Handbook on Plants and Cell Tissue Culture
<table>
<thead>
<tr>
<th><strong>Code:</strong></th>
<th>ENI157</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Format:</strong></td>
<td>Paperback</td>
</tr>
<tr>
<td><strong>Indian Price:</strong></td>
<td>1275</td>
</tr>
<tr>
<td><strong>US Price:</strong></td>
<td>125</td>
</tr>
<tr>
<td><strong>Pages:</strong></td>
<td>640</td>
</tr>
<tr>
<td><strong>ISBN:</strong></td>
<td>8178330296</td>
</tr>
<tr>
<td><strong>Publisher:</strong></td>
<td>Select</td>
</tr>
</tbody>
</table>
Plants cell tissue culture is a rapidly developing technology which holds promise of restructuring agricultural and forestry practices. During the last two decades cell culture have made considerable advanced in the field of agriculture, horticulture, plant breeding, forestry, somatic cell genetics, phytopathology etc. Plant cells can be grown in isolation from intact plants in tissue culture systems. The cells have the characteristics of callus cells, rather than other plant cell types. These are the cells that appear on cut surfaces when a plant is wounded and which gradually cover and seal the damaged area. Plant cells and tissue culture are often used for the production of primary and secondary metabolites. Plant tissue cultures can be initiated from almost any part of a plant. The physiological state of the plant does have an influence on its response to attempts to initiate tissue culture. The parent plant must be healthy and free from obvious signs of disease or decay. The source, termed explant, may be dictated by the reason for carrying out the tissue culture. Younger tissue contains a higher proportion of actively dividing cells and is more responsive to a callus initiation programme. The plants themselves must be actively growing, and not about to enter a period of dormancy. Plant tissue culture is used widely in plant science; it also has a number of commercial applications. Tissue culture is employed in; micropropagation, elimination of pathogens from plant materials, germoplasm storage, production of somaclonal varients, embryo rescue, production of haploids, production of artificial seeds, production of secondary metabolities, production of transgenic plants etc.

Some of the fundamentals of the book are plant tissue culture, basic requirements for tissue culture laboratory, surface sterilization of explant materials, development of tissue culture techniques, principles of cell culture cell, special factors influencing growth and metabolism, media for culturing cells and tissues, sterilisation procedures, design and equipment of a tissue culture laboratory, isolation method for microorganisms for culture, culture preservation and stability, genetic modification of industrial microorganisms mutation etc.

The present book discuss about the methods, culture preservation and stability procedures, storage and transportation of plant cell tissue culture. This book is an invaluable resource for research workers, students, technocrats, entrepreneurs, institutional libraries etc.
1. PLANT TISSUE CULTURE

Historical Events in Plant Tissue Culture

Basic Requirements for Tissue Culture Laboratory
1. Area for Medium Preparation
2. A Sterile Room
3. Glasswares and Other Instruments
4. A Constant Temperature Room
5. A Shaker System

Formulation of Tissue Culture Medium
1. Composition of M.S. Medium
2. Preparation of M.S. Medium

Collection of Explant Materials
Surface Sterilization of Explant Materials
Preparation of Explants and inculcation
Incubation of Culture Flasks

2. SUBCULTURE OF CALLUS

Regeneration of Plants from Callus
Organogentic Method
Embryogenesis Method

3. NUCELLUS CULTURE

4. EMBRYO CULTURE

Uses of Embryo Culture

5. MERISTEM CULTURE

Uses of Meristem Culture

6. ANOTHER CULTURE

Procedure For Anther Culture
Uses of Anther Culture

7. SUSPENSION CULTURE

Methods For Growth Measurement
Experiments to Assess the Cell Viability
Uses of Suspension Culture

8. DEVELOPMENT OF TISSUE CULTURE TECHNIQUES

9. PRINCIPLES OF CELL CULTURE

CELL

Fine Cell Structure
Nuclear-Plasmic Relationships
Cellular Activity

CELL DIVISION

CELL TYPES AND TISSUES

BEHAVIOUR OF CELLS IN CULTURE GROWTH,
DIFFERENTIATION AND METABOLISM

Primary And Established Cell Lines
The Nature Of Cell Alteration Or Transformation

