OCCASIONAL PAPERS

Museum of Texas Tech University

Number 228 *6 October 2003*

PREPARATIONS OF MAMMALIAN KARYOTYPES UNDER FIELD CONDITIONS

Front cover: Karyotype of male *Micronycteris hirsutus* collected from Trinidad.

Preparations of Mammalian Karyotypes under Field Conditions

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Karyotypic preparations that reveal the morphology of chromosomes without chromosomal overlap are valuable for many types of studies, including species identification, systematics, G- and C-band homology, genome organization, identification of rearrangements, fluorescent in situ hybridization resolution (FISH), etc. For many years, our laboratory has been concerned with the production of in vivo bone marrow preparations that are made under field conditions, usually involving wild caught mammals that are immediately processed to produce karyotypic preparations. There have been a number of papers that describe such methods (Hoy and Berlowitz, 1931; Ford and Hamerton, 1956; Patton, 1967; Robbins and Baker, 1978; Lee and Elder, 1980; Baker et al., 1982; Christidis, 1985; Baker and Qumsiyeh, 1988; Hafner and Sandquist, 1989). Over the past few years, we have standardized procedures using disposable products to reduce variation in quality of chromosomal spreads. This paper describes the methods we have found to be most consistently productive. The most significant change to our previous methods concerns incubation time in the hypotonic solution and concentration of cells during hypotonic treatment. The methods described have produced quality results that have served our research interests. Our paper is directed toward preparation of karyotypes of mammals; however, these methods can be adapted for other vertebrates, especially birds and some herptiles. For those embarking on field preparations of karyotypes, a review of the above-cited papers is encouraged.

Additional or other papers are concerned with specific problems such as blaze-drying spreads (Scherz, 1962), increasing the mitotic index (Lee and Elder, 1980), as well as, karyotyping postmortem individuals (Hafner and Sandquist, 1989). Chromosomal banding techniques are reviewed in Sumner (1990). An historical perspective of cytogenetics in mammals is provided by Baker and Hafner (1994) and Hsu (1979). The book by Hsu (1979) is difficult to locate, but it certainly is a pleasure to read.

Herein, we describe our procedures in highlighted steps and then discuss issues that relate to each step in paragraphs referenced back to those procedures. We also include a list of supplies, equipment, and chemicals needed for a field trip where karyotypes are prepared.

Methods

Yeast stress to increase mitotic index. Where possible, we employ the yeast stress method described by Lee and Elder (1980). A 1:1 mixture of dry baker's yeast and sugar is diluted 1:7 with H_2O, shaken to dissolve, and incubated at human body temperature (inside the belt/waistband of the karyotyper) until the yeast is active. Then the specimen is injected subcutaneously in the hip or shoulder with 0.1cc of the yeast culture on day one. A second equal injection from a fresh yeast and sugar mixture is made 24 hours later. The specimen is sacrificed 48 hours after the first injection. Sites of injection are varied between the two injections.

Mitotic inhibitor. Animals are injected intraperitoneally with vinblastine sulfate (Velban) at a concentration of 0.02% (10ug in 50ml of dH_2O). The amount injected depends on the weight of the specimen. Very small individuals, such as bats that weigh about 5g, would be injected with 0.03ml. A 10-15g individual would be injected with 0.01ml per 2g of body weight. Larger individuals would be given a maximum of 0.15ml, even for a large skunk, squirrel, raccoon or opossum.

In vivo incubation time. For rodents, phyllostomid bats, shrews and other species that have a fairly stable body temperature, we obtain best results with a Velban treatment of 45 minutes to an hour. Incubation times from 30 minutes to 2 hours produce acceptable results. For vespertilionid bats, hedgehogs or other species that go into a deep torpor, arousal of specimen and continued activity may be necessary to improve the mitotic index.

Hypotonic solution and aliquots. For hypotonic swelling of mitotic cells, we use 0.075M KCl in a 15ml disposable centrifuge tube with a screw cap. For several minutes prior to sacrifice of the individual, the centrifuge tube containing the hypotonic solution is kept inside of the belt next to the body of the karyotyper to ensure that the temperature of the hypotonic solution approaches human body temperature.

The volume of the hypotonic solution relative to the amount of bone marrow is important. For a 3-5g bat, the optimal volume of hypotonic solution is 3ml, even if the bone marrow is flushed from both humeri. For larger bats or rodents that provide a larger volume of bone marrow, the volume of hypotonic solution would be increased to 10ml or more, and if both humeri (or femora) are used, then separate tubes with the same or variations of the amount of hypotonic solution can be used.

