Radnoti Tissue-Organ Bath Principles

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Introduction

Isolated tissue and organ preparations have been in use for over one hundred years. These preparations provide researchers with convenient biological models that are independent of the systemic influences of *in vivo* preparations.

Isolated tissue-organ experiments can generally be run in groups of 2, 4, 8 or more sample preparations, thereby enabling a high throughput in most laboratories. These *in vitro* preparations are also more readily instrumented and can be easily subjected to controlled changes in perfusate, oxygen availability, drug administration etc than is otherwise possible in the intact animal.

The increased development and use of transgenic animal models, particularly mice, has extended the scope of isolated tissue and organ preparations by creating models that can express normal or pathological genetic sequences. In turn, isolated preparations can allow molecular biologists to quantify the physiological (phenotypical) effects of altered genetic sequences.

Basic Overview of Principles

Experimental Design

To ensure a successful experiment, a suitable organ bath configuration must be constructed that permits optimal performance of the tissue in a controlled environment whilst allowing for suitable instrumentation. Before performing any experiment the following should be considered:

- The basic hypotheses and specific aims of the research project.
- The species and the types of tissues that would be suitable research models.
- The physiological response that may be induced by the experimental protocol.
- The methods, instruments, physiological measurements and statistics that will be required to measure and analyze these responses.

A careful review of the literature, combined with a comprehensive analysis of the research problem, is important in designing a successful research project.

Organ Bath Apparatus

The basic requirements for any isolated tissue preparation include provisions for temperature control and oxygen and substrate delivery. A water-jacketed organ bath provides a stable and easily adjustable way of temperature control. Substrates and other nutrients that are required to sustain tissue function are provided via a physiological solution, similar in chemical composition to plasma.

Oxygen delivery to the tissue is provided by saturating the physiological solution with a gas mixture (carbogen) delivered by a glass frit (needle). Additional experimental requirements may include electrical stimulation, changes in flow, pressure or distention. Transducers/probes used in organ bath setups will vary depending on the type of tissue and physiological parameters to be investigated. Instruments are available to monitor:

- Changes in force.
- Flow or pressure.
- The release of chemical substances into the perfusate or alteration of perfusate characteristics.
- Oxygen consumption or carbon dioxide production.
- Electrophysiological alterations.

These responses can be recorded and analyzed easily using PowerLab and Chart.

Selection of Physiological Buffer Solution and Aeration Techniques

The buffer solution(s) chosen for the dissection and maintenance of the tissue is important as it will affect the viability of the preparation and hence experimental protocol.

Artificial Solutions

While whole blood may be used from a donor, there are many associated complications and variables to be considered. Aeration is an issue as normal bubbling can destroy the protein components of blood and plasma. The natural chemical components of blood, such as hormones, plasma metabolites etc, may also interfere with the tissue's physiological response and will need to be taken into consideration. Because of these limitations, most isolated tissue preparations use an artificial plasma-like buffer solution to maintain tissue viability.

Because almost all artificial solutions are water based, the water purity is of primary importance and should at least be the equivalent of double distilled water with organic contaminants removed. Modern reverse osmosis systems or glass stills passing on water to charcoal and ion exchange resin filters provide high quality, purified water. Some buffer recipes add 0.1-5 mM EDTA for chelation (removal) of unwanted heavy metals that may be added from salts or leached from metal tubing or other sources.

Reagents used in the production of buffers should be of high quality the equivalent of USP or Analytical Reagent grade. There are a number of widely used physiological salt solutions, such as Ringer's, Tyrode's and Krebs-Henseleit (see Table 1) and their salt concentrations are often modified to resemble those in the donor's plasma.

Table 1

	Ringer's mM	**Tyrode's mM**	**Kreb's Henseleit mM**
$CaCl_2$	1.4	1.1	2.5
KCl	5.6	2.7	4.7
NaCl	154	137	118
$MgCCl_26H_20$		0.5	
$NaHCO_3$		12	25
$NaH_2PO_4H_2O$		0.4	
$MgSO_4$			1.2
KH_2PO_4			1.2
Glucose		5.5	5.5
Na-EDTA			0.5

