# DIFFUSION TESTING FUNDAMENTALS

INTRODUCTION

When deciding which type of diffusion cell system you need, or before running an experiment with your new diffusion cell or system, it is important to not only be familiar with the equipment but have a well planned experimental protocol with clear objectives. What do you want to know about a particular compound or membrane? Does it matter which type of membrane you use? What membranes are available that will fit your needs? How will you interpret and present the results? There are many parameters to consider, and working out the details beforehand will reduce the number of failed attempts and produce consistency between experiments that is crucial when comparing results. This paper will address basic concepts of diffusion, describe various materials from which to choose for your diffusion study, and describe the components of diffusion cells and diffusion systems.
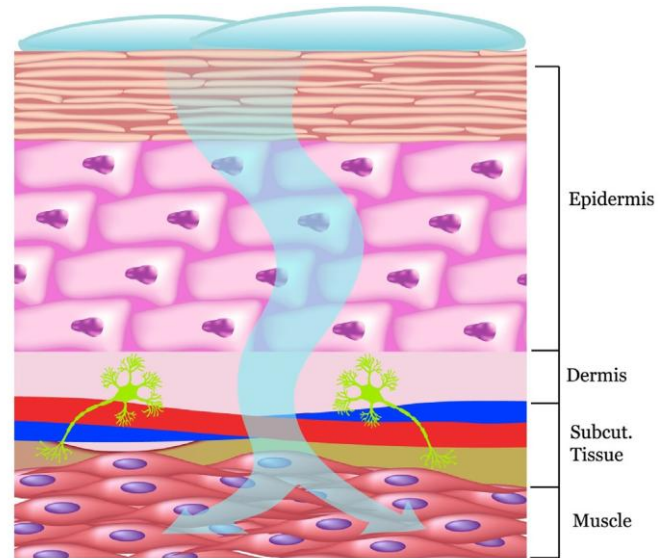
BACKGROUND/TERMINOLOGY

Diffusion is, by definition, the random movement of molecules through a domain driven by a concentration gradient, from high concentration to low concentration. *In vitro* diffusion is generally passive diffusion of a permeant from a vehicle in the donor chamber, through an artificial or biological membrane into a receptor fluid in a receptor chamber, disregarding delivery systems such as iontophoresis and microneedles. The *permeant* is the molecular species moving through or into the tissue/membrane. *Permeation* is the movement of the permeant through the membrane that encompasses first partitioning the membrane and then diffusion through the membrane. Penetration can occur into the membrane without necessarily diffusing, or passing through, the membrane.

Flux is the amount of permeant crossing a membrane per unit area into the circulating system per unit time, and for *in vitro* permeation this "system" is the receptor chamber, expressed in units of mass/area/time. Similarly, accumulation is the amount of permeant crossing a membrane within a certain time, expressed in units of mass/area. *Diffusivity* is a property of the permeant and is a measure of how easily it penetrates a specific membrane expressed in units of area/time. The *permeability coefficient* (K_p) describes the rate of permeant penetration per unit concentration expressed in distance/time.

Any compound applied to either tissue or an artificial membrane will have a *lag time*, the time it takes to permeate through the membrane and diffuse into the receptor fluid and then finally reach a steady state of diffusion. The lag time is the period during which the rate of permeation across the membrane is increasing. *Steady state* is reached when there is a consistent, unchanging movement of the permeant through the membrane. The amount of time it takes to achieve steady state will depend on several factors, the permeability of the tissue or membrane being studied, the properties of the compound itself, and the flow rate of the receptor fluid if flow-thru type diffusion cells are being used.

The *permeability barrier* is a lipid barrier dependent on the amounts, types, and organizations of lipids present in a tissue. Permeability of a particular tissue does not directly correlate to the thickness of that tissue. For example, skin structure varies to some degree over the human body and different permeants exhibit different rank orders for various skin sites.
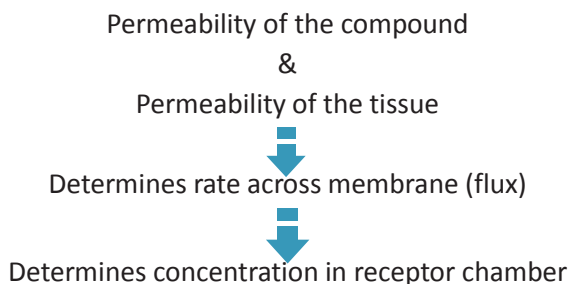
I. EXPERIMENTAL   DESIGN/EQUIPMENT/PRINCIPLES
Choice of diffusion cells: static or continuous flow

Static cell (vertical or Side-Bi-Side):

Description: Franz type cell or Side-Bi-Side Cell, fixed volume receptor chamber, controlled temperature, port to sample receptor fluid, stirred receptor fluid (Side-Bi-Side Cells allow stirring of both the donor and receptor chambers.)

