# Short Interval Monitoring of Glucose in Zucker Diabetic Fatty (ZDF) Rats

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*Microdialysis and ultrafiltration sampling techniques provide a method of studying glucose dynamics in the Zucker Diabetic Fatty rat without the use of blood sampling.*

Rodent models of disease are very useful for studying disease pathophysiology and also for testing safety and effectiveness of potential treatments. One model of Type II diabetes is the Zucker diabetic fatty rat (ZDF/Gmi™). These rats have been developed by Genetic Models, INC of Indianapolis.

Human Type II diabetes, also known as non-insulin dependent diabetes (NIDDM), is the most prevalent form of diabetes constituting about 80% of the diabetes cases. Its time of onset is during the middle to later years of life. It is often preceded by obesity, wide glycemic excursions often resulting in hypoglycemic episodes, and insulin resistance. There are about five million type II diabetics in the U.S. with a possible additional five million undiagnosed cases. The study of the pathophysiology of type II diabetes in rodents could have a significant effect on preventing or moderating the debilitating effects of the disease in the human population.

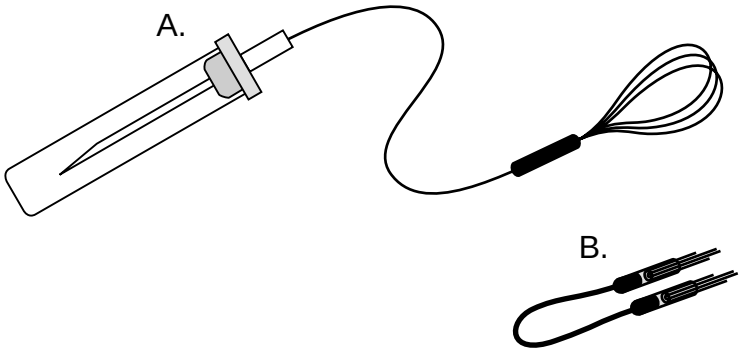
The obese Zucker rat was first described in 1961 (1). These rats carried a recessive *fa* gene for obesity. They were insulin resistant, hyperinsulinemic and glucose intolerant but did not become frankly diabetic. In a colony of these rats maintained at Indiana University School of Medicine, some of the obese rats developed diabetes. These were initially described by Clark *et. al.* (2-4). The ZDF model was developed by selective inbreeding for the diabetic trait (5,6). The glucose levels of young obese male rats are normal. Between 7 and 10 weeks of age glucose levels rise sharply and then more gradually after 10 weeks of age. Female obese rats do not usually become diabetic, but diabetes can be induced by manipulation of dietary glucose and fat. The ZDF model is hypercholesterolemic and hypertriglyceidemic (6). Studies of the model have also shown some vascular changes in the retina and some changes in peripheral nerves.

Studies on these diabetic rat models frequently involve monitoring glucose concentrations. The traditional method of obtaining a sample for measurement of glucose is to obtain blood from the tail by snipping the end of the tail. An alternative method would be to cannulate a jugular vein and maintain a heparinized vascular access. Both of these methods place limitations on the number of samples which can be collected and thus on the amount and frequency of data collected. Removing blood from the tail is painful and stressful to the animal. Stress can cause the release of stress hormones which may elevate glucose creating artifacts in the data. Vascular sampling is less stressful to the animal, but maintaining patent vascular access over long periods of time is difficult. Repeated vascular sampling also increases the probability of introducing a systemic infection. Because of the small size of rodents there is a limitation placed on the number of samples which can be obtained by the small blood volume. Repeated sampling can cause anemia and affect the general health of the animal. This can be partially alleviated in vascular access sampling by separating the plasma and returning the cells.

Blood sampling for glucose measurement is also done during glucose tolerance tests to evaluate the progress or severity of the diabetes. During this test four or five samples may be taken to obtain the shape of the curve. There are some questions about the pathophysiology of this model which might be addressed by more frequent sampling of glucose. These include the basal insulin release patterns of

**F1**

*Ultrafiltrate probe with Vacutainer™ collection (A). DL-5 microdialysis probe (B).*




obese *vs.* non-obese. Release of frequent small quantities of insulin *vs.* less frequent larger releases should be reflected in basal glucose fluctuations. This possibility could be investigated by monitoring glucose levels at 5 minute intervals or less. Progressive changes in glucose tolerance or meal response could be monitored by continuous glucose sampling. The facility for this amount of data collection is not available through blood sampling.