Do Cultured Cells Differentiate?
KINETICS OF CELL GROWTH
(a) Established cell lines
(b) Primary cell lines
The cell cycle
Interaction among cells
Genetics of cultured cells
METABOLISM
Carbohydrate metabolism
Synthetic mechanisms
Protein Metabolism
Lipid metabolism
Nucleic acids
Structural elements
Relation of metabolism to growth
SPECIAL FACTORS INFLUENCING GROWTH AND METABOLISM
THE CELL AND ITS ENVIRONMENT PRESUMABLY
Temperature
Osmotic pressure
Hydrogen ion concentration
Other inorganic ions
Carbohydrates
Gases
Amino acids
Vitamins
Proteins and peptides
Supplementary metabolites
Hormones
Other specific factors
The matrix
Balance among factors
MEDIA FOR CULTURING CELLS AND TISSUES
I. NATURAL MEDIA
PLASMA
BLEEDING FROM THE WING
BLEEDING FROM THE HEART
BLEEDING FROM THE CAROTID ARTERY
COLLAGEN
BIOLOGICAL FLUIDS
Preparation of serum
Placental cord serum
Amniotic fluid
Ascitic and pleural fluid
Aqueous humour
Serum ultrafiltrates
Dialysed serum
Insect haemolymph
Coconut water (coconut milk)
TISSUE EXTRACTS
The preparation of embryo extract
Preparation of chick embryo extract
Preparation of embryo extract from young embryos
The preparation of bovine embryo extract
Ultrafiltrates of embryo extract
Other tissue extracts
Other media of biological origin
MEDIA FOR CULTURING CELLS AND TISSUES
II. DEFINED MEDIA
MEDIA FOR TISSUES FROM WARMBLOODED VERTEBRATES
Solubility of materials.
Compatibility of components
Purity of materials.
Chemical instability
Stock solutions.
BALANCED SALT SOLUTIONS
Materials
Preparing a balanced salt solution
PARTIALLY COMPLETE SYNTHETIC AND COMPLETE MEDIA
Preparation of Eagles Medium
MEDIA FOR CULTURE OF TISSUES FROM COLD BLOODED VERTEBRATES
MEDIA FOR INVERTEBRATE TISSUES
MEDIA FOR PLANT TISSUES
10. PREPARATION OF MATERIALS
PREPARATION OF APPARATUS
Glassware
Plastic vessels
Stoppers for culture vessels
Rubber tubing
Instruments, etc
CLEANING PROCEDURES GLASSWARE
Detergents
Alkalies
Oxidising acids
Ultrasonics
Special problems
Automatic washing machines
PREVENTION OF CONTAMINATION
I. STERILISATION PROCEDURES
Sterilisation by dry heat
Sterilisation by moist heat
Radiations
Antiseptics
Antibiotics
Filtration
Storage of sterile materials
Chronic contamination (especially PPLO and L forms)
Sterility testing
Elimination of contamination
Outbreaks of contamination
PREVENTION OF CONTAMINATION
II. ASEPTIC TECHNIQUE
Contamination from tissue
Contamination from the air
Contamination from the operator
DESIGN AND EQUIPMENT OF A TISSUE CULTURE LABORATORY
Sterilisation and cleaning facilities
Sterile working area
Storage for media
Incubator facilities
Special glassware and apparatus
General equipment
Special apparatus
Coverslip techniques
Rollertube techniques
Organ culture
Handling of strains
Sources of materials
LABORATORY DESIGN
A singleroom unit
Laboratory suite for tissue culture
Sterilisation room
The preparation room
The aseptic room
Aseptic cubicle
Hot room
General facilities
11. PRIMARY EXPLANATION TECHNIQUES
I. TISSUE CULTURES
SLIDE CULTURES
THE PREPARATION OF SLIDE CULTURE
Single coverslip with plasma clot
Maximow double coverslip method with plasma clot
Single coverslip with liquid medium. Laying and hanging drop cultures
AFTERCARE OF SLIDE CULTURES
Washing and feeding double coverslip cultures
Patching
Transferring coverslips cultures