Sacrifice and tissue collection. The individual is sacrificed by means that have been approved by the Animal Use and Care Committee for the university or governing body that approves research protocols. Immediately after the animal has expired, the humerus of the bat or the femur of the rodent, marsupial, etc. is removed and cleaned of muscle tissue. The proximal and distal ends of the bone are removed without disruption of the red marrow pellet and the bone marrow is flushed directly into the centrifuge tube with a subset of the warmed hypotonic solution that has been drawn into a syringe fitted with a needle that matches the internal diameter of the bone. Different sized needles are required: a 25 or 23 gauge needle works well for small bones and a 21 or 18 gauge needle works well for larger bones. Seal one end of the bone by inserting the end of the needle and squeezing the junction of bone and needle between the thumb and the index finger.

Aspiration of bone marrow. Immediately after the bone marrow has been flushed out, the cell suspension is aspirated vigorously with a disposable plastic transfer pipette (Fisherbrand Transfer Pipets Disposable Polyethylene, Cat. No. 13-711-7) to break up the bone marrow into a single cell suspension. Roughness with the cell suspension is permissible at this time.

In vitro incubation of bone marrow cells. After aspiration, the cap is replaced and tightened and the centrifuge tube is incubated for 14 minutes against the body of the karyotyper.

Adding Carnoy's fixative. After incubation, a full transfer pipette (about 2ml) of freshly mixed Carnoy's fixative (3 parts absolute methanol: 1 part glacial acetic acid) is added to the cell suspension solution by slowly introducing the fixative at the bottom of the centrifuge tube. This solution is gently mixed until all fixative has been dispensed from the pipette.

Centrifugation. The cell suspension is centrifuged for 2 minutes at 1000-2000 rpm.

Washing the cell button. The supernatant is decanted and approximately 3ml of fresh fixative is added to the cell button by pipetting down the side of the centrifuge tube. The cell button is gently resuspended. Rough or vigorous pipetting will rupture metaphase cells that may provide excellent karyotypes if they remain unbroken.

Centrifugation, washing of cells and removal of water from the cell suspension. Repeated 2 or 3 more times. Critical to producing karyotypes that have the chromosomes spread out and non-overlapping is the removal of the water that swelled the cell during the hypotonic treatment. This removal of water from the cell is accomplished by replacing the hypotonic solution with multiple washes of fixative. Ultimately the cell should remain swollen but be filled with non-hydrated fixative.

Final volume of fixative relative to concentration of cells. After decanting the supernatant for the third time, the volume of the cell button is appraised and fresh fixative is added in a volume that results in a cell suspension that produces the proper concentration of cells on a microscope slide when 2 drops of the suspension are blaze-dried.

Preparation of slide. High quality microscope slides are hand cleaned with Kimwipes to remove grease or other contaminants. Slides are then labeled with pencil on frosted end and typically, we make one slide that is stained with Giemsa and used for initial observations in the field.

Two drops of cell suspension are dropped onto a cleaned microscope slide. The drops are placed on the slide about 1.5 cm apart and the cell suspension is allowed to spread over the surface until it reaches the edges of the slide. At the time the suspension reaches both sides of the slide, the cell suspension is ignited by a flame while holding a match beneath the slide, but with the flame reaching the top surface of the slide so that the methanol ignites. The cell suspension is allowed to burn until the flame quenches itself. At that time, any remaining droplets of glacial acetic acid can be permitted to dry or removed by flinging them from the surface of the slide with a flick of the wrist.

Staining of slide. Phosphate buffer (35-40ml) and a transfer pipette (2ml) of stock Giemsa solution are placed in a plastic Coplin jar and properly mixed by aspiration. The flame-dried, freshly prepared slide is placed in this stain mixture for 8 minutes. After 8 minutes, the stain is driven from the Coplin jar by adding distilled water to the jar until all traces of Giemsa are undetectable in the solution. At this stage, the slide is removed from the Coplin jar and placed on a paper towel to dry. After drying, the slide can be examined for quality and results. Slides should be placed in a slide box and protected from dust until returned to the laboratory.

Transfer and storage. The remaining cell suspension is then transferred to a 2ml Eppendorf tube, which has been properly labeled on the side and the top of the tube. The Eppendorf tube is sealed and wrapped in parafilm. The cell suspension is maintained at room temperature and returned to the laboratory where it is stored in a –20° Celsius freezer until additional slides are needed.