An alternative to blood sampling which can potentially increase the amount of data obtainable from small rodent models such as the ZDF rat is membrane probe sampling (7). Microdialysis (MD) and ultrafiltration (UF) are two techniques which utilize such membrane probes to sample the extracelluar space. In MD, an isotonic solution is pumped through the membrane and low molecular weight molecules diffuse across the membrane in response to a concentration gradient. In UF, a negative pressure is applied to the probe, and extracellular fluid is drawn through the membrane under the driving force of the pressure gradient. The recovery of an analyte, which is the ratio of the concentration of the analyte in the sample to the concentration in the extracellular fluid, is affected by different factors with each technique. In MD, the recovery is related to membrane surface area and flow rate. In both techniques recovery is influenced by temperature and analyte-membrane interactions. In MD, the volume of sample obtained per unit time is determined by setting the perfusion flow rate, so it can be easily controlled by the investigator. In UF, the flow rate is determined by the membrane surface area. In UF the recovery of low molecular weight hydrophilic molecules is close to 100%. The UF glucose concentration, therefore, is the same as the extracellular fluid glucose concentration. One of the major benefits of the UF technique is that concentrations can be determined directly without any recovery calculations. MD probes with a long membrane can be used with a low flow rate to give high recovery rates. There are a number of methods to independently determine MD in vivo recovery rates. One of the simplest methods is to use both a UF and a MD probe and compare the UF to MD sample concentrations. Once the recovery rate is known, the sample concentration can be related to the extracellular fluid concentration. The simultaneous use of MD and UF probes permits the investigator to take advantage of the benefits of each technique simultaneously. The MD can provide for frequent sampling and the UF can provide the information for calculating the actual interstitial concentration in the MD samples.

The ideal probe implantation site for glucose measurement in the rat is the subcutaneous tissue. This provides a large easily accessible space for implantation of long membrane probes which optimize recovery for MD and sample volume for UF. Previous studies in dogs and humans have demonstrated that subcutaneous UF and blood glucose are the same (8-10). Previous studies in streptozotocin diabetic mice have also demonstrated the utility of membrane probes for long-term glucose monitoring of small rodents(11).

Coupling membrane probe sampling with a method of glucose analysis, which requires small sample volumes, permits sampling intervals on the order of three to five minutes. The Bioanalytical Systems immobilized enzyme reactor (IMER) method of glucose analysis (12,13) requires only 1 to 2 µL of sample. This is a liquid chromatography flow injection method. The sample is injected into the chromatograph. It flows through the IMER which contains glucose oxidase. A byproduct of the enzymatic reaction is $H_2O_2$. The $H_2O_2$ is then reduced at a post-column peroxidase electrode. The current produced at the peroxidase electrode is proportional to the original glucose concentration.

In this study, littermate lean and obese ZDF rats, which were received from Genetic Models, INC, were studied with UF and MD probes at various stages to demonstrate the utility of membrane probe sampling for glucose tracking in these models.

## *Materials and Methods*

### Rats

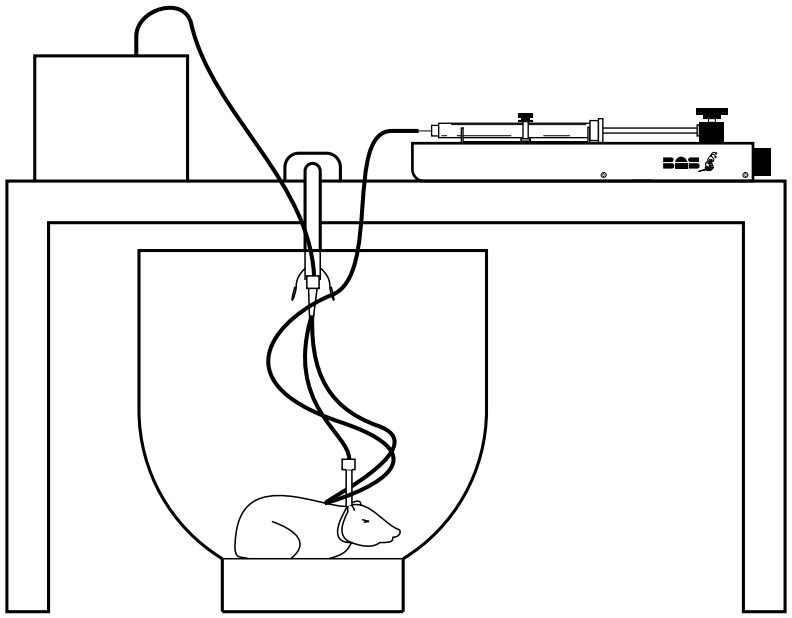
Male Zucker, ZDF rats were received from Genetic Models, INC., (P.O. Box 68737, Indianapolis, IN 46268-0737). For this study two 2-month-old littermates, one lean and one fatty, and one 6-month-old fatty rat were used.

### Probe implantation

An ultrafiltration and microdialysis probe were implanted subcutaneously on opposite sides about 1 cm off the midline down the back of the rat according to the procedure described previously (14). The UF probe was a UF-3-12 (MF-7023, BAS) ***(F1a)*** which contained of three fibers, each 12 cm long in a looped construction, making the implantable length of the probe 6 cm.

**F2**

*The BAS Beekeeper is a system to house awake and active rodents during in vivo sampling experiments. A balanced swivel arm keeps tubing lines free and safely removed from the animal.*



The MD probe was a DL-5 (MF-7051, BAS) ***(F1b)*** which contained one 5 cm fiber. This was implanted in a looped configuration making an implantable length of 2.5 cm. All probes were sterilized with ethylene oxide.