CARREL FLASK TECHNIQUE
PREPARATION OF CULTURES
Renewal of medium
The transfer of tissue
TESTTUBE CULTURES
Plasma clot technique
Feeding testtube cultures.
Patching testtube cultures
Transfer of cultures from testtube
Culture of primary explants in roller tubes without plasma.
Flying coverslips in test tubes
THREEDIMENSIONAL SUBSTRATES
PRIMARY EXPLANTATION TECHNIQUES
II. ORGAN AND EMBRYO CULTURE
Organ cultures on plasma clots
Cultures on agar
Fluid media
PREPARING AN ORGAN CULTURE ON A CELLULOSE ACETATE RAFT
SETTING UP AN ORGAN CULTURE OF EMBRYONIC LIMB BONES ON A GRID
Set up apparatus
Prepare dishes
Prepare explants
Set up explants (e.g. chick limb bones)
Subculture (The medium should be changed every 48 hours.)
CHOPPED TISSUE TECHNIQUE
Cultivation of poliomyelitis virus in minced tissue suspensions
CUTTING CHICK EMBRYONIC HEART EXPLANTS BY MEANS OF THE McILWAIN TISSUE CHOPPER
WHOLE EMBRYO CULTURE
Culture of preimplantation mammalian embryos
Culture of postimplantation mammalian embryos
PRIMARY EXPLANTATION TECHNIQUES
III. DISAGGREGATION METHODS
PREPARATION OF CELL SUSPENSIONS FROM FRESH TISSUES
Disaggregation of embryonic limb buds
Preparation of trypsinised embryonic carcass
Trypsinibation of monkey kidney tissue
Preparation of primary human amnion cells
Trypsinibation procedure
Trypsinibation in the cold
Cloning of primarily disaggregated cells
12. CELL LINES
STATIC CULTURE METHODS
SUSPENDING CELLS FROM A MONOLAYER CULTURE
INOCULATION OF NEW VESSELS
FEEDING AND MAINTENANCE
Agar slope cultures
SUSPENSION CULTURES
Media for suspension cultures
Gas phase
General methods
General management of suspension cultures
Batch cultures
Continuous medium replacement
GROWTH OF PLANT CELLS IN SUSPENSION
CLONING CELLS
Cloning of HeLa cells by the dilution technique
Agar suspension technique
Cloning in fibrin gels
Cloning cells by the isolation technique
Technique
Characterisation of cell lines
SPECIAL ASPECTS OF HANDLING PRIMARY CELL LINES
General maintenance
Seed stocks
13. ISOLATION METHOD FOR MICROORGANISMS FOR CULTURE
SOURCES OF ORGANISMS AND SOME SAMPLING STRATEGIES
DIRECT ISOLATION METHODS
Pretreatment of Samples
DILUTION AND INCUBATION OF SAMPLES
Media Considerations
ENRICHMENT CULTURE METHODS
Baiting Methods
General Chemical Enrichment
Specialized Enrichment Systems and their Applications
Enrichments from sea water
Enrichments for biomass production
Enrichments for nitratereducing bacteria
Enrichments in complex media
Biodegradation
Heterogeneous continuous flow systems
14. CULTURE PRESERVATION AND STABILITY
PROCEDURES PRIOR TO SELECTING A PRESERVATION METHOD
Object of Preservation
Good Record Keeping of Previous Treatment and Lineage
Notation of Reported Characteristics of a Culture
Culture Preservation and Stability
DETERMINANTS FOR CULTURE IDENTITY,
CHARACTERISTICS AND PURITY
Authenticated Cultures Confirmation of Stated Traits
Morphological
Biochemical
Physiological
Research and Development Strains
Elimination of leaky mutants
Assurance of auxotrophic traits (elimination of mixed genetic bag)
Selective pressure for maintaining specific culture traits
Longterm Storage
Cost efficiency
Minimal maintenance
Endurance of label
Precise inventory system
Shortterm Storage
Ease of sample preparation
Label reliability
Economic aspects
Reliability
Ease of retrieval
Rapid retrieval

