **Addressed.**

References:

1. Please ensure references have a consistent format.

Table of Materials:

1. *Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.* **Addressed.**
2. *Please remove trademark (™) and registered (®) symbols from the Table and Materials.* **Addressed.**

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript details tissue collection and archival protocol for the Bat1K initiative which aims to sequence a chromosome-level genome for all extant bats. I enjoyed reading this manuscript, it was very well written, and I felt the described methods were overall practiced and thorough.

Thank you for your consideration.

Major Concerns:

(1) My first issue is the intensity of the protocol. This was touched on in the discussion (L700-717), as the proposed methods may not be practical in the field due to (a) the number of animals being processed, (b) environmental conditions, or (c) available resources (personnel, experience, tools, financial). Beyond time, personnel, and abilities, the Postcranial dissections for RNA protocol requires >18 tubes per animal (2 pectoral muscle, lung, 2 heart (1 RNA/1 LN2), 2+ liver, pancreas, gall bladder, stomach, spleen, small intestine, large intestine, kidney, bladder, testes/uterus/ovaries, 2 skin), and each subheading requires additional tubes/samples.

I would like to see the authors briefly outline 'priorities' for field dissections. For example, if full dissections, as described, are not possible for all animals, would it be best to fully process one specimen of each species collected? Or a male and female of each species collected (if available)? Then what would reduced-sampling look like? What tissues should be prioritized? With the number of tissues proposed, would it be unrealistic to preserve the whole animal either in EtOH, frozen, or RNAlater?

We have provided a section regarding prioritization under less ideal conditions.

(2) What is the data archival protocol for the Bat1K initiative? Where will the associated specimen data be archived and digitized and where will the specimens and associated tissue tubes be permanently housed and made available to the scientific community? These are key details currently missing from the manuscript that will ensure the longevity of this ambitious project.

Similarly, is there a plan for integration with existing collections resources? The physical resources required for the proposed protocols are expensive, require many trained personnel, and ample space to store these samples at -80. To avoid reinventing the wheel, collaborations with existing agencies that specialize in field work and collections may benefit this initiative by providing consistency and a breadth of sampling not possible for independent researchers.
As a team, we discussed what should be described at this point. We added a paragraph regarding this in the last paragraph of the discussion.

(3) I remain unconvinced that this manuscript and the protocols therein are relevant to only bat tissue collection and preservation - Why are similar methods not applicable for other taxa? Particularly other mammals? But also, other vertebrates?
We added a sentence to the end of the introduction stating the relevance for other taxa.

I agree that a lack of quality tissues is limiting scientific inquiry, and particularly third-gen genomic investigations, but is it the standardization of collection methods that are problematic or the lack available specimens? Perhaps a combination of both? Per L97, technology changes rapidly and the desired collection procedures are expected to change in concert -
As part of the Bat1K initiative, it is critical that all scientists have a standardized way of collecting the tissue to facilitate sequencing. While sampling efforts for certain taxa are also necessary, the purpose of this manuscript is to get inform those collecting tissues of the optimal and preferred approaches at the time.

Have you explored the application of Biometrica tubes (https://www.biomatrica.com/) room-temp DNA stable tubes (or similar products: DNAstable [Sigma-Aldrich] or Gentregra [Nova Biostorage]). This may be an especially promising options for countries lacking developed cryobanks and repositories.
Thanks for the suggestion. Our team has not yet explored the application of such vials, but we do see their potential. We made note of it in the protocol that these resources exist, with the caveat that we have not validated these materials.

Minor Concerns:
A few additional methodological details will improve clarity:
L144: Has this been tested? **We removed this sentence because it was repetitive with the paragraph below where there are multiple citations.**
L159: define or provide recipe for 'indicator silica' for integration into museums and active collecting agencies
We described indicator silica as the following: "(*i.e.* a type of desiccant made of silica gel beads that changes color when moisture is observed)".