FRANZ CELL

SIDE-BI-SIDE CELL
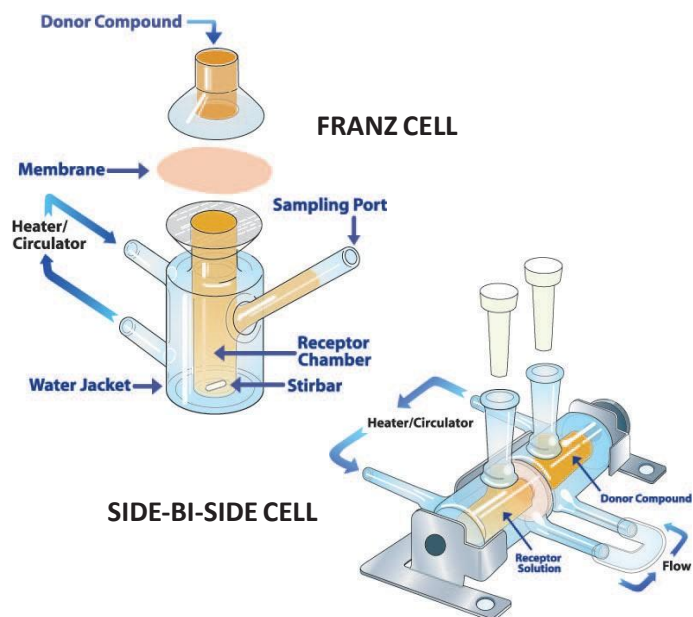
Uses: Evaluating compound uptake into a membrane, finite dose permeation, steady state flux of compounds (either alone or in formulations.)

Permeability of the compound
&
Permeability of the tissue
↓
Determines rate across membrane (flux)
↓
Determines concentration in receptor chamber
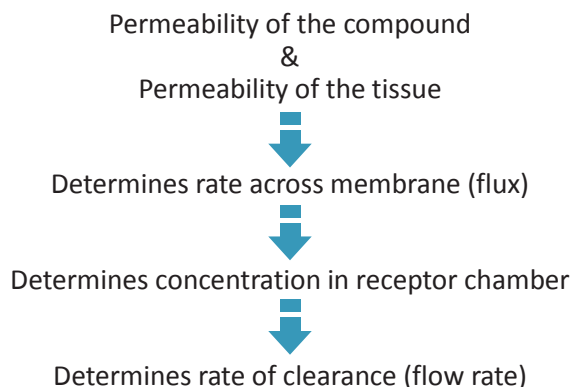
Considerations:

1. If you are using a highly permeable compound with a large volume receptor chamber, the large volume reduces the gradient, so build up of the compound in the receptor part is not a problem (sink conditions are maintained).

2. If you are using a highly permeable compound with a small receptor chamber, the buildup of compound reduces the concentration gradient and therefore slows the flux of the compound (non-sink conditions).

3. If you are using a low permeability compound, then detection of the compound in a large volume receptor chamber can be a problem.

Continuous flow cell or flow-thru cell:

Description: Franz type cell or In-Line Cell, fixed volume receptor chamber, controlled temperature - Franz type cells are stirred, In-Line Cells have continuous flow which causes turbulence in the receptor chamber and simulates stirring, flow rate is adjustable, permits automated sampling.

IN-LINE CELL
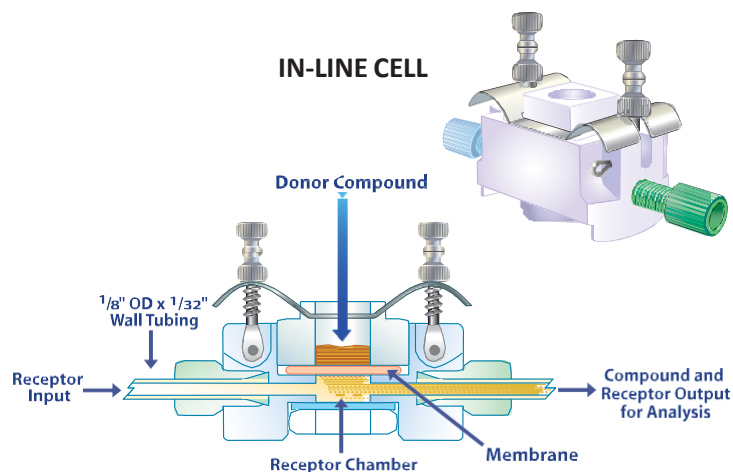
Uses: Mimics in-vivo (flow equates blood flow), evaluating compound uptake into membrane, finite or infinite dose permeation, steady state flux and K_p of compounds (either alone or in formulations.)