SELECTION OF MAINTENANCE CONDITIONS AND PROCEDURES FOR IMPLEMENTATION, BASED ON CULTURE USE
Longterm Storage
Analytical organisms
Comparison strains
Manufacturing plant cultures
Shortterm Storage
New metabolite producers for investigative studies
Clones from populations for improved metabolite producers
Working stocks of analytical organisms

CULTURE RESTORATION AND GROWTH CONSIDERATIONS
Restoration
Concentration of inocula
Nutrition
Osmotic (rehydration)
Temperature (rehydration and/or rate of melting)
Growth
Requirements
Temperature
Aeration (including dissolved gases)
Duration
Verification of Purity
15. GENETIC MODIFICATION OF INDUSTRIAL MICROORGANISMS

MUTATION
- DNA Repair Mechanisms
- Mutagen Specificity
- Survival Curves and Optimum Conditions for the Use of a Mutagen and Expression of Mutations
- Site Specific Mutagenesis
- Applications of Mutation to Antibiotic-producing Microorganisms

RECOMBINATION
- Protoplast Fusion
- Conjugation and Natural Plasmids
- Transformation
- Transduction
- Sexuality and Parasexuality in Fungi
- Recombinant DNA Technology
- Transposable Elements
- Applications of Recombination to Antibiotic-producing Microorganisms

GENETICS AND SCREENING

16. IN VITRO RECOMBINANT DNA TECHNOLOGY

GENERATION AND CLONING OF DNA FRAGMENTS
- Fragmentation of DNA
- Class II restriction enzymes
- Random DNA fragments and the generation of genomic libraries
- Enrichment for specific DNA sequences
- Synthesis of cDNA
- Chemical synthesis of DNA
- Covalent Linkage of DNA Fragments to Vector Molecules
- Ligation to vector molecules
- Methods favouring formation of hybrid DNA molecules
- Modification of DNA Extremities
- Isolation of Recombinant Molecules and Interspecies DNA Transfer
- Transformation and transfection
- In vitro packaging

CLONING VECTORS
- Plasmid Vectors
- Vectors Derived from Bacteriophage I
- Phage vectors
- Cosmids vectors
- Special Purpose Cloning Vectors
- Expression vectors
- Single-stranded phage vectors
- Plasmid vectors for subcloning and sequencing
- Vectors for the detection of transcription and translation signals
- Vector Systems for Organisms other than E. coli

DETECTION AND ANALYSIS OF CLONES
- Screening Recombinant Clones
Nucleic acid homology
Translation in vitro
Immunological screening
Characterization of Cloned DNA
Isolation of cloned DNA
Physical characterization of cloned fragments
Characterization of products expressed by cloned fragments
MANIPULATION OF CLONED GENES IN VITRO
Mutagenesis
Generation of deletions and insertions
Point mutations
Efficient Expression of Cloned Genes
Constructions that maximize expression
Secretion of cloned products
17. NUTRITIONAL REQUIREMENTS OF MICROORGANISMS
BACTERIA AND FUNGI
Macronutrients
Carbon
Nitrogen
Hydrogen
Oxygen
Phosphorus
Sulfur
Potassium
Magnesium
Micronutrients
Growth requirements
Effects of trace elements
Addition of trace elements
Chelation
Growth Factors
Vitamins
Amino acids
Miscellaneous growth factors
ALGAE
Macronutrients
Carbon, oxygen and hydrogen
Nitrogen
Phosphorus and sulfur
Potassium and magnesium
Micronutrients
Growth Factors
PROTOZOA
18. DESIGN, PREPARATION AND STERILIZATION OF FERMENTATION MEDIA
MEDIUM DESIGN
MEDIUM PREPARATION
STERILIZATION
19. NUTRIENT UPTAKE AND ASSIMILATION
NUTRIENT UPTAKE
Simple Diffusion
Transport Systems
Facilitated diffusion
Active transport
Redundancy of Transport Systems
ASSIMILATION
Assimilation of Carbon
Assimilation of Nitrogen
Control of nitrogen assimilation
Assimilation of Other Elements
20. MODES OF GROWTH OF BACTERIA AND FUNGI
GROWTH OF UNICELLULAR ORGANISMS
Cocci
Grampositive Rods
Gramnegative Rods
Budding Yeasts (Saccharomyces)
THE CELL CYCLE
GROWTH OF FILAMENTOUS ORGANISMS
Germination of Fungal Spores
Hyphal Morphology
Growth of Individual Hyphae
The extension zone
Cytology of the nonextending part of fungal hyphae
The peripheral growth zone
Growth of Mycelia
YEASTMYCELIAL DIMORPHISM
COLONY GROWTH
Growth of Colonies on Solid Media
Growth of Colonies in Liquid Media
EFFECT OF GROWTH RATE AND OTHER VARIABLES ON CELL COMPOSITION AND MORPHOLOGY
Unicellular Organisms
Fungi and Actinomycetes
21. MIXED CULTURE AND MIXED SUBSTRATE SYSTEMS
MIXED CULTURES
Methods of Study
Enrichment of Mixed Cultures
Analysis of Twospecies Systems
Analysis of Multispecies Communities
Kinetics of Mixed Cultures
Genetic Interactions
Mixed Culture Processes
Spontaneous mixed culture processes
Defined mixed cultures
Contamination and Degradation
Contamination
Industrial fermentations with unstable strains
Environmental Biotechnology
MIXED SUBSTRATES
Patterns of Mixed Substrate Utilization
Control of Mixed Substrate Utilization in Batch Culture
Control by regulation of substrate transport
Control by regulation of enzyme synthesis
Control by regulation of enzyme activity
Mixed Substrate Utilization in Continuous Culture
Double substrate limited growth
Efficiency of growth on mixed substrates
COMETABOLISM
Cometabolism in the Environment
Technological Potential
22. PROTOPLAST TECHNOLOGY
ISOLATION OF PROTOPLASTS
1. Mechanical Method
2. Enzymatic Method
MAINTENANCE OF PROTOPLASTS
Viability Tests for Protoplasts
1. FAD Test
2. Phenol Safranin Test
3. ColflourWhite Test
4. Microscopic Observation of Cytoplasmic Streaming
Plant Regeneration from Protoplasts
Applications of Protoplast Culture
PROTOPLAST FUSION
Methods of Protoplast Fusion
Selection of Hybrid protoplasts
Regeneration of Plantlets
Uses of Protoplast Fusion
INVITRO MUTATION BREEDING
Induction of invitro Mutagenesis
Uses of Invitro Mutation Breeding
23. GERMLASM STORAGE
GERMLASM STORAGE BY CRYOPRESERVATION
1. Collection of Plant Materials
2. Addition of Cryoprotective Agents
3. Freezing Treatment
4. Longterm Cold Storage
REUSE OF PRESERVED TISSUE
1. Thawing
2. Removal of Cryogen
3. Callus Induction
4. Plant Regeneration
Achievements
Advantages of Cryopreservation

