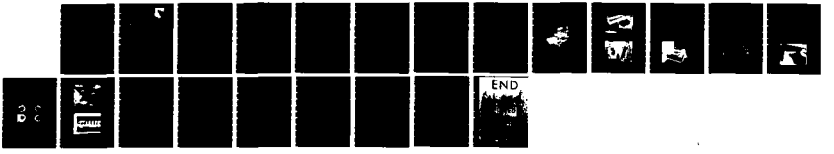
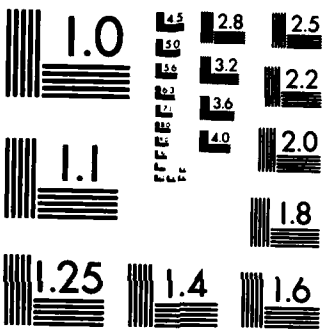
AD-A137 010 CRYOMICROTOME APPLICATIONS: TECHNIQUES FOR THE STUDY OF 1/1
SKELETAL MATERIAL..(U) AIR FORCE AEROSPACE MEDICAL
RESEARCH LAB WRIGHT-PATTERSON AFB.. K C SMITH ET AL.
UNCLASSIFIED SEP 83 AFAMRL-TR-83-074 AFAMRL-TR-83-074 F/G 14/2 NL





MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AFAMRL-TR-83-074

AD A137010

CRYOMICROTOME APPLICATIONS: TECHNIQUES FOR THE STUDY OF SKELETAL MATERIALS

KATHERINE C. SMITH
CLARENCE M. OLOFF
LEON E. KAZARIAN

SEPTEMBER 1983

DTIC FILE COPY

Approved for public release; distribution unlimited.

AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
AIR FORCE SYSTEMS COMMAND
WRIGHT-PATTERSON AIR FORCE BASE, OHIO 45433

NOTICES

When US Government drawings, specifications, or other data are used for any purpose other than a definitely related Government procurement operation, the Government thereby incurs no responsibility nor any obligation whatsoever, and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data, is not to be regarded by implication or otherwise, as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use, or sell any patented invention that may in any way be related thereto.

Please do not request copies of this report from Air Force Aerospace Medical Research Laboratory. Additional copies may be purchased from:

National Technical Information Service
5285 Port Royal Road
Springfield, Virginia 22161

Federal Government agencies and their contractors registered with Defense Technical Information Center should direct requests for copies of this report to:

Defense Technical Information Center
Cameron Station
Alexandria, Virginia 22314

TECHNICAL REVIEW AND APPROVAL

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

James C. Rock

JAMES C ROCK, LT COL, USAF, BSC
Associate Director
Biodynamics & Bioengineering Division

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER: AFAMRL-TR-83-074	2. GOVT ACCESSION NO.: AD-A137010
	3. RECIPIENT'S CATALOG NUMBER
4. TITLE *(and Subtitle)*: CRYOMICROTOME APPLICATIONS: Techniques for the Study of Skeletal Materials	5. TYPE OF REPORT & PERIOD COVERED: Technical Report
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): Katherine C. Smith, Clarence M. Oloff, Leon E. Kazarian	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Air Force Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson AFB, Ohio 45433	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 7231-14-16, 62202F
11. CONTROLLING OFFICE NAME AND ADDRESS	12. REPORT DATE: July 1983
	13. NUMBER OF PAGES: 17
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)*: Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

AFAMRL CONTACT: Ms Katherine C. Smith, AFAMRL/BBD, Ext 513 255 3242

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

Cryomicrotomy
Histology
Skeletal System
Hard Tissue Analysis

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The LKB 2250 PMV Cryomicrotome is a large sledge-type microtome designed for sectioning both undecalcified bone and specimens of large size. The maximum specimen size is 150 X 450 mm, sufficient for an entire rabbit or small monkey.

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

Freshly harvested specimens are quick-frozen in a solution of hexane cooled with dry ice and can be prepared for immediate sectioning or stored at -20°C without adverse effects until they are sectioned.

Sections collected for staining and histology are cut at a thickness of 10-12 um and are collected on transparent tape. The sections are fixed, allowed to dry, and then stained by an appropriate procedure. Stained sections remain on tape and are mounted on glass slides using Euparal as the mounting adhesive.

Sections suitable for scanning electron microscopy are cut at 75-100 um and freeze-dried for approximately 24 hrs. Tissue to be studied is mounted on aluminum pin type mounts and sputter-coated with a 100 nm layer of gold. Specimens are observed and photographed using an Autoscan Scanning Electron Microscope.

Photography is an additional procedure used with the cryomicrotome. A view camera is set up to take 4 X 5 inch color negatives of the top of the frozen specimen block. The negatives are reproduced as 8 X 10 inch color prints and are highly detailed photographs showing gross anatomy and structural geometry of the specimen through the entire plane of sectioning.