Permeability of the compound
&
Permeability of the tissue
↓
Determines rate across membrane (flux)
↓
Determines concentration in receptor chamber
↓
Determines rate of clearance (flow rate)

Considerations:

1. If you are using a highly permeable compound, the high flow rate necessary to clear the receptor chamber to maintain gradient can result in a large volume of permeant to analyze.

2. If you are using a low permeability compound, the flow rate necessary to clear the receptor chamber can result in a large volume of permeant for which detection can be a problem.

3. A smaller receptor chamber requires less permeant to clear the receptor chamber which results in less permeant volumes and better detection.

II. MEMBRANE TYPES

1. **Human tissue** *ex vivo:* It may be difficult to obtain sufficient quantities of normal, healthy human tissue to perform permeability experiments with large enough sample sizes for statistical analysis. Many laboratories use human cadaver skin obtained from accredited U.S. human tissue banks. There might be ethical and legal considerations in obtaining human tissue biopsies and surgical specimens.

2. **Small animals:** Models such as rats, mice, and rabbits have traditionally been used in research for human tissue permeability as they are usually inexpensive to purchase and maintain. Drawbacks are the tissue areas are usually thinner than human skin and have a different morphology resulting in higher compound permeabilities.

3. **Large animals:** Monkeys, dogs, pigs, and other large animals have also been used extensively but may be expensive to purchase, especially in the cases of monkeys and dogs. Pig and monkey soft tissue is very similar to human soft tissue in terms of morphology and function, therefore is widely used as a surrogate for human skin.

4. **Polymeric membranes:** These kinds of membranes are usually used for *in vitro* release testing (IVRT). According to the FDA SUPAC-SS (May 1997), any "appropriate inert and commercially available synthetic membranes such as polysulfone, cellulose acetate/nitrate mixed ester," ... "of appropriate size to fit the diffusion cell diameter" can be used. Usually hydrophilic polymeric membranes with a pore size of 0.45 μm are used. Once set during the IVRT method developing phase, the same membrane should be used for the entire duration of the project.

Polymeric membrane

5. **Human skin equivalents (HSEs):** These are tissues engineered 3D skin constructs (such as MatTek products) that utilize a combination of cultured human skin cells and extracellular matrix components under controlled culture conditions. The advantage of these tissues is that viability can be maintained. The main disadvantage is that the permeability of the simulated tissue is generally higher than that of *in vivo*.

Artificial membranes and animal models can be valuable predictors for compounds and formulations for *in vivo* use. You can address metabolism, penetration, and distribution of the compound of interest, but be careful about making direct claims for that compound in a specific model to human *in vivo* use.

Human skin equivalent

Stratum Corneum

Epidermis

Dermis

Viable Fibroblasts

Photo courtesy of MatTek Corp.
http://www.mattek.com/ pages/products/ epidermft
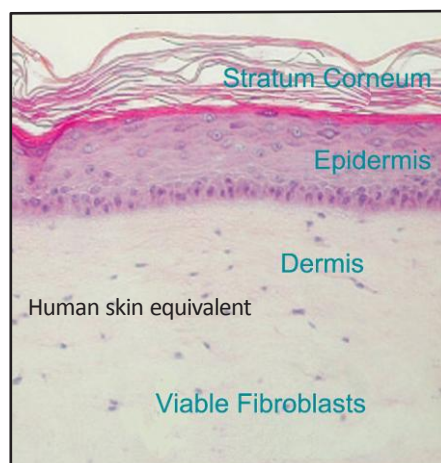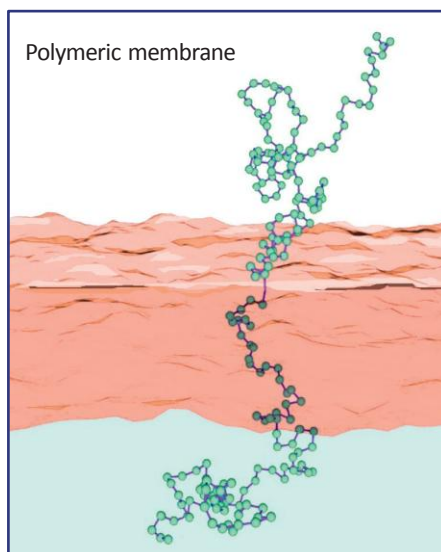
III. DONOR FORMULATIONS