STORAGE OF GERM PLASM OF POTATO

24. GENETIC ENGINEERING THROUGH THE TRANSFER OF CELL ORGANELLES
1. Isolation of Cell Organelles
2. Isolation of Protoplasts
3. Induction of protoplast to uptake cell Organelles
4. Selection of Transformed Protoplast
5. Regeneration of Plantlets

Advantages of Organelle Uptake Method

SUBPROTOPLASTS
Production of Cybrids
Applications of Cybrids

25. SPECIAL CONSIDERATIONS FOR DIFFERENT TISSUES

VERTEBRATE TISSUES
Embryonic tissues

DISSECTION OF THE CHICK EMBRYO
Chick embryonic limbbones for organ culture

MAMMALIAN EMBRYONIC TISSUES

ADULT TISSUES
PREPARATION OF EXPLANTS OF THE BUFFY COAT
Culture of peripheral blood leucocytes
Human skin fibroblasts

PROLONGED CULTURE OF DIFFERENTIATED CELLS

CULTIVATION OF TISSUES FROM COLDBLOODED VERTEBRATES

CULTURE OF INVERTEBRATE TISSUES
Arthropods
Other invertebrates

STORAGE OF TISSUE BEFORE CULTURING

CULTURE OF PLANT TISSUES
Preparation of tissues from plants
Culture of tomato roots
Culture of carrot callus

26. CULTIVATION OF CELLS IN VIVO TRANSPLANTATION

Transplantation into embryos

PROCEDURE
Transplantation into tolerant chimeras
Transplantation into genetically similar hosts
Transplantation into nonvascular areas

Procedure for anterior eye chamber implantation
Procedure for brain implantation
Diffusion chambers
Transplantation to irradiated and cortisonetreated
animals
scites tumours
Maintenance of sterility

27. LARGESCALE CULTURE METHODS
Preparation and sterilisation of apparatus
Preparation and sterilisation of media
Cells and media