DISCUSSION

Yeast stress. This method greatly increases the mitotic index. We have found the method described by Lee & Elder (1980) works very well when the specimen is adapted to a controlled laboratory environment and is able to eat and drink. We have had trouble with bats even when they were adapted to being caged and are eating plenty of food. Bat specimens typically die within 24 hours if yeast is injected under the skin of the body. We have had some success by adding a small amount of yeast to form a bubble under the skin of the forearm. We seldom attempt to yeast stress a bat in the field because the failure rate is too high. We also have had problems yeast stressing *Monodelphis* and species such as shrews that frequently do not survive the use of this method. We often must adapt the treatment to accommodate a species' individual response to stress.

Mitotic inhibitor. Over the years, we have continually reduced the amount of vinblastine sulfate injected into a specimen. If too much mitotic inhibitor is given, the chromosomes over contract and are more difficult to work with. This is especially important for G-banding and in situ hybridization. Tightly contracted chromosomes result in the loss of fine details and less resolution of banding patterns. Other mitotic inhibitors, such as colchicine, can be substituted.

For *Reithrodontomys humulus* and for some pocket gophers of the genus *Geomys*, the chromosomes of most spreads are over contracted even without Velban treatment. The point of the above discussion is that you may have to adapt your methods to the biology of individual species.

In vivo incubation time. Because normal karyotyping of multiple animals under field conditions does not result in easy application of a precise in vivo incubation schedule, the time of in vivo incubation will vary. For most species, after 2 hours the chromatids of metaphase chromosomes begin to separate too much and in most cases where the time of incubation for a specimen is beyond 2 hours, the karyotypic preparations are compromised. As long as the incubation time is kept between 30 minutes and 2 hours, the preparations are adequate. We try to have an in vivo time of about 1 hour.

Hypotonic solution. Our results indicate that for this step, the temperature approaching that of human body temperature is critical. The proper time of incubation in hypotonic and the concentration of bone marrow cells relative to the volume of hypotonic are also important. Of these three, the two most important are the incubation time and the concentration of bone marrow cells. The in vitro time should be near 14 minutes and not much longer.

If cell buttons will be archived for future use, preparing a large cell button in 2 or more separate tubes can be advantageous. However, if the goal is to produce a maximum of 2 or 3 slides, then the treatment of large quantities of bone marrow has no advantage.

Sacrifice of individual. The issue here is to be sure that your Animal Use & Care Committee approves of the methods that you are employing and that you have a valid approval number for your protocol. Anaesthetization of the individual prior to sacrifice is possible and this certainly resolves issues relative to pain.

For bats, try not to cut the humerus too close to the elbow joint in order that the forearm can be properly measured on the voucher specimen. Examination of the femur or humerus often reveals areas of red bone marrow as well as yellow bone marrow. The desired mitotic cells are present in the red bone marrow, and it's best not to flush out the yellow bone marrow if possible. For each animal, concentrate on flushing the red bone marrow if it is visible in the shaft of the bone.

Aspiration of bone marrow. Immediately after the bone marrow is flushed into the hypotonic, the cells are not swollen and can be handled roughly. Assuming you do not slosh the hypotonic out of the tube, being too rough on the cells with the transfer pipet may be difficult. Nonetheless, the goal is to be just rough enough so that the bone marrow is separated into individual cells. Large chunks of muscle or bone that inadvertently appear in the hypotonic should be removed with the pipet at this time.

In vitro incubation of bone marrow cells. The shift to 14 minutes is a significant change in our protocol and although longer time periods some times give good results for rodents, the 14-minute incubation works really well for all species that we have tried. For bats, we experimented with incubation periods of 20 minutes to 35 minutes and found that all of these produced substantially fewer spreads than the 14-minute incubation. When we shortened the incubation period to 14 minutes, we increased the mitotic index and even then, there are a number of cells that are swollen to a point of fragmentation. Every effort is made to keep the incubation time between 12 and 16 minutes. This level of incubation seems to produce consistently reasonable results.

Adding Carnoy's fixative. After hypotonic incubation, the cells are extremely fragile and all pipetting and centrifugation etc. should be conducted with care in order not to break the cells. We typically add Carnoy's fixative to the bottom of the centrifuge tube and gently disrupt any material that has settled at the bottom of the tube. Mixing the hypotonic and fixative reduces the likelihood that cells will clump together. Sometimes at this stage, there is floating cellular debris on the top of the hypotonic solution that often contains lipids and cellular matrix that can be pipetted and removed from the hypotonic solution. Pipetting this material results in a cleaner cell button when slides are prepared.