The compound of interest can be prepared in a variety of formulations - liquid solutions, suspensions, creams, gels, ointments, lotions, pastes, powders, or adhesive patches. The formulation chosen should mimic the real world application as much as possible. Varying the formulation, concentration, and adding permeability enhancers can also help you gain valuable information about the permeant characteristics and behavior.

1. **Application - infinite dose/finite dose:** The concentration of the permeant will depend on the study aims.

a) An infinite dose, one in which the permeant will not be depleted from the donor formulation over the course of the experiment, will produce fundamental permeation behavior and is used when testing that behavior in the presence of permeability enhancers.

b) A finite dose mimics the amount of permeant that would be practical in actual use; for example, a specific amount of drug to be administered to a patient. The aim would be to determine what amount of the permeant needs to be incorporated in a specific vehicle that will deliver the required amount of drug to the patient in order to be effective.

c) An alternative application would be to mimic application of a compound that would in actuality temporarily remain on the tissue; for example, a salve applied to the skin or a drug to the inside of the mouth. The permeant would be applied to the tissue for a short period of time, it would then be removed, and samples would be collected for a length of time following permeation.

2. **Vehicle formulation:** Also named blank or control formulation, vehicle formulation is the application dose without the active pharmaceutical ingredient (API) or drug agent. The vehicle in which you choose to incorporate the permeant of interest, whether liquid, semi-solid or paste, will again depend on your study aims. There are several things to consider:

a) Aqueous solutions and phosphate buffers are good options for getting basic permeation data.

b) Lipophilic compounds have a low aqueous solubility and may require alternative vehicles such as water/alcohol mixtures or propylene glycol. It must be noted that these additives may interact with the structure and permeability barrier of the tissue and thus change the flux of the permeant.

c) Inert materials can affect the flux of the permeant through both artificial and animal membranes.

d) The nature of the vehicle can alter the potency of certain enhancers. That is, the same enhancer can affect the penetration of a specific permeant differently depending on the vehicle it is incorporated in.

e) Selection of buffer is important to control the degree of permeant ionization. Adjusting pH can also fully dissociate the permeant into charged ions.

f) The thermodynamic activity (the driving force for permeation) of the same concentration of permeant can change drastically by modifying the vehicle components.

3. **Permeant detection:** Consideration must be given to the method of permeant detection. This can be performed by chromatographic or spectroscopic methodologies, or the use of radio-labeled permeants. A discussion of advantages and problems associated with these methods can be found under Section V. SAMPLING.

4. **Other considerations:** For each formulation tested, a minimum of three parallel cells should be used for every run. Due to biological variability in skin, in many cases 5-6 replicates should be run per group. Evaporation of compounds, solvents, or formulations from donor chambers may occur, especially if the cells are maintained at high temperatures.

IV. RECEPTOR MEDIA

Selection of a receptor solution depends on the nature of the permeant and the type of diffusion cell used. An ideal receptor medium for an *in vitro* permeability experiment should mimic the *in vivo* situation. Flow-thru systems minimize accumulation in the receptor chamber, thus aqueous receptor fluid is usually adequate. This could be a concern for static cells where the permeant is not continually cleared.

Solubility of a compound should be taken into consideration so that the compound is at its desired form in the donor formulation as well as being delivered at a certain amount in the receptor medium; which would affect the diffusion gradient and could slow diffusion.

Aqueous receptors are common for hydrophilic and moderately lipophilic permeants. Phosphate buffer saline (PBS, pH 7.4) should be used if the permeant is ionizable. For un-ionizable permeants, a solubilizer added to the receptor medium should be sufficient. More lipophilic permeants or permeants with low aqueous solubility require additional solubilizing agents such as surfactants, protein (bovine serum albumin) or organic solvents (ethanol/water systems). The effect of these solubilizers on the penetration of your compound of interest needs to be considered as these solubilizers may cause damage to the barrier or changes in the tissue which can alter the permeability results. They should, therefore, be used at the minimum concentration necessary to solubilize the permeant.