APPARATUS FOR MASSIVE CULTURE OF CELLS ON GLASS SURFACES
Largescal Roux flask cultures
Roller bottle methods
Solid matrix perfusion systems.
The multiple surface tissue culture propagator

MASSIVE SCALE SUSPENSION CULTURE
Culture vessels

CONTROL OF CULTURE CONDITIONS
Temperature
pH
Oxygen

28. PRESERVATION, STORAGE AND TRANSPORTATION OF LIVING TISSUES AND CELLS
Maintenance at slightly reduced temperatures
Maintenance at refrigerator temperature
Preservation by freezing
Equipment
General Procedure
Transportation of cells

29. MORPHOLOGICAL STUDIES
Morphological Studies
COMMON FIXATION AND STAINING TECHNIQUES
FOR TISSUE CULTURE MATERIAL
I. Commonly used fixatives
II. Routine stains
III. Special histochemical stains
Chromosome spreading technique
Determining the mitotic coefficient
Planimetry
Examination of living cells
Photography

PERFUSION OR CIRCUMFUSION CHAMBERS
Timelapse cinemicrography

QUANTITATIVE OPTICAL METHODS
Auto radiography
Preparation of cultures for electron microscopy

30. APPLICATIONS OF TISSUE CULTURE
I. Micropropagation
2. Elimination of Pathogens
3. Germplasm Storage
4. Somaclonal Variation
5. Embryo Rescue
6. The Production of Haploids
7. Artificial Seeds
Types of Artificial Seeds.
8. Production of Secondary Metabolites
9. Production of Somatic Hybrids
10. Transgenic Plants
Secondary Metabolites
Culture of Plant Cells for the Extraction of Secondary Metabolites
1. Designing of Bioreactor
2. Selection of Explant Source
3. Surface Sterilization
4. Preparation of Explant
5. Callus Culture
6. Suspension Culture
7. Cell Plating
8. Testing for Biosynthetic Activity
9. Culture of more Productive Clones
10. Extraction of Secondary Metabolites
Biotransformation In Plant Cells
Elicitor dependent Biosynthesis
Immobilization of Plant Cells
Hairy Root Clones
31. LIST OF CULTURE
NCTC 109 AND NCTC 135
32. SOURCES OF MATERIALS FOR TISSUE CULTURE
General suppliers of laboratory apparatus
General glassware (in addition to above firms)
General biological products and biochemicals
General chemicals
Special tissue culture media
Suppliers of cell cultures

Sample Chapter:
Now that very many cell-strains have been isolated, one of the major problems confronting some laboratories is the maintenance of the large number of cultures necessary to carry several strains simultaneously. A further difficulty is the tendency for cell-types to change after they have been kept in a state of rapid proliferation for some years. Similar problems have been encountered in the handling of micro-organisms and the answers which have been suggested for the maintenance and storage of cells are very similar to those, which have been adopted by bacteriologists. All the methods depend on the maintenance of the cells at reduced temperatures but there are some differences in technique depending on the temperature used.

Cells can be maintained at slightly reduced temperatures, refrigerator temperatures or very low temperatures (-70°C to -190°C). Room temperature and refrigerator storage are sometimes used for short-term maintenance but for long-term storage very low temperatures are necessary. In the deep-frozen state metabolism is, of course, suspended completely.

Maintenance at slightly reduced temperatures
Most mammalian cells and tissue fragments will survive indefinitely at 30°C provided the medium is renewed when required. By this means the frequency of feeding can be greatly reduced. Thus, compared with 37°C, the intervals between feeding can be about three times as long.

The temperature can be still further reduced and many cells will survive unharmed at about 20°C. At this temperature very infrequent renewal of medium is required, although the cells rarely continue to behave quite normally if their normal incubation temperature is of the order of 37°C. They tend to round up and may leave the glass and sometimes they become packed with fat droplets. However, if the medium is renewed and the temperature raised to normal once more the cells very rapidly return to normal after a short lag period.

Maintenance at refrigerator temperature
Some reports have appeared concerning the storage of tissue in the refrigerator (2 - 6°C). It would appear that sheets of cells and single cells do not survive well for more than a few days in these conditions but that tissue fragments will survive for several weeks in a nutrient medium. This may be due to the high oxygen tension, which can develop in the medium. The main practical use of this method is in the preservation of surviving tissue before explanation, usually only for a few days. The tissue should be cut into pieces of a few cubic millimeters and stored in a nutrient medium.