The LKB 2250 PMV Cryomicrotome has a wide range of applications that allows the operator to combine a variety of techniques to extract a great deal of information from a single specimen.

PREFACE

This work was performed in support of work unit 72311416 "Biomaterials / Kinematics Research" at the Biodynamic Effects Branch (BBD), Biodynamics and Bioengineering Division, Air Force Aerospace Medical Research Laboratory. The assistance of the Technical Photography Division (4950/RMP) is greatly appreciated.

Summary

This report outlines procedures used with the LKB 2250 PMV Cryomicrotome.

Techniques discussed involve specimen preparation, specimen sectioning for both light and electron microscopy, photographic procedures and techniques for sectioning plastic embedded specimens, now being developed.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure

INTRODUCTION

The LKB 2250 PMV Cryomicrotome (LKB Inc. Rockville, Maryland) is a large sledge-type microtome designed for sectioning both undecalcified bone, and specimens of large size. The largest freezing frame is 150mm X 450mm and will accommodate, for example, an entire rabbit or small monkey.

An LKB 2250 Cryomicrotome is in use at the Biodynamic Effects Branch, Biodynamics and Bioengineering Division of the Air Force Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio (Fig. 1). The Biodynamic Effects Branch is involved in research in the areas of biomaterials and biokinematics especially as related to the skeletal system and the unique capabilities of the LKB cryomicrotome make it a valuable tool for studies of this type.

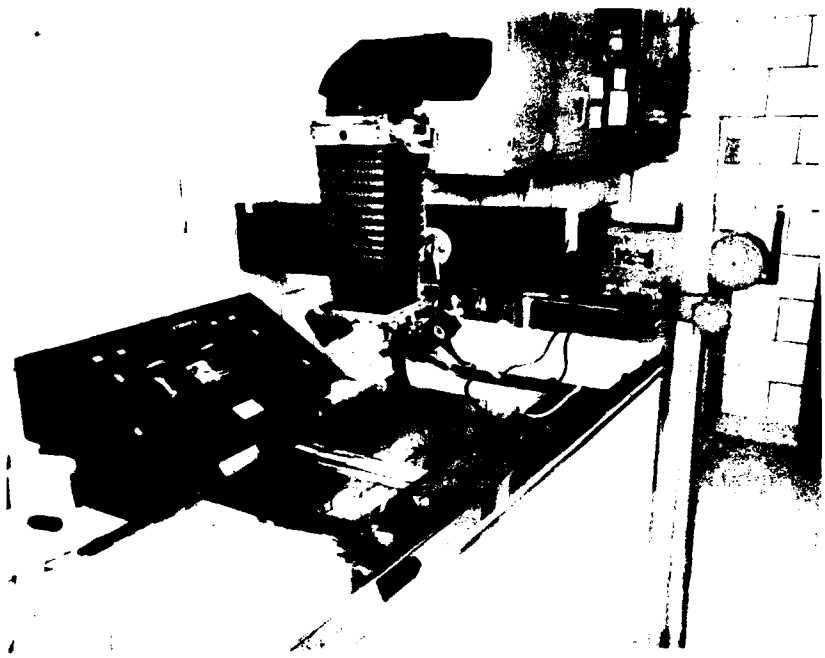


Fig. 1 View of the LKB 2250 PMV Cryomicrotome, including the control panel and photographic set-up.

SPECIMEN PREPARATION AND FREEZING

The cryostatic unit of the LKB 2250 has a temperature range of -5°C to -30°C and for most purposes the cabinet temperature is kept at -20°C.

Immediately after they are harvested specimens are quick frozen in hexane cooled with dry ice at a temperature of approximately -70°C. Soft tissue quality is greatly affected by freezing time as slower freezing results in greater ice crystal formation and thus damage to the tissue. Once quick-frozen, specimens can be prepared for sectioning immediately or they can be stored at -20°C without damaging effects until they are sectioned.