V. SAMPLING

The best method of sampling in terms of time intervals, frequency, and volume, and the best method of reporting the permeation of a compound of interest will depend on your research question. Do you want to know how much compound crosses a membrane at short time intervals, the total amount of compound that crosses the membrane over a long sampling period, or the total amount of compound found within the membrane after a given exposure period? Do you want to calculate flux, accumulation or permeability constant (K_p)? The following addresses factors that will help you determine these parameters.

Flux and permeability can be determined using radio-labeled or unlabeled compounds. The calculation of each parameter can be found in the next section. The flux of a compound can be converted to an amount of compound (in ng or µg) per time interval. Regardless of how your analysis will be performed, you need to know the time points of the specific samples to report the results as either of two types

of accumulation: 1 - The total quantity of the compound in the collected sample per time interval, regardless of volume, and 2 - As the concentration of the compound, i.e. the amount of compound per standard volume of sample.

Radio-labeled compounds can be detected in very small quantities and in a large volume of receptor medium. Analysis is usually quite rapid and accurate. The entire collected volume can be easily counted using a scintillation counter, and then the flux or K_p calculated from the counts per minute (cpm) obtained can be converted to the amount of compound in any given sample at any given time interval by knowing the specific activity of the labeled compound. Many compounds are readily available and inexpensive to purchase, but other compounds, such as pharmaceuticals, are very expensive to synthesize and may have handling and storage issues. For example, a radio-label could be unstable in the donor formulation.

Non-radio-labeled compounds can also be used in permeability experiments and detected with methods including high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), and fluorescence. These methods are versatile; they can be adapted to many permeants, and you can often conduct multiple analyses. It is possible to determine if the same species of compound that permeated the membrane was that which was applied. The disadvantage to these detection methods is that very small quantities of compound can go undetected or under-detected because of the large volume in the receptor chambers of static cells or large quantities collected from flow-thru cells. As small samples are required for quantification by HPLC or ELISA, you need to take aliquots of the sample and then calculate the total compound in a given sample over a given interval. You can also decrease the volume of the sample by drying it down to a total quantity of sample that can then be analyzed.

As the results of your experiment will be reported as the amount of compound penetrating a membrane over time (see Section VI.), the volume of aliquots taken for analysis and the time intervals between samples needs to be precise to obtain reliable data. When using a static system, the receptor fluid volume will be the same for all the diffusion cells, therefore, the amount of compound determined in an aliquot can be easily calculated for that specific volume. The samples should be taken quickly and consecutively from all receptor chambers so that the time interval is consistent for all samples. If there is a delay of minutes between taking samples instead of seconds, this should be taken into account when performing calculations. When using a fraction collector with automated sampling, the time

intervals for the samples from all the cells will be exactly the same. There may, however, be slight variations in the volume of receptor fluid pumped through each diffusion cell into the collection vials. This is due to slight variations of the tubing element diameters or clamping pressures in the pump cassettes but is not a concern if the total volume of sample is being analyzed. If aliquots are taken for analysis, then the volume of each collected sample must be measured so that the total amount of compound in the sample can be calculated precisely. For example, if a 100 µl aliquot of sample is determined to contain 2 pg of compound, then there would be 20 pg total in a 1 mL sample but 24 pg in a 1.2 mL sample.

It may be necessary to obtain a small volume of receptor fluid over a long period of time with your continuous flow system. This would occur when you want to reduce the amount of sample for analysis to avoid pipetting or to detect a compound that you know would only penetrate in very small quantities over a period of time. In essence, you are converting to a "static" system. In this instance you would turn the pump off for a period of time and then quickly purge the receptor chamber by running a specific volume through it to obtain your sample. This is a valid method if you report the time interval correctly. In this case, the time interval would be from the time the pump was turned off through the end of the purging of the receptor chamber. Keep in mind with this method that the driving force for diffusion is the concentration gradient. If the membrane is very permeable, the compound could accumulate quite rapidly in the receptor medium, consequently increasing the concentration in the receptor chamber and thus slowing diffusion. You must also be sure to adequately purge all the compound that has accumulated in the receptor chamber.

A buildup of compound in the tissue followed by a slow release into the receptor medium can occur. This has been demonstrated in experiments where a compound of interest formulated in a liquid, gel or paste is applied to a membrane and then removed after a short period of time but samples are collected after the removal. The compound may continue to diffuse for some time up to several hours after removal of the compound from the membrane surface. This can be attributed to a reservoir effect. The upper layers of the tissue absorb the compound fairly rapidly, and once saturated, the compound diffuses through the deeper layers of the tissue and into the receptor medium at a fairly constant rate. Again, as long as the concentration gradient remains, the diffusion will continue.