In most of these solutions, phosphates (or sulfates) are also added. Besides adjusting the pH and increasing buffering capacity, the presence of carbonates, bicarbonates and phosphates aid in maintaining normal anion homeostasis. As an alternative, buffering capacity can be created through the use of synthetic buffers like HEPES or MES in place of bi carbonates with 100% oxygen used for aeration. It is also possible to use these synthetic buffers in addition to bicarbonate, and then adjust the sodium balance appropriately to maintain osmolarity. The most common metabolic substrate is glucose, with pyruvate, lactate and fatty acids also added. Often salts other than calcium are kept as a concentrated stock in one container, whilst calcium and glucose are kept as a concentrated stock in another container. These stocks are then diluted and combined just prior to use. This reduces precipitation of calcium phosphate and retards bacterial growth by having hyperosmotic solutions. To reduce tissue edema, albumin, polyvinylpyrrolidine, dextran or other plasma expanders can be added. Note that the presence of albumin or other readily denatured or poorly soluble compounds will require indirect aeration, such as that provided by a membrane oxygenator, to prevent foaming and precipitation. Always adjust the pH of the solution while it is at the bath temperature selected and aerated.

Oxygenation of Perfusate

Bicarbonate buffers, such as Tyrode's and Krebs-Henseleit, contain chemicals that are based on those naturally occurring in mammalian blood, and are most effective when in equilibrium with relatively high levels of carbon dioxide. Therefore, carbogen (95% oxygen/5% carbon dioxide) gas is often used to aerate the perfusate. The use of 95-100% oxygen rather than atmospheric levels of 20% is required to increase the oxygen content of the solution and thereby compensate for the lack of hemoglobin or other oxygen carriers.

The buffer reservoir and organ bath systems are usually continuously aerated to ensure that the dissolved oxygen levels are adequately maintained for the tissue preparation. Gas flow to the buffer is normally controlled with a two-stage regulator designed for the gas mixture used. Flow rates of 0.5 to 2 liters per minute and/or pressures of 1 to 2 psi are adequate for most preparations. Aeration in organ baths are controlled via a needle valve and should be at a steady level providing a continuous, stable line of fine bubbles that do not cause a "boiling" effect in the bath. Higher flow rates will only serve to increase evaporation, disturb the preparation, induce signal artifact and may even damage fragile components of the organ bath.

Tissue Dissection/Collection

With any organ bath preparation, the time between the removal of the tissue and provision of a nutrious/oxygenated solution is important. Wherever possible, it is recommended to have the donor animal in the laboratory, close to the equipment setup. For some preparations, tissues may only be available from commercial processors (i.e. abattoirs) and, therefore, field dissections are necessary. For these types of preparations, dissection time, transport and storage is important and the following points will help to ensure successful collection and experimentation.

- Ensure efficient and fast dissection and collection of the tissue.
- Ensure minimal handling of the tissue.
- Where possible, larger sections of tissue should be collected and smaller sections dissected within the laboratory.
- An adequate supply of chilled perfusate solution may be used for storage, if available.
- Tissue should be transported on ice.

Dissection in the laboratory should be performed in a dedicated area near a sink with running water that is located out of the stream of traffic. For a fast and successful collection of the tissue, the following should be considered:

- A comfortable chair and appropriate benchspace.
- Well lit dissecting area and magnifiers (where necessary)
- The level of magnification should be chosen to clearly distinguish areas of interest and the magnifying lens should have a good depth of field; a zoom lens is ideal.
- Contrasting background may be used on the dissecting table to aid in identification of the tissue.

Once tissues are prepared, they may be stored for later use. Certain tissue samples, such as papillary muscles and smooth muscle strips and rings, can be stored in containers of chilled physiological salt solutions at 4° C for a few hours.

Dissecting Bath

The dissection bath is usually a low flat dish that is water-jacketed and aerated, allowing easy access to the tissue for dissection and visualization with a microscope or magnifier. The dissection is often performed at a low temperature (4o C) either using chilled perfusate solution or by placing the dissecting bath in a container of ice. The reduced temperature is used to decrease tissue damage by slowing down metabolic processes, thereby reducing the requirements for oxygen and nutrients, as well as reducing enzyme activity. The dissecting bath can also be coated with a layer of silicone plastic (Sylgard) that allows the tissue to be pinned to the bottom of the dish to facilitate dissection.

Dissecting Equipment

The dissecting equipment used should be carefully selected and maintained separate from other equipment in the laboratory to avoid chemical or biological contamination. Instruments made of high quality stainless steel have the advantage of ease in maintenance and cleaning, whilst carbon steel can be used when a sharper tip or edge is needed. Instruments made of titanium and other metals, glasses, ceramics and plastics are also suitable. Forceps and scissors should have tips sheathed with a soft plastic Tygon or silicone tubing for protection when stored. The instruments should be carefully and thoroughly cleaned, preferably using low phosphate soaps, followed by thorough rinsing with distilled water and completely dried before storage.