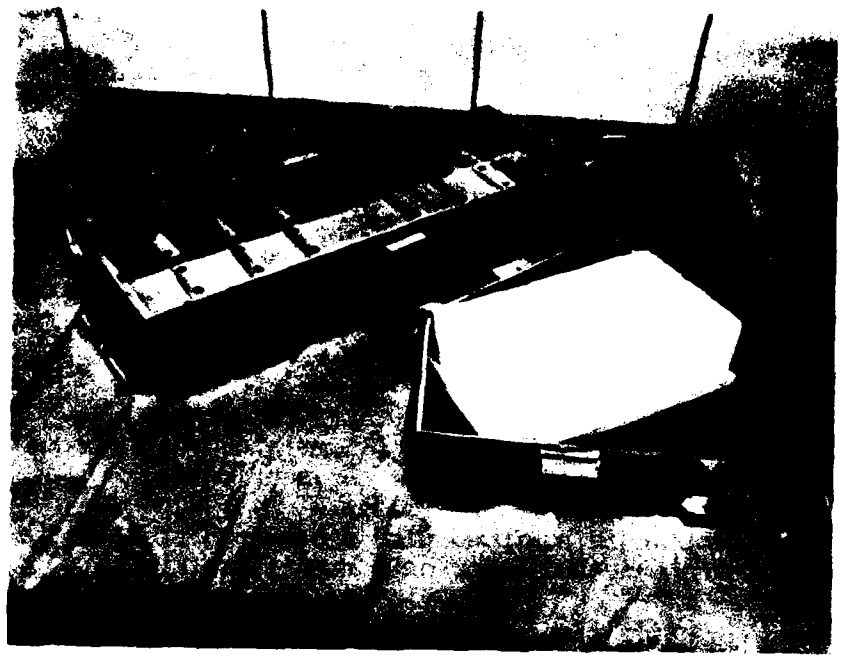


Fig. 2 An example of the freezing frames and stages used with the cryomicrotome. Right: 200 X 120 mm stage with frozen carboxmethyl cellulose block attached. Left: 450 X 150 mm stage and freezing frame.

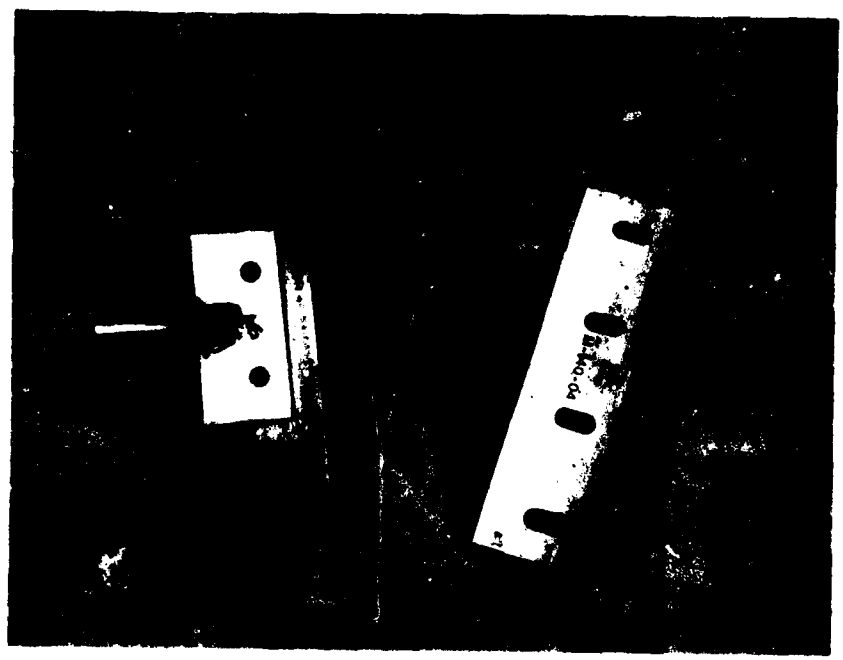


Fig. 3 Right: Steel knife used for sectioning hard tissues. Left: knife attached to holder of the Shandon Autosharp 5 microtome knife sharpener.

In preparation for sectioning, specimens are embedded in a viscous solution of cold sodium carboxymethyl cellulose (CMC) (3). The viscosity of the CMC solution can be varied according to specimen composition (hard vs. soft tissue) in order to produce a frozen block with approximately the same hardness throughout. Generally a 20-25 ml% (V/V) aqueous solution of CMC is used in this laboratory with good results. Several microtome stages with removable freezing frames are available from the manufacturer, ranging in size from 150 X 450 mm down to 20 X 40 mm, and will accommodate a variety of specimens (Fig. 2).

The microtome stage with freezing frame attached is lowered into the quick-freeze solution slowly to allow the CMC block to freeze completely from the bottom up and from the sides inward. After the block is completely frozen, it is removed from the hexane and stored in a plastic bag inside the cryomicrotome chamber. Generally, the block is left overnight to allow the temperature to equilibrate with that of the cryo-chamber. The stage is firmly attached to the microtome sledge by bolts (150mm X 450 mm frame), or by a ball joint system (all other frames) to prepare for sectioning.

The LKB cryomicrotome is equipped with a hardened steel knife that is designed for sectioning bone and other hard materials (Fig. 3). Knives are available with either a 20° or 35° facet angle, with the 35° angle recommended for use with bone. A knife resharpening service is offered by LKB Inc., however, a Shandon Autosharp 5 microtome knife sharpener (Shandon Southern Instruments Inc., Sewickley, Pennsylvania) with a modified knife holder is used in this laboratory with very good results (Fig. 4).