Carnoy's fixative. When spreads are not being produced so that each chromosome can be observed, one potential problem is the fixative. The fixative needs to be made fresh for each 3 or 4 animals and is never allowed to sit more than 30 minutes or so. If either the methanol or the glacial acetic is not of high quality, or if it has been exposed to air in a moist environment and allowed to hydrate, the fixative may not produce high quality spreads. Exposure to environment can be reduced by covering the container with parafilm or by plugging the top of the container with a Kimwipe. Slides of spreads that have not been properly fixed often appear to have cytoplasm associated with the cell and when stained with Giemsa, pick up a fairly strong blue color. The outline of individual chromosomes is fuzzy in improperly fixed cells.

Centrifugation. Longer periods of centrifugation theoretically might break the cells, but if this is true, breakage is not evident in our work. The main issue is to make sure that you have produced a cell button that will remain at the bottom of the centrifuge

tube when you decant the supernatant, but not compacted to the point where resuspension cannot be achieved with gentle aspiration. We prefer a swinging bucket centrifuge rather than a fixed angle rotor because if the centrifuge tube is rough, some cells may break when they contact and slide down the sides of the tube in a fixed angle rotor centrifuge wash. Our preferred method of karyotyping involves an electric centrifuge. Sometimes, however, electricity is not available and at these times, a hand-cranked centrifuge works very well. Such a backup in the kit can be useful.

Washing the cell button. Again, the cells are very fragile and care must be taken not to break them.

Centrifugation, washing of cells and removal of water from the cell suspension. If the cell button appears white and the supernatant is clear when viewed in the tube, then multiple repetitions may not be necessary. However, fixation must be completed.

Final volume of fixative relative to concentration of cells. This hit or miss process is best appreciated after considerable experience. One good thing about the process is that if cells are too concentrated, or alternatively, not concentrated enough on the microscope slide, it is possible to centrifuge the cell button again and to resuspend the cell button in a volume of fixative that produces a more appropriate concentration of cells. If cells are too concentrated on the slide, this results in chromosomal spreads that are too clumped together and this condition can be alleviated to some extent by reducing the concentration of cells in an increased amount of fixative before it is applied to the slide.

Preparation of slide. If necessary, ethanol (95% concentration) can be used to clean microscope slides.

Staining of slide. The temperature of the stain can affect quality of the spread. Best results are obtained at 30°C (70°F), but if stain is too weak a warmer temperature can increase the staining intensity. Warmer temperatures however result in greater background on the slide. The water that is used to flush the stain after 8 minutes can be tap water if distilled water is in short supply. The reason that the slide is not directly removed from the stain is because there is often a film of stain floating on the surface of the staining solution and if this film attaches to the slide it can disrupt viewing of major regions of the slide. By flushing the stain from the Coplin jar before the slide is removed, then the film is not present to attach to the slide.

The volume of stain in the Coplin jar is best limited to the non-labeled portion of the slides being stained. If there are 10 slides in a Coplin jar, considerably less volume of phosphate buffer is needed to cover the working portion of the slide than if only a single slide is being stained.

Transfer and storage. If cell suspensions are carried on an airplane, a common problem is that the liquid in the Eppendorf microcentrifuge tubes leaks. The best solution that we have found is to make sure that the cell suspensions are upright so that if there is a loss of volume from the Eppendorf, it is air rather than cell suspension. The total volume of air in the Eppendorf is best kept at a very low level even if that means that cell suspensions will have to be recentrifuged and concentrated once slides are made after returning to the laboratory. At this time, tubes should be checked for adequacy of labeling to ensure that the numbers and information are legible.

Other issues related to karyotyping. (1) Two chemicals (glacial acetic acid and absolute methanol) that are required for karyotyping are not allowed on commercial airlines. Because many field trips involve flight to field locations, preplanning and proper arrangements are essential for ensuring that these high quality chemicals will be available at the field site. Usually a colleague or collaborator located in the country of destination can purchase these if sufficient time is provided. In the case of a crisis, we have found these chemicals in hospitals and/or universities, although this usually has a low probability of success.

(2) Because of customs and regulations, possessing letters to document the ownership of the karyotyping kit, centrifuges, microscopes, etc. is strongly recommended. Letters documenting collaborations and agreements, collecting permits, and approved animal use and care protocols are also valuable assets while conducting research.

Voucher specimens. We do not feel that it is possible to overstate the case relative to the need of voucher specimens to be archived in an accredited museum collection. Further, our history indicates that it is extremely difficult to adequately cross-reference voucher specimens to microscope slides, cell buttons, tissues archived for DNA and other studies, but necessary to ensure the value of research efforts.