Sterilization of the instruments is not usually required unless there is a problem of biological contamination/ infection or a requirement for a sterile preparation. Instruments can be sterilized by autoclaving.

Selection and Use of the Tissue/Organ Bath

After the tissue or organ is dissected, it is placed in a tissue-organ bath. Vertical baths require less space to set up than horizontal units; however, horizontal units permit the use of regular or inverted microscopes to visualize the preparation. The bath may be a simple water-jacketed container with an aeration tube and drain. The size/volume of the bath should be selected to accommodate the tissue and any additional instrumentation. However, the bath volume should be minimized to conserve drugs added to the bath. The bath should be securely mounted on a rigid stand to reduce any vibration artifacts.

To maintain perfusate temperature, the solution may be delivered to the tissue chamber via a warming coil positioned within a heated water chamber. The reservoir may also be heated to facilitate temperature maintenance.

Stimulation

Stimulation of tissues may be achieved by a number of methods. Field stimulation may be performed in which ring or plate electrodes are placed near the tissue and a current passed between the electrodes. Alternatively, the tissue can be stimulated directly by attaching electrodes to the tissue. This method is used to stimulate the tissue via a nerve trunk that innervates the tissue i.e. the isolated diaphragm dissected with the phrenic nerve attached to a stimulating electrode.

Perfusate Supply

The pre-warmed perfusate can be added from an external reservoir or beaker and passed through a pre-warming coil and water-jacketed tubing prior to entering the tissue bath. The solution may be drained from the bath using gravity or suction. If a solution must continuously flow through the bath, an overflow port connected to a vacuum or gravity drainage system can remove the solution. Drugs can be added directly to the bath, either via manual pipettes or through the use of syringe pumps or injectors.

Acclimatization/Stabilization

A period of acclimatization or tissue stabilization is generally required to allow the tissue to adjust after the dissection trauma, handling, washing out of metabolites, anesthetics and other products used or produced during tissue preparation and mounting. This period can vary from 10-15 minutes for an isolated heart to several hours for isolated smooth muscle. The duration of the acclimatization will depend on the tissue sample, storage, surgical procedures and experimental protocols. During this time, the tissue may be gradually stretched or undergo pre-treatment with selected agonists or antagonists (to limit or increase physiologic and pharmacological responses).

Perfused Preparations

Preparations with one or more functional blood vessels have the option of being perfused at either a constant pressure or constant flow, with or without recirculation. Perfused systems may be configured in a non-recirculating mode, where the solution only passes through the organ once, or in a recirculating mode, where the solution passes through the organ many times. In both tissues and organs, the non-recirculating mode is useful to remove (wash out) drugs added to the sample and also permits a fresh supply of solution to be available. Recirculating the solution is useful when the experimenter wishes to:

a) Measure the increase in a substance released at low levels by the organ, such as metabolites or neurotransmitters;
b) Add rare or expensive materials, such as radioisotopes, experimental drugs, etc., to the solution and allow the tissue to equilibrate; and,
c) Limit the amount of solution required when the volume of solution necessary to maintain the organ in a single pass system would be excessive, as in a liver preparation.

There are a number of negative and positive aspects to either constant flow or constant pressure systems.

Constant flow preparations are easier to monitor changes in the resistance of blood vessels because with a constant flow rate, any constriction or dilation of the coronary vessels is measured as a change in pressure using a pressure transducer. The production, release, efflux or uptake of substances is also easier to determine and calculate with a constant flow system. A disadvantage of the constant flow system is that if the internal requirements for nutrients in the tissue are not met at a set flow rate, the tissue or organ cannot compensate for this shortfall through vasodilation.

Measurements of changes in vessel diameter in a constant pressure system require flow measurements that can be obtained using a graduated cylinder, drop counters, fraction collectors, flow indicators, magnetic or ultrasonic flow probes. Drug titrations in constant pressure systems require the use of pre-mixed solutions in reservoirs and, therefore, a large amount of drug may be required. Constant pressure systems can also require the repositioning of the elevated reservoir to maintain a constant pressure head, or the use of a refilling pump or pressurized refilling system. In a constant pressure system, the vessels can dilate and increase the total amount of perfusate flowing through the tissue or organ, thereby satisfying changes in tissue oxygen and substrate demands.