Preservation by freezing
In several fields advantage has been taken of the observation that deep-frozen tissue may remain viable. This principle was applied to the storage of viruses and bacteria but when it was applied to animal cells they did not survive. At first this was thought to be due to laceration of the cell membranes by ice crystals but more recent evidence suggests that the cause may be osmotic changes which give rise to irreversible changes in lipoprotein complexes resulting in splitting of membranes within the cell. In any event the answer to the preservation of living animal cells proved to be the addition of a substance such as glycerol or ethylene glycol to the medium and slow freezing. The technique was worked out in some detail, by Smith who demonstrated the survival of ovarian granulosa cells after deep freezing. Scherer and Hoogasian demonstrated its effectiveness with some stock lines of cultured cells and since then it has been widely applied.

The following principles summarise generally accepted current practice.
1. The cells should be in a healthy state before freezing.
2. They should be suspended in growth medium containing glycerol or dimethyl sulphoxide (5-15 per cent.) shortly before freezing, and sealed in a gas-light ampoule.
3. Freezing to -70° should be controlled over a period of about an hour or preferably longer. A cooling rate of one degree a minute is recommended.
4. Storage temperature should be maintained at -70° or lower.
5. Thawing should be rapid (2 - 3 minutes).

Details of the technique are given below.

Equipment
1. Refrigerator.-Cells may be stored in a low-temperature deep-freeze cabinet operating at a temperature in the region of -70° C. (e.g. Revco deep-freeze) or in a dry-ice chest at about the same temperature. However, viability tends to be lost over a period of months at these temperatures. At temperatures of less than -90°C no progressive loss of viability can be detected over very long periods and these very low temperatures are therefore recommended. Liquid nitrogen is usually used as the refrigerant. It has a temperature of -196°C and the overlying vapour in a well-insulated vessel gives temperatures within the range of -150° to -180°. Suitable containers (often called liquid nitrogen refrigerators) are manufactured by several companies. The Linde division of the Union Carbide Company manufactures a range of suitable vessels, including a small one with a capacity of 972 ampoules, which is suitable for a relatively small laboratory.
2. Devices for recovery of cells.-Cells are usually stored in 1 ml. ampoules in liquid nitrogen refrigerators. Most methods for marking ampoules are unsatisfactory. Hence, it is important that records be kept meticulously and that ampoules be stored systematically. The liquid nitrogen refrigerator amounts to a Dewar flask, similar to a large Thermos flask. The contents are distributed to a series of containers, usually deep cans, like pipette canisters, which can be recovered individually by some device or another. Within these containers the ampoules are held either in tubes or clipped to 'canes'. A cane is a metal rod bearing a number of clips to which individual ampoules can be attached. Each cane or tube carries a reference number.
3. Cooling devices.-In order to ensure a steady slow rate of cooling several devices have been developed. The simplest can be made from a block of expanded polystyrene insulating material about four inches thick. This is sliced down the middle to give a top and bottom half and matching cavities are then made in each half with a large cork borer to hold ampoules. Ampoules are placed in the cavities, the two halves held together with an elastic band, and the whole device placed in a Revco deep-freeze cabinet for two hours. The cells are transferred direct to the liquid nitrogen refrigerator.

A similar device, invented by Greaves and his colleagues, consists of a modified stopper for a liquid nitrogen refrigerator and operates on exactly the same principle. It is available from the Linde company. For meticulous large-scale work programmed cooling devices have been developed but these are not necessary for the ordinary laboratory. Simpler methods than the ones mentioned above are also available. For instance, reasonably good results can be obtained by packing the ampoules in cotton-wool in an ice-cream carton and putting them first in the 4° refrigerator for one hour then in the -20° freezer for one hour before transferring them to the coldest temperature available. Results are not so good by this method but in a laboratory not equipped to handle cell-freezing routinely it provides a satisfactory makeshift.
4. Other equipment.-A large Dewar flask will be required to store or transport liquid nitrogen if a liquid nitrogen refrigerator is used. (It may be remarked that it is best to have a well-established routine of topping-up every week, although the reservoir of liquid nitrogen may be sufficient for many weeks.)