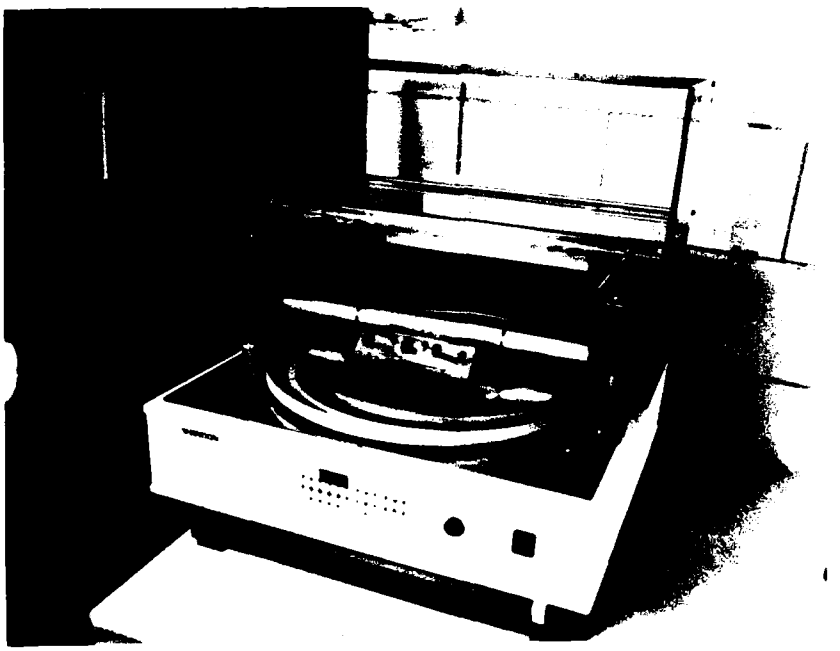


Fig. 4 The Shandon autosharp 5 microtome knife sharpener with knife in position for sharpening.

SPECIMEN SECTIONING

LIGHT MICROSCOPY. The knife feed control on the LKB cryomicrotome regulates section thickness. The control can be set from 1 to 999 μm in 1 μm increments, however, when sectioning undecalcified bone the maximum thickness is approximately 300 μm. Attempts to cut thicker sections, especially of cortical bone, can result in damage to the microtome knife edge.

Sections collected for staining and histology are cut at a thickness of 10-12 μm and are collected on transparent tape (3M type 688, 800 or 810) (Fig. 5). Specimens generally have not been fixed prior to sectioning so they are fixed soon after they are collected (1). The type of fixative used is very important; alcohol tends to cause contraction of tissue while acetic acid causes expansion especially of collagen (7). After trying a variety of fixative solutions, the following fixative (15) was chosen to be most suitable for the sections cut in this laboratory:

Acetic-Alcohol-Formalin

95% ethyl alcohol	70 ml
10% formalin	29 ml
glacial acetic acid	1 ml

Sections are placed in the fixative for approximately 30 seconds and then gently rinsed in tap water. The sections are allowed to dry thoroughly before they are stained.

Fig. 5 A fixed, unstained 10 μm section of spinal column, collected on tape.

Most histological staining procedures can be easily adapted for use with sections cut on the cryomicrotome. In general, it is desirable to use a fairly rapid staining sequence employing aqueous stains. Although the tape used for specimen collection can withstand a wide variety of chemicals, it cannot be exposed to xylene or similar compounds because this results in breakdown of the adhesive.

The majority of stains used in this laboratory are specific for bone and related connective tissue. The following stains are routinely used (see appendix): Mallory-Heidenhain stain for connective tissue, von Kossa method for calcium, Alcian Blue method for acid mucopolysaccharides, Picro Ponceau with Hematoxylin for collagen, Safranin O-Fast Green for cartilage and routine Hematoxylin-Eosin. It is not necessary to rehydrate sections prior to staining because the tissue is fresh-frozen and has not been dehydrated. After staining, sections are dehydrated in a graded series of alcohol (50%, 70%, 95%) prior to mounting on slides (Fig. 6). The mounting adhesive used is Euparal, a mixture of oil of eucalyptus, gum sandarac, salol, paraldehyde, menthol and camphor. As mentioned previously, xylene affects the tape on which the sections are collected and for that reason xylene-based mounting media must be avoided. A thin layer of adhesive is spread directly on the slide and the trimmed tape containing the stained section is placed on the slide, with the section facing up. Additional Euparal is dropped onto the section with an eye-dropper or pasteur pipet and the cover glass is slowly lowered onto the slide. The adhesive must be allowed to spread out in a continuous layer under the cover glass as it is being lowered or bubbles tend to form under the cover glass. Slides are allowed to dry for approximately one week before they are placed in slide boxes for storage.

Fig. 6 A stained 12 μm mid-sagital section of spinal column, alcian blue.