L156-157: Is that data included with this study? If not, it will need a citation. How are you measuring stress?
We added a citation here.
L181: Can you briefly define or clarify what you're calling a 'cell buffer' (or is this a proper noun?) and the concentration/methods of generating the proposed 'agarose plug'
This section was unclear. It is now edited with more clarification.
L282: May benefit from adding cloth, before 'bat bag' to make the manuscript accessible to general collectors or field biologists
Addressed.
L404: Describe the hepatic duct (physical and relative location).
We added the following description of the hepatic duct: "The hepatic duct, which functions to drain bile from the liver, connects the liver to pancreas and small intestine. This vessel is easily identifiable by probing the inferior/posterior portion of the liver and tracing the vessel in a posterior manner. The duct is often a greenish color as well."
L411: collecting only portions of the small and large intestines prevents screening for endoparasites. Is there a reason these parts of the organism should not be examined by a parasitologist in the field? Or taken whole for later examination? **We added two sentences describing the potential to look for endoparasites.**

Additional minor concerns:
L87: Add citation. **Added.**
L134: Add citations for AllProtect and RNAlater. **These are described in the Table of Materials and Reagents.**
L267-270: Based on the way this currently reads, I thought 'This detail' referred to the avoidance of harp trapping at periods of cave emergence, however the sentence reverts back to discussing mist nets - could use some reorganization.
This paragraph was rearranged.
L275-277: Is the enzymatic degradation of tissues post thoracic compression unique to bats?
We clarified this statement.
L289 - can you provide an average time based on bat size?
We clarified length of time based on body size.
L324: (2.2) Higher densities of mitochondrion in muscle tissue may lead to a loss of reads for the nuclear genome.
We added this note.
L332: language switched from 'LN2' to 'liquid nitrogen' - both correct, just maintain consistency.
Corrected.
L333: Citation for brain tissue providing the most HMW DNA?
Citation added.
L340: is ice sufficient or would dry ice or LN2 be preferable or equally beneficial?
The purpose of the ice of is to chill the RNAlater. While the vials are cooling, they should be kept on ice if possible. If dry ice or LN2 is used, then the RNA later will freeze and not sufficiently penetrate the tissue when added.
L351: The location of this cut could use more detail. Further, this dissection method appears to

forfeit all morphological skull characters? Do you have a recommendation for subsampling whole skulls to retain
We added a final step 14 in this section to read: "This protocol forfeits most of the skull. However, teeth, and particularly those from the mandible, can be recovered and may be useful for species diagnostics. Bone tissue can be stored in 1X phosphate-buffered saline (PBS)."
L394: this step of the dissection also destroys morphological characters of the post-cranial skeleton. **We made this apparent and added the note: "This forfeits the skeletal frame. If bone is to be preserved, carefully dissect from skin in muscle and store in 1X PBS after soft tissue dissection is complete."**
L362: additional description of the cribriform plate would be helpful since this bone is critical to orientation. Perhaps adding a photograph? **We added a sentence about this. Visualization of this will be clarified in the filmed portion of the content.**
L601 and L604: degree symbol changes - please check consistency throughout the manuscript. **Addressed.**

Reviewer #2
Major Concerns:
I would have appreciated more discussion about the types of cells likely to be obtained by cell culture (e.g. fibroblasts) and also mention of that fact that the culture method described in the manuscript selects for adherent cells. It is possible that some researchers may be interested in culturing non-adherent cells from different organs and may not want to culture fibroblasts from wing punches. I understand that a protocol may not have been developed for this in bats, but citations of references to primary culture of suspension cells may be useful. Furthermore, no mention is made of one of the major benefits of performing cell culture, namely karyotype analysis, which is important to point out. Also, it was recommended that live cells be counted using an automated cell counter, but this may not be available in many labs, and live/dead cells can also easily be counted using a hemocytometer, which is much cheaper and more widely available, and should therefore be described.
We have added a few sentences in the introduction to comment on this. Edits made in line 268-269 The protocol we provide selects for adherent cells. The combination of the source tissue and growth media we use makes this protocol suitable to select and grow fibroblasts. 689 by using an hemocytometer as described in detail in the reference

Based on the researchers' experience, I would also have been interested in some type of table or sentence describing where they managed to source liquid nitrogen while in a field-type setting, as researchers might find this information useful.
We addressed this in the discussion.

Minor Concerns:
I noted that this manuscript needs some language editing - in general the English usage is good, but there are some odd phrases like '...standardized methods for high quality tissue acquisition and preservation are essential in order to extend these methods BEYOND non-model organisms.'

which means the opposite of what was intended i.e. to extend these methods to non-model organisms. Correction of these errors will improve the clarity of the manuscript.

Corrected. We reviewed the remaining part of the manuscript and tried to improve for clarity.

Supplemental Material for *Journal of Visualized Experiments*

Best practices for tissue collection of bats for -*omics* analyses and primary cell culture

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Protocols for Bat Capture:
All methods described here have been approved by the Institutional Animal Care and Use Committee (IACUC) of Stony Brook University.