General Procedure
The procedure for preparing twelve ampoules of a cell strain is described.
1. Harvest 1-2x108 cells, which must be growing exponentially) by a standard method, such as trypsinisation. Resuspend in 21.5 ml. of fresh growth medium. Open ampoules.
2. Add 2.5 ml. dimethyl sulphoxide (to give a final concentration of 10 per cent.). Mix quickly and dispense approximately 2 ml rapidly to each ampoule. Store ampoules on ice.

3. Seal ampoules (practice may be required). Return to ice. (3A. Test ampoules by submerging in a solution of crystal violet in methyl alcohol. If the ampoules are leaky some dye will be sucked in. This step is unnecessary if the operator is proficient.)

4. Place ampoules in cooling device and leave 1-2 hours to cool to below -30°C.

5. Rapidly transfer ampoules to liquid nitrogen refrigerator. (Thawing at this stage will kill the cells.)

6. Complete records.

7. Within the next few days remove two ampoules, thaw as described below, and test for viability by dye exclusion or plating efficiency. If viability is good confirm the record. If it is bad prepare a new batch of cells to replace the bad batch.

8. To recover cells from the deep-freeze proceed as follows. Prepare a beaker by filling it with water at 37°C and provide it with a cover. Put on protective goggles. (Occasionally a defective ampoule may fill with liquid nitrogen and explode on thawing.) Remove an ampoule with forceps and slip it under the cover into the beaker. When it has thawed open the ampoule and transfer the contents to a suitable vessel. Adding medium slowly at first, make the volume up to at least 25 ml. Transfer to culture vessels and incubate.

In the procedure described above dimethyl sulphoxide was recommended as the protecting agent. In our experience better results are obtained with it than with glycerol and this is probably the general experience. The reason may be that it can diffuse in and out of cells more readily with the result that there is less osmotic damage when it is added to medium or diluted out.

Transportation of cells

It is now common practice to transport samples of cell strains over long distances. The availability of airfreight and reliable mail services reduces the problem to the relatively simple one of maintaining the cells alive for, at the most, two days in transit.

It has to be accepted that the culture vessel will be agitated during handling so that there is no point in attempting to maintain the cells as a monolayer on the wall of a vessel partially filled with medium which may slop about. If it is desirable to despatch them as a monolayer practically all the medium should be removed from the vessel or else it should be completely filled. Alternatively the cells may be transported as a suspension.

The main hazard in transporting cells is exposure to extremes of temperature. Thus, in winter if cells are sent by ordinary mail it is possible that they will be frozen. If they are sent by airfreight at any time of the year the same may happen due to low temperatures in the uninsulated freight compartment of a high-flying aircraft. In summer in many parts of the world the air temperature alone may rise to levels lethal to cells, while they may be killed rapidly even in temperate climates if the sun shines directly on the package containing them. However, awareness of these factors is usually all that is necessary to ensure that adequate steps are taken to deal with them.

At most times of the year in Great Britain it has been found adequate to send the cells, either as a suspension or as a monolayer with about 2 ml of medium in the bottle, by Express letter post. An ordinary prescription bottle or a large test-tube is used. It is protected, with some wadding and wrapped up securely in a parcel which is despatched in the late afternoon. From most parts of the country this ensures delivery first thing the following morning. In cold or hot weather, it is necessary to insulate the package much more thoroughly. If it is to be sent over a distance in the winter it is advisable to send it by passenger train with an arrangement to have it collected at the other end.

If cells have to be carried over a longer distance, it is best to send them by air. In making arrangements for
international shipments it is advisable to consult a government organisation to avoid unnecessary customs delay. Transatlantic shipments to Great Britain from the United States have been arranged by the United Kingdom Treasury and Supply Delegation by arrangement with the Medical Research Council. It should be emphasised to those responsible that the package must not be exposed to extremes of temperature. In countries such as the United States, in which extremes of temperature are encountered, it is necessary to send cells in insulated packages most of the year. Foam plastics, powdered cork and cotton are effective insulators.

If for any reason cells have to be transported over a long distance by slower methods this can be achieved satisfactorily by filling the culture vessel completely with fresh medium immediately before transportation and then keeping them at a temperature of about 20°C. By this means cells have been known to survive in a suitcase for a period of at least three weeks. Where possible the other methods described are to be preferred.