SCANNING ELECTRON MICROSCOPY. Sections for scanning electron microscopy are cut at a thickness of 75-100 μm and as before, sections are collected on tape. As the sections are collected, they are left in the cryo-chamber to freeze-dry, usually for 24 hours. Normally the LKB 2250 cryomicrotome goes through an automatic defrost cycle every 12 hours, however, it is possible to override this by using the Dehydration button on the control panel. The cryo-chamber is thus prevented from defrosting and the specimens are maintained in a cold, dry environment during dehydration. After dehydration is complete, the sections and tape are trimmed to the appropriate size and mounted, tissue up, on aluminum pin type mounts coated with conductive carbon paint (Fig. 7). The sections are further dehydrated and then sputter-coated with a 100 nm layer of gold in an EFFA vacuum evaporator (E. F. Fullam, Inc. Schenectady, NY). The coated specimens are observed and photographed using an Autoscan Scanning Electron Microscope (Perkin-Elmer ETEC, Hayward, CA) and Polaroid Type 55 positive/negative film.

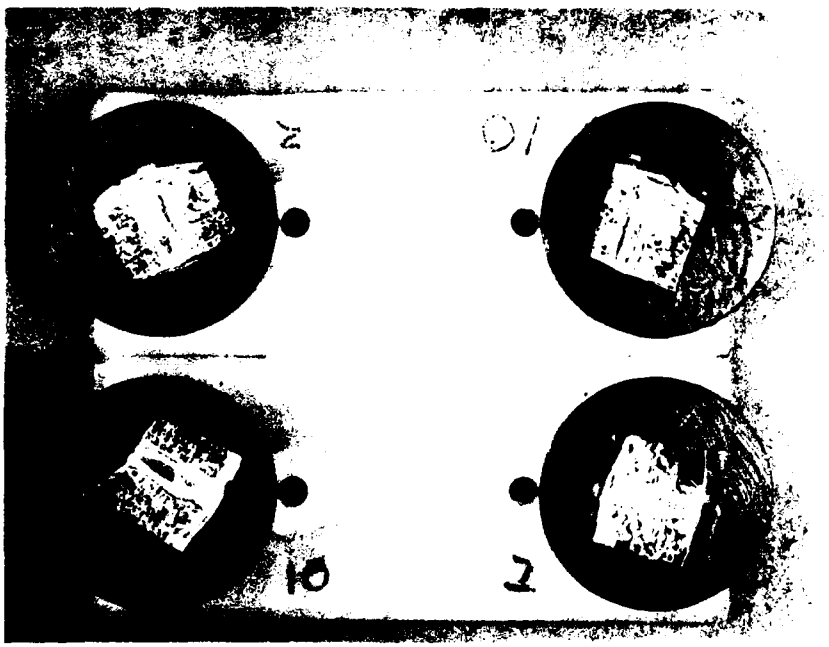


Fig. 7 Trimmed freeze-dried, 100 μm sections of intervetebral discs and adjacent vertebral bodies. Specimens are mounted on aluminum pin type mounts coated with conductive carbon paint

PHOTOGRAPHIC PROCEDURE

Photography of the sectioned block face can be used as an alternative or an adjunct to the collection of sections (13, 14). A view camera with an 8 1/2 inch lens, mounted on an adjustable stand, is positioned above the sliding windows of the cryo-chamber and focused on the top of the section block (Fig. 8). The camera is set up to take either 4 X 5 inch Polaroid photographs or 4 X 5 inch color negatives. Polaroids are generally taken only to ensure that the camera focus and exposure settings are correct. Color negatives are taken at predetermined intervals as the block is sectioned, for example, every time the

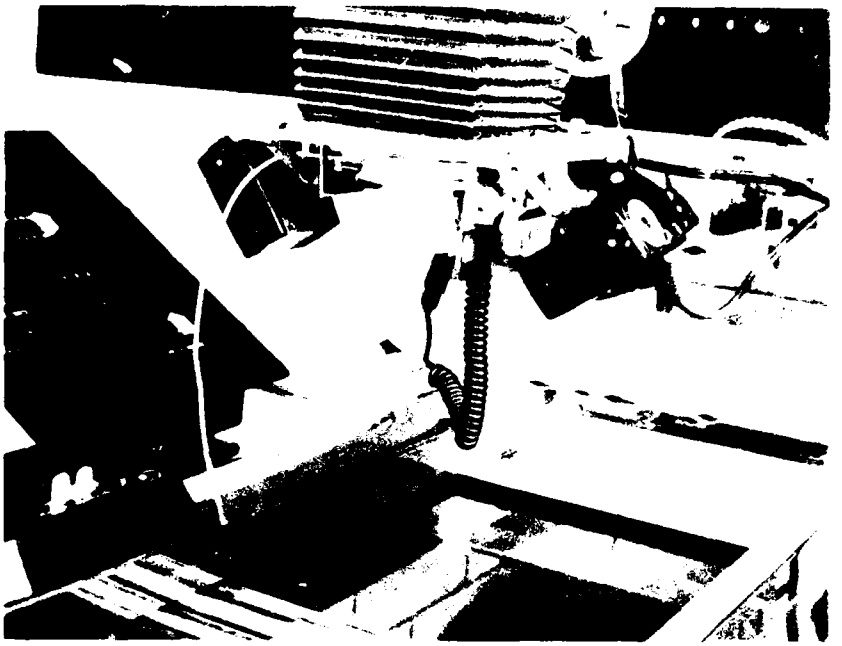


Fig. 8 View camera mounted on adjustable stand, used for photography of the section block face.