The frequency of samples collected will be determined by the goals of your experiment. If you have no prior concept of the permeability of a membrane to a particular compound, it is initially prudent to take small samples over a long period of time to determine the onset of steady state flux and the overall permeability. If you know the membrane is highly permeable to the compound, you will want to take very short and frequent samples. If you only want to know the total amount of compound to cross the membrane in a given time period, you can take fewer samples with longer time intervals. It is best to do several replicates for a given compound and condition, and then average the amount of compound collected at each time interval for those replicates. You can then plot these results over the entire experimental period, which will provide you with of reliable data. For example, one scenario would be to collect for very short intervals in the beginning of an experiment, such as 15 minute intervals for one hour, 30 minute intervals for 2 hours and then 60 minute intervals for several hours. This will allow you to determine how quickly a drug permeates, when it reaches steady state flux, and finally the total quantity of compound permeating the tissue over the entire sampling period. Using this you can calculate flux or K_p for whatever time period is of interest for this compound. You can determine the amount of radio-labeled compound still in the tissue by autoradiography or by solubilizing the tissue and counting in a scintillation counter. You can also identify compound in the tissue by grinding and/or homogenizing it in liquid and centrifuging off the liquid to be analyzed with HPLC, ELISA, Luminex or pyrosequencing.

VI. CALCULATING FLUX AND PERMEABILITY COEFFICIENT (K_P)

Flux (J) is the amount of permeant crossing the membrane per time. It is given in units of mass/area/time or in units of radioactivity/area/time. Note: If the permeant was applied in a finite dose, then you can only calculate a flux by the formula: $J = Q/(A \bullet t)$ where Q is the quantity of compound traversing the membrane in time t, and A is the area of exposed membrane in cm^2. Units of flux are quantity/cm^2/min.

Examples: ($\mu g/cm^2/h$) or ($pg/cm^2/min$)
($cpm/cm^2/min$)

Steady state flux (J_{ss}) is the amount of permeant crossing the membrane at a constant rate; this occurs after the lag phase when the amount continues to increase. When the amounts measured at successive sampling intervals are not significantly different, this is considered steady state.

$J_{ss} = Q/(A \bullet t)$ where Q is the quantity of compound transported through the membrane in time t, and A is the area of exposed membrane in cm^2. The unit of steady state flux is quantity/($cm^2 \bullet hr$).

If the amount of permeant applied to the membrane was an infinite dose, then the permeability constant (K_p) can be calculated from the relationship $K_p = Q/[A \bullet t \bullet (C_o - C_i)]$ where Q is the quantity of compound transported through the membrane in time t (min), C_o and C_i are the concentrations of the compound on the outer side (donor side) and the inner side (receptor side) of the membrane respectively, and A is the area of exposed membrane in cm^2. Usually C_o can be simplified as the donor concentration and C_i as 0. The units of K_p are cm/min or cm/hr.

VII. METHODOLOGY

However you design your experiments, you should be consistent and precise in your methods. It is imperative to become familiar with your equipment, to set some baseline values for the membranes or tissues you plan to use, and to run several compounds if possible to determine if your detection method will be adequate. Initially run some simple experiments with as few variables as possible. Once you have set baseline values, it will be easier to ascertain that your experiment is running smoothly or if there is a problem with the methodology.

Be consistent with preparation of the tissue and membranes. Mount the tissue carefully to avoid overstretching or damaging it by perforation. Specimens must be large enough that there is an edge of tissue which can be securely clamped in the diffusion cell around the orifice. Care must be taken to ensure that tissue does not protrude into the receptor chamber which could block the flow of receptor fluid.

Use tissue as soon as possible after excision or freeze it at -80°C until you do use it. Keep notes on the time from harvest to use when excising tissue from live animals.

Be precise in compound preparation, receptor medium preparation, pipetting samples, and sampling intervals. Changing the pH of or adding solubilizers to your solutions can affect the penetration of some compounds. You should be aware of this and be able to justify your methods.

Temperature can affect the results of your experiment so you must be consistent in using room temperature, skin temperature, or body temperature when comparing compounds. Report temperature in your methodologies. Always attempt to reproduce the *in vivo* situation as closely as possible.

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