Measurements of Tissue and Organ Activity

Control Tissues

When first developing or establishing a preparation, it is important to have a series of positive and negative controls to determine how well the preparation is functioning. These controls may be mechanical, pharmacological or physiological in nature. For example, acetylcholine has been used to vasodilate isolated vessel preparations (that have tone) to determine the presence of functional endothelium that can generate nitric oxide. The stability of the baseline activity, the response to agonists in the presence and absence of antagonists, the effective dose response range, onset of the response, the effect of stretching, oxygen consumption and carbon dioxide production can be used to determine how well a preparation is functioning and how it compares to another preparation. These controls may be repeated at the beginning, middle and end of the experimental series to monitor changes in tissue or organ performance. The use of standard controls also allows quality control and trouble shooting of the preparation once the protocols have been selected.

Force Measurements

To measure muscular activity, the organ, muscle strips or rings are attached by wire or silk suture to a force transducer. This transducer converts the force generated by the muscle into an electrical signal that is then recorded using PowerLab and Chart. The muscle is usually preloaded with a weight or pre-stretched using a tissue tensioner or micrometer. The wire or suture connecting the muscle to the transducer should not touch the bath walls and should be in line with the transducer. The tissue should also be positioned out of the stream of any aerating bubbles from the frit as these will contribute to noise artifacts in the recording.

Isometric or isotonic measurements can be made with appropriate transducers. In an isometric measurement, the muscle length remains constant as the force changes, whilst in an isotonic measurement, the muscle shortens against a constant force. Isometric measurements, which are measured as grams or milliNewtons are more commonly used in pharmacological experiments. Isotonic measurements made at high speed can be used to measure properties such as shortening velocities in tissues. The output range of the transducer should be selected to optimize the size of the signal without plateauing (cropping) the maximum response. The frequency response of the transducer can be adjusted to dampen external vibrations and motion artifacts (noise), but this adjustment may reduce the ability to detect rapid force changes.

Flow Measurements, Electrical Activity and Bioassays

There are other measurements of muscular activity. A blood vessel or intestinal smooth muscle can be cannulated and constriction or relaxation of the vessel measured as a change in flow or pressure. Electrical activity can be measured in muscular (and also neural) preparations through the use of glass microelectrodes inserted into the cells of the preparation.

By equilibrating radioisotopes with the sample, the experimenter can follow drug metabolism, the formation of carbon dioxide, the formation and degradation of second messengers such as inositol phosphates and ion fluxes (^{45}Ca, ^{86}Rb, etc.) into and out of the tissue. These studies may be end point assays, in which the sample is washed out and/or extracted at a given time, or performed on a continuous basis, using fraction collectors or in-line detectors. The tissue may also release chemicals, produced by or stored in its cells, into the bath solution. The presence and identification of these chemicals can be measured by assaying the solution, either continuously in situ or as aliquots removed from the bath. Ion selective electrodes that detect changes in pH, anions or cations, nitric oxide, carbon dioxide or oxygen can be inserted either directly into the bath or be placed in flowthrough adapters that are inserted into the perfusion tubing used to add or remove solution from the bath or sample. In a similar fashion, potentiometric electrodes can be used to determine the release of catecholamines and their chemical identity. A number of biochemical and molecular biological techniques can be used in isolated tissue systems, such as 1 and 2 dimensional gel electrophoresis, Western blots, RT-PCR, etc. Optical methods for determining intracellular ion activities with fluorescent indicators are also possible, and the production of free radicals can be determined through bioluminescent assays that introduce luminol or other agents into tissues. Both vertical and horizontal baths can have optical ports to be used with microscopes or with fiber optic cables placed in line with the tissue samples to permit these above measurements to be made.

A classical way to detect the release of chemicals from tissues is the bioassay. A bioassay for released substances can be performed by exposing another tissue to the released chemicals, causing that second tissue to respond by contracting or causing another type of easily measurable response. The second tissue may be in a standard or specialized bath and can be pre-treated with agonists or antagonist to modify its response characteristics.

Limitations of isolated tissue preparations

With all *in vitro* preparations, consideration of the limitations in the technique should always be onsidered in the experimental protocol. Some of these limitations are listed below and should be taken into consideration.

- Damage to the tissue during dissection and tissue preparation.
- Difficulty in exactly reproducing the normal physiological environment. The tissue will not last indefinitely and may not respond identically to in situ or in vivo preparations.
- If a tissue is not perfused through its blood vessels or is not inherently porous, delivery of oxygen and nutrients is restricted.
- Tissues with high oxygen and nutrient demands, such as cardiac muscle, may have a hypoxic core if the tissue is > 0.5 mm thick due to limited diffusion.
- If the tissue is not thin, then the requirements for nutrients and oxygen must be low as in the case of smooth muscle.

It should be noted that the delivery of oxygen can be limiting in highly active tissues like heart and liver, even if these organs are perfused through their vascular bed.