Permissions

Collecting material from vertebrates is tightly regulated by law in most countries, and researchers embarking on collecting material must ensure that they follow the correct procedures. Depending on the country, research permissions and visas are often required to gain access to field sites and perform surveys. Additional permits are often needed to cover experimental procedures, and to export and import tissue. Most countries are signatories to CITES (the Convention on International Trade in Endangered Species), which regulates the transportation of material of many plant and animal species across international borders (see below for further details). Similarly, many nations (but not the United States) are adopting the measures set out in The Nagoya Protocol, which forms part of the 1992 Convention on Biological Diversity. The Protocol aims to protect access to genetic resources, and to ensure that countries from where such resources originated share any benefits arising therefrom.

Aside from national regulations, tissue collection is often also regulated within regions, and also within institutions. Many research institutions require researchers to obtain ethics clearance prior to commencing work. Finally, to ensure that the correct permissions have been obtained, and procedures followed, many academic journals now require details of both institutional care committees and permits. As an example of one country's requirements, here we summarize the permissions needed to collect tissues for researchers employed in the United States. Federal regulations require every research institution to have a board (called the Institutional Animal Care and Use Committee or IACUC) to evaluate protocols for handling of any vertebrate, including bats. This is in addition to any permits for research, collection or export that you may need to conduct work in a given state or country. A non-exhaustive, US-based list includes:

1. Regulations require a Centers for Disease Control (CDC) permit to transport any bat tissues both into the United States and within the country.
2. Importation or export of zoological specimens —with the exception of urine or fecal samples— requires a Fish and Wildlife Service (FWS) form to be filed at the port of entry or exit. For importation, this form requires export permits from the country of origin, in addition to detailed information on the quantity and nature of the sample.
3. A United States Department of Agriculture (USDA) permit may be needed if the tissues originate from a species that is known to harbor livestock pathogens. Usually, documentation that specimens come from the wild and are not known to harbor pathogens is sufficient to demonstrate you do not need this permit.
4. The Convention on International Trade of Endangered Species (CITES) regulates the international movement of samples from all CITES-listed species, including a few bat species. Note this is unrelated to IUCN classification, but instead is an internationally agreed-upon list of species. If the species sampled is CITES-listed, a CITES import permit is needed, as well as a CITES export permit from the country of origin of the samples.

Finally, we note that permissions required can change rapidly without notification, and we urge researchers to remain vigilant to stay attuned to these changes. This may require directly calling regulators to ensure the collection and research complies with all relevant regulations.

Capture and handling

A note on minimal safety precautions: All people handling bats should be trained by a bat-competent researcher and vaccinated for rabies with a course of pre-exposure injections (Note: if bitten during handling, a further course of post-exposure injections is still necessary). The use of protective hand-wear such as thick, but pliable, leather gloves is essential. The best compromise between dexterity and protection is to always wear one leather glove, and to use a cotton bat bag to engage the teeth of the bat (taking care that the layers of material are not thin enough that the teeth can go all the way through) and keep the other hand ungloved to disentangle the net.

Bat Capture

1. Set up traps prior to dusk and open the nets immediately prior to bat emergence. Never leave a net or trap open overnight without frequent inspection.

2. During emergence, at dusk, all mist nets must be checked every 10 minutes. Take care to thoroughly inspect the net or trap a bat might be trapped on a corner, or at the bottom and be missed.

3. Carefully remove the bat (Fig. 1) and keep the bat in a bat bag until it can be safely processed. There must be enough bat bags to allow captures to be held singly, and a system to process them so no one bat spends too much time in handling. Hang the bat bags separately from one another, rather than hung in clumps, until ready for processing (Fig. 3). Otherwise, bats can easily overheat or become dehydrated.

4. Fecal matter readily accumulates while bats are in bat bags. Collect in AllProtect or silica gel to keep dry. Wash bat bags prior to, and during the fieldwork campaign. Empty bags before reuse during the night to avoid disease transmission among bats and between bats and researchers.

5. Separate bat bags into the ones containing bats to be released at the end of a session (so that released bats do not get captured again by mist nets before these are taken down), and those to be collected for tissue. Holding bags can be labeled by pen or by strips of paper tucked under the draw strings (Fig. 3), so that the contents of each bag is known without having to re-open. Bags held overnight should be hung out of the reach of animals, away from morning sunshine, and away from air conditioning.