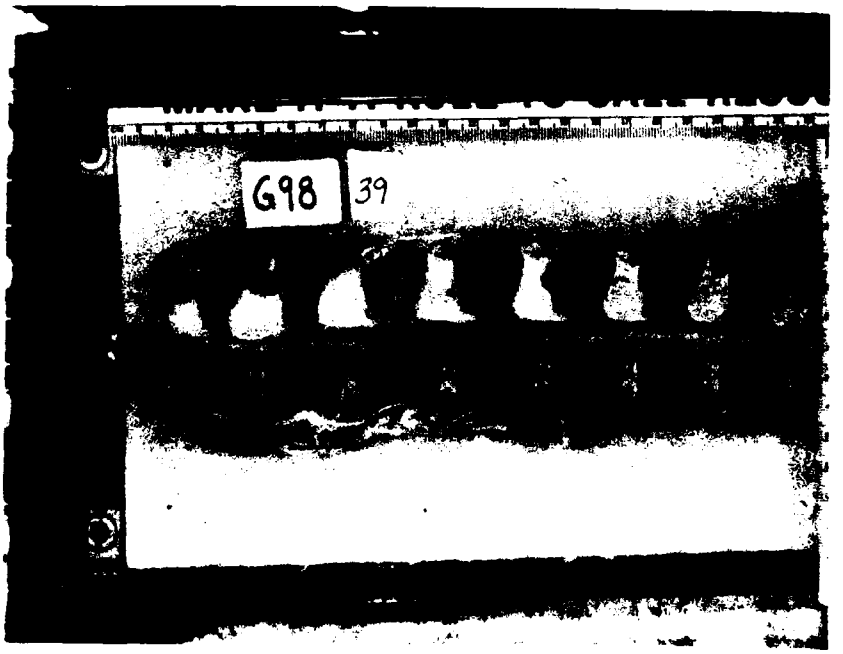


Fig. 9 Example of photograph taken with view camera. Animal is identified as G98 and photograph is number 39 of the sequence.

depth of the block has been reduced by 500 μm, a photograph is taken. The negatives are developed and printed as 8 X 10 inch color prints, resulting in a series of photographs detailing gross anatomy and structural geometry of the specimen through the entire plane of sectioning (Fig. 9). Thin and/or thick sections can be collected at the same levels as the photographs to yield a variety of data on the macro-as well as micro-anatomy and histology of a specimen.

OTHER TECHNIQUES

The LKB Cryomicrotome can also be operated at room temperature as a regular microtome. A technique for sectioning plastic-embedded undecalcified tetracycline-labeled bone for histomorphometric evaluation (5, 18) is now under investigation. Bone samples to be sectioned are fixed in 95% ethanol and placed in methyl methacrylate monomer for 2 to 3 weeks to ensure complete infiltration. The samples are then embedded in partially polymerized methyl methacrylate and cured at room temperature. Once the block is completely cured and hardened it can be sectioned at room temperature on the LKB at a thickness of 5 to 10 μm (4). This procedure should offer a relatively simple yet effective method for sectioning undecalcified bone (especially trabecular) for histomorphometric analysis. Further information on this technique will be reported at a later time.

CONCLUSION

The LKB 2250 Cryomicrotome is a valuable research tool with a wide range of applications. Its capacity to section undecalcified bone makes it especially suited to investigations involving the skeletal system and to studies focusing on the geometric interrelationship of anatomical structures (13,14). The unique capabilities of the LKB 2250 Cryomicrotome allow the operator to combine the use of light microscopy, scanning electron microscopy and macro-photography to extract a great deal of information from a single specimen.

APPENDIX A
STAINING PROCEDURES

Mallory - Heidenhain Stain: Rapid One - Step Method (2)

Solution:

Distilled water	200.0 ml
Dissolve each of below before adding next stain:	
Phosphotungstic acid	1.0 g
Orange G, C.I. 16230	2.0 g
Aniline blue, WS, C.I. 42780	1.0 g
Acid fuchsin, C.I. 42685	3.0 g

Procedure:

1. Stain: 5 minutes
2. Wash in running water: 3-5 seconds
3. Dehydrate rapidly and mount

Results:

collagen-blue
ground substance of cartilage and bones-shades of blue
mucus, amyloid, other hyaline substances-shades of blue
erythrocytes, myelin-yellow
elastin-pale pink or yellow

von Kossa, Modified from Mallory (10) (7)

Solutions:

silver nitrate, $Ag\ NO_3$	1.0 g
distilled water	100.0 ml

Fresh solution best: discard after 1 week
Safranin 0, 0.1% aqueous

Procedures:

1. Treat with silver nitrate in dark: 15 minutes
2. Rinse thoroughly in distilled water
3. Expose section to bright light: 15 minutes
4. Wash thoroughly in distilled water
5. Counterstain in safranin: 2 minutes
6. Dehydrate and mount

Results

calcium deposits-dard brown to black
nuclei and other tissue elements-shades of red and rose

Alcian Blue-Acid Fuchsin (modified) (8, 11, 16)

Solutions:

alcian blue 8GX, C.I. 74240	0.5 g
acetic acid, 3% aqueous	500.0 ml
acid fuchsin, C.I. 42685	1.0 g
picric acid, saturated, aqueous	500.0 ml

Procedures:

1. Stain in alcian blue: 30 minutes
2. Rinse thoroughly in running water: 2 minutes
3. Stain in acid fuchsin: 5 minutes
4. Wash in 1% acetic acid: 1 minute
5. Dehydrate in 95% alcohol and mount

Results:

acid mucopolysaccharides-blue green
nuclei-red
bone-red
ligament-brick red

Picro-Ponceau with Hematoxylin (6)

Solutions:

Hematoxylin
Picro-ponceau:

ponceau S, C.I. 27195, 1% aqueous	10.0 ml
picric acid, saturated aqueous	86.0 ml
acetic acid, 1% aqueous	4.0 ml

Procedures:

1. Stain in hematoxylin: 7 minutes
2. Wash thoroughly in running water until sections are deep blue: 10 minutes or longer
3. Stain in picro-ponceau: 3 minutes
4. Dip several times in 70% alcohol
5. Dehydrate in 95% alcohol, 2 changes to ensure complete removal of excess picric acid and mount

Results:

nuclei-brown to brownish or bluish black
collagenous and reticular fibers-red
elastic fibers, muscle fibers, erythrocytes, epithelia-yellow
bone-brown to yellowish brown

Safranin O-Fast Green after Lillie (9) modified by Van Sickle (17)

Solutions:

fast green, aqueous 1:5000 (add 1-2 drops concentrated acetic acid to intensify color if necessary)
Safranin O, 0.1% aqueous

Procedures:

1. Stain in fast green: 3 minutes
2. Rinse in 1% acetic acid: 30 seconds
3. Stain in safranin O: 5 minutes
4. Dehydrate in 95% alcohol, 2 changes and mount

Results:

cartilage - red
collagen, bone - bluish green

Hematoxylin-Eosin (7)

Solutions:
hematoxylin
eosin Y, 1% aqueous or alcoholic

Procedure:
1. Stain in hematoxylin: 3 minutes
2. Wash in running water until blue: 3-5 minutes
3. Stain in eosin: 1 minute
4. Dehydrate and mount

Results:
nuclei-deep blue
cytoplasmic structures shades of red
cartilage and calcium deposits-dark blue

REFERENCES

1. Appelgren, L. E., B. Widell and S. Ingvarsson (1978). Frozen whole body sections: improved morphology by thawing before staining. LKB Application Note 312.

2. Cason, J. E. (1950). A rapid one-step Mallory-Heidenhain stain for connective tissue. Stain Technology, 25: 225-226.

3. d'Argy, R. (1977). Tissue pieces from whole body sections used for chromatographic separation of metabolites. In: Conference on Autoradiography in Pharmacology and Toxicology, Upsalla, Acta Pharmacology and Toxicology, Upsalla, Acta Pharmacology and Toxicol. 41 (supp. 1): 16-17.

4. Eurell, J. DVM, Ph.D., Department of Metabolism, University of Florida. Personal communication.

5. Frost, H. M. (1969). Tetracycline-based analysis of bone remodeling. Calc. Tiss. Res. 3: 211-317.

6. Gurr, E. (1956). A Practical Manual of Medical and Biological Staining Techniques. Interscience Publishers Inc., New York.

7. Humason, G. L. (1967). Animal Tissue Techniques. W. H. Freeman and Co., San Francisco.

8. Johnson, F. B. (1957). Manual of Histologic and Special Staining Techniques. Armed Forces Institute of Pathology, Washington, D. C. p. 143.

9. Lillie, R. D. (1965) Histopathologic Technic and Practical Histochemistry, Ed. 3. McGraw-Hill Book Co., New York.

10. Mallory, F. B. (1944). Pathological Techniques. W. B. Saunders Co., Philadelphia.

11. Mowry, R. W. (1956). Alcian blue technique for histochemical study of acidic carbohydrates. Journal of Histochemistry and Cytochemistry, 4: 407.

12. Nussbaum, N. S. (1981). Cartilage of the intervertebral disc end-plate, a histological, histochemical, and fine structure study. AFAMRL-TR-81-122.

13. Rauschning, W. (1979). Popliteal cysts and their relation to the gastrocnemio-semimembranosus bursa. Upplands Grafiska AB, Uppsala. Doctoral dissertation.