ACKNOWLEDGMENTS

For editorial assistance, we thank John Bickham, Hugh Genoways, Joyce Genoways, Mary Maltbie, Jim Patton, Calvin Porter, and Brenda Rodgers. We acknowledge Marcy Revelez for field assistance. We acknowledge the field parties for the 2001 Sowell Expedition to Ecuador and Honduras, and the 2000 Field Methods course led by Robert Bradley to Mexico. Supported in part by Texas Tech University, the Biological Database line item and the NIH grant GM38737 to Holly Wichman.

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Karyotyping Kit List

As listed, the kit will karyotype 50-100 individuals. The limiting factors are the available volume of absolute methanol and glacial acetic acid and the number of 15 ml falcon tubes included in the kit.

_____1 double decker fishing tackle kit, hard-sided (24 x 38 x 53 cm, or larger)
_____2 plastic boxes with snap lids that fit in kit (Plano Tackle Box System)* for Falcon and Eppendorf tubes
_____10 - 1cc tuberculin syringes
_____10 - 3cc syringes
_____2 - 10cc syringes
_____5 of each: 25, 23, 22, 21, 20, 18, 16 gauge needles
_____Timers and batteries
_____50 ml tube of 1:1 dry baker's yeast: sugar mixture
_____2 prong-3 prong plug adapter
_____Ketamine
_____Bone/wire cutters
_____Scissors – variety of sizes including Joyce Chen kitchen scissors
_____Forceps
_____Wine cork with needles
_____Flashlight
_____Permanent ink pens (Sharpies, Uniballs)
_____Pencils
_____Styrofoam or plastic 15 ml tube rack
_____Styrofoam or plastic 2 ml Eppendorf tube rack
_____2 boxes of Microscopes Slides
_____Tape for labeling tubes etc.
_____Matches
_____Plastic Coplin jar with screw lid (glass is acceptable, but not preferred)
_____Stock Giemsa stain – 250 ml round plastic jar
_____3 – 500 ml amber rectangle plastic bottles for absolute methanol (filled when appropriate)
_____1 – 500 ml opaque rectangle plastic bottle for glacial acetic acid (filled when appropriate)
_____1 – 250 ml wash bottle filled with 95% EtOH
_____200 - Disposable plastic transfer pipettes (extras elsewhere stored in quart size plastic bags)
_____balance for weighing (electronic preferred)
_____Kimwipes
_____1 L rectangle plastic bottle for KCl
_____1 L rectangle plastic bottle for PO_4 buffer (fill line marked, not packed in kit, but used once buffer is mixed)
_____KCL (5.58 g units in Eppendorf tubes to mix 1L)
_____PO_4 buffer – pH 7.0 - 7.2. Mix 0.469 g NaH_2PO_4 and 0.937 g NA_2HPO_4 in 1 L of distilled water. Extra buffer salts weighed to the above proportions are stored in Eppendorf microcentrifuge tubes.
_____50 – 15 ml Falcon conical centrifuge tubes with caps (in plastic box with snap lid, extras elsewhere)
_____100 - 2 ml flat top Eppendorf microcentrifuge tubes (in plastic box with snap lid, extras elsewhere)**
_____1 – 50 ml Falcon conical tube to mix Velban
_____Velban – 1 mixed (10 ug to 50 ml water), 1 10 ug ampule of lyophylized powder
_____Sterile H_2O to mix Velban
_____2 - 30 ml empty sterile serum vials from American Pharmaceutical Partners, Inc. for mixed Velban
_____50 ml graduated cylinder – plastic (preferably cut down from 100 ml)
_____Parafilm
_____Gallon size Ziploc freezer bags
_____Quart size Ziploc freezer bags

In addition: surge protector strip, centrifuge with 15 ml rotor, extra methanol (70 animals per Liter) and acetic acid (200 animals per Liter), slide boxes, freezer boxes for 2 ml Eppendorfs for cell buttons, paper towels, extension cord, microscope optional, materials needed for record keeping, sheets of absorbent bench paper, and labels and tags for tubes and voucher specimens.

*Various sizes with dividers suitable for needles, syringes, Eppendorf tubes, KCL and buffer aliquots, etc.

**Extra space will be needed to accommodate all supplies listed above. An additional tackle or storage box to organize extra materials is recommended.

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Layout and Design: Jacqueline B. Chavez
Cover Design: Robert J. Baker

This book was set in Times New Roman and printed on acid-free paper that meets the guidelines for permanence and durability of the Committee on Production Guidelines for Book Longevity of the Council on Library Resources.

Printed: 6 October, 2003

Library of Congress Cataloging-in-Publication Data

Occasional Papers, Number 228
Series Editor: Robert J. Baker

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ISSN 0149-175X

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