A note on bat care during human handling: Handling is very stressful for wild bats and species vary widely in their fragility. For these reasons it is important to plan ahead to minimize handling and any stress as much as is possible. Most bats should be released as soon as possible at the site of capture. Some of the subsequent steps (sexing, measuring)

can be carried out at the moment of capture if there are enough pairs of skilled hands to do so. If bats need to be held (*e.g*., to release the next day, or for a few hours before dissection), they need to be supplied with water and in some cases food. This is especially critical if bats have been captured before feeding (*e.g*., at dusk). In general, smaller bats are more delicate and will require more energy per unit mass than larger ones. For this reason, and if held for the evening before release or dissection, it may become necessary to supplement food. Water can be supplied to bats in their bat bags by moistening a ball of cotton with abundant fresh water, which also prevents dehydration. Nectar feeding bats (*e.g*., *Glossophaga*, *Lonchophylla*) have high energetic demands and need to be fed even if held for an hour or so. These bats will readily drink a solution of sugar water from a vial or even the top of a bottle. Delicate insectivorous bats, such as *Micronycteris*, may also require feeding. Collecting crickets, moths, grubs, or caterpillars while capturing the bats can help prepare for feeding. The insects need to shake or move (*e.g.*, with dissecting tweezers) to get the bat to eat. Mealworms, either alive or dead but soaked in water, can be used. Small fruit-eating bats can be fed ripe banana. Note that feces from bats that have been fed should not be subsequently used for dietary analyses using DNA.

Measurements, condition, and sex

All bats, including those to be released, should be processed. The following steps are relevant to both bats to be released and those to be dissected.

1. Before taking the bat out of the bag, weigh it. An electronic kitchen scale, or pesolas are the standard for this. After taking the bat out, measure it using a ruler (with a mm scale) or a pair of calipers. The standard mammal measurements are the following: body length (tip of snout to start of tail), tail, foot (base of foot to tip of claw), ear (base to tip), and forearm. This last measurement is often used in diagnosis and cannot be obtained from dry specimens.

2. Age the bat. Bats can be identified as juveniles on the basis of below adult-size forearm length (e.g., when comparing to a field guide). Additionally, they often possess cartilaginous zones between their long finger bones due incomplete ossification, which can be seen by examining the wing against a bright light. In young bats, the knuckles of the finger bones are also typically tapered and the membrane itself feels tacky or sticky to the touch. Young bats that are not fully weaned are sometimes found attached to their mother's body.

3. Sex the bat. Although bats tend to have distinct penises, in some lineages determining sex can be complicated by the presence of a protruding labia, which superficially can be confused with a penis (Fig. 4). Similarly, in some bats of the superfamily Rhinolophoidea the presence of a distended false nipple in the pubic region can, without due care, also be misidentified as a penis. A handheld magnifying lens to look for a longer slit opening the vulva can be used, or if the bat is dissected, check for sex once more at that time.

4. Determine the reproductive status of the bat.

4.1. If the bat is an adult female, check for signs of pregnancy or lactation. Gently feel the abdomen, a hard lump may indicate a fetus.
4.2. Darkened and enlarged nipples surrounded by white mammary tissue are all indicators of late pregnancy/lactation (Fig. 5).
    4.2.1. Pregnant and lactating bats have much greater metabolic demands than others. Release the bat as soon as possible if one is not intending to dissect.
4.3. Determine the status of the testes. Male bats in reproductive condition have particularly large and descended testes (Fig. 6). Usually non-reproductive males do not exhibit easily visible testes.

Figure Legends

Figure S1. Bat bags, many containing recently trapped bats, hanging on a string to keep samples easily sorted. Strips of paper attached to bat bag ties indicate the time of capture and tentative ID to ensure bats are not unnecessarily handled. Photo by Stephen Rossiter.

Figure S2. An example of confusing protruding labia in *Noctilio leporinus*. This bat is a female. Photo by Jon Flanders.

Figure S3. A dark, as opposed to pink, and enlarged nipple indicates lactation in this *Phyllops falcatus*. Photo by Laurel Yohe.

Figure S4. The reproductive condition of males should be noted depending on the state of testes. This bat is in reproductive condition. Photo by Laurel Yohe.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);FigS1_bat_bags.png

Supplemental Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);figS2_noctilio.png

Supplemental Figure 3

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Supplemental Figure 4

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