14. Rauschning, W. (1979). Serial cryosectioning of human knee-joint specimens for a study of functional anatomy. The LKB Instrument Journal 26 (3): 47-50.

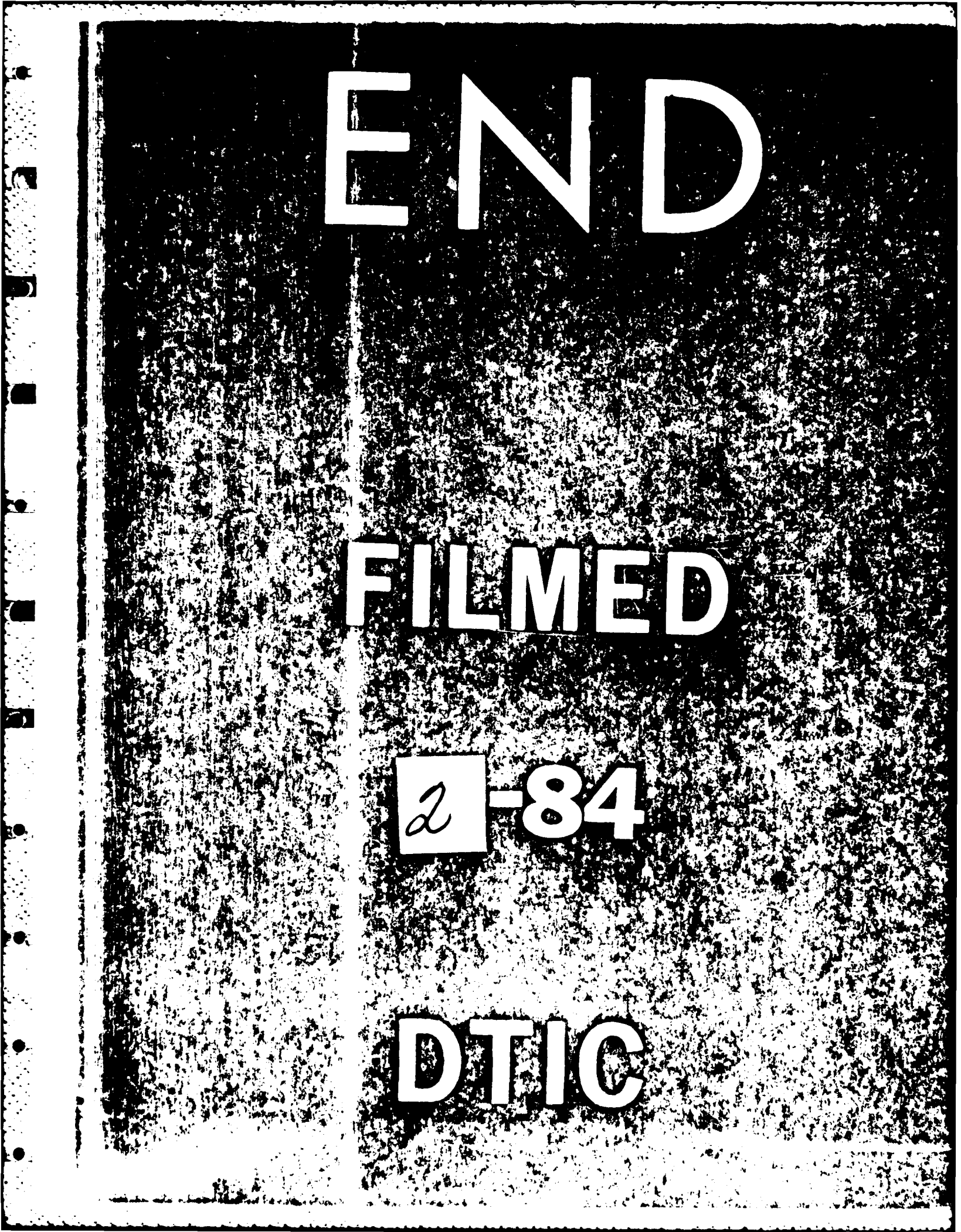
15. Sato, F. and H. Iri (1978). Studies on surgical section rapid freezing techniques. In particular, problems for surgical specimen preparation using a cryostat. Japanese J. of Clin. Pathology 26 (2): 125-131.

16. Steedman, H. F. (1950). Alcian blue 8G: a new stain for mucin. Quarterly J. of Microscopical Science 91: 477-479.

17. Van Sickle, D. C., DVM, Ph.D., Department of Anatomy, Purdue University. Personal Communication.

18. Villanueva, A. R. (1980). Histomorphometric quantitative analysis of bone, In: Theory and Practice of Histotechnology. Ed. by D. C. Sheehan and B. B. Hrapchak. C. V. Mosby Co., St. Louis, p. 108-117.

☆ U.S. GOVERNMENT PRINTING OFFICE: 1984-759-106/1314


END
FILMED
2-84
DTIC