The rats were anesthetized by intraperitoneal injection of a 10:1 mixture of Ketamine (100 mg/mL) and Xylazine (100 mg/mL) at a dose of 0.1mL/100g. For the MD probe implantation, two small (3 mm) incisions were made, one at the neck and another at the 4 cm posterior. For the UF probe implantation, incisions were at the neck and at the 8 cm posterior. The probes were each placed inside an introducer (MR-5313, BAS). The introducer was inserted into the proximal incision and tunneled subcutaneously to the distal incision. The introducer was withdrawn from the distal incision, leaving the probes in place. The incisions were sutured. The proximal sutures also served to anchor the probes. A needle hub was attached to the UF probe for vacutainer sampling.

### Awake animal system setup

The rats were placed in the BAS BeeKeeper (MD-1575, BAS) ***(F2)***, consisting of a large polycarbonate bowl, swivel, balance arm, tether system, and table. This animal system allows the animal to move around freely and practice normal rodent patterns of eating and sleeping. Overall, it minimizes stress to the animal and allows for continuous sample collection. Before the animal was placed in the awake animal system the tubing and swivel components were sterilized by perfusion for one hour with Gericide cold sterilant followed by one hour of sterile Ringer's solution to wash out the sterilant. This procedure prevents introduction of bacteria into the samples which could consume the glucose and give erroneous data. After the rat was in the awake animal system, the microdialysis tubing was connected to the liquid swivel and the probe was perfused with sterile Ringer's solution using the modular BAS microdialysis pump (MD-1000, MD-1001, BAS). Various flow rates were used for different sampling intervals. For frequent sample collection: 5 µL/min. and 7µL/min. were used. For long interval sampling: 2 µL/min. was used and for overnight or weekend sampling: 0.2 to 1 µL/min. were used so that the syringe would not have to be refilled as often. Microdialysis samples were collected in a fraction collector. Since the two channels of the swivel were being used for the microdialysis perfusion and collection the ultrafiltration samples were collected by hub and vacutainer. Sample collection by hub and vacutainer necessitates longer sampling times because of the interruption to sampling cause by breaking the vacuum at the time of sampling. It does allow for simultaneous MD and UF sampling.

### Glucose Studies

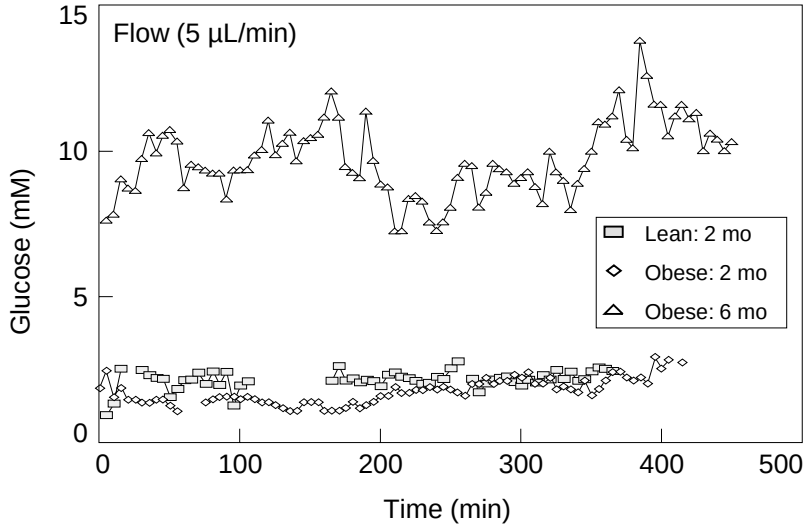
To demonstrate the utility of membrane sampling techniques, a number of different sampling protocols were used: 1) To study short term variations in glucose which may reflect patterns of insulin release, 5 minute samples were collected under fasting conditions. 2) To demonstrate the utility of membrane probe sampling in traditional tests, rats which had been fasted overnight, were given glucose orally at a dose of 2 g/kg. Sampling was at 15 minute intervals. 3) To demonstrate the effect of a bolus of food, rats that had been sampled under fasting conditions were fed a bolus of food. Sampling was continued at 5 minute intervals. 4) To follow normal glucose fluctuations under normal conditions, the rats were allowed food *ad libitum* and glucose was sampled at 5 minute intervals.

### Glucose analysis

Glucose was analyzed on a BAS 480 liquid chromatograph by flow injection analysis using a glucose immobilized enzyme reactor (MF-6152, BAS) (12) preceded by a Phase II ODS, 3 µM, 40 x 3.2mm column. Peroxide generated by the IMER was detected by a "wired" peroxidase electrode (13). The mobile phase was 20 mM phosphate containing 750 µL/L 1,5 di methyl hexylamine, 5 mL Kathon (CF-2150, BAS), pH 5.5. This is an extremely sensitive method which requires only 1 to 2 µL of sample. The samples are diluted with mobile phase 50 to 400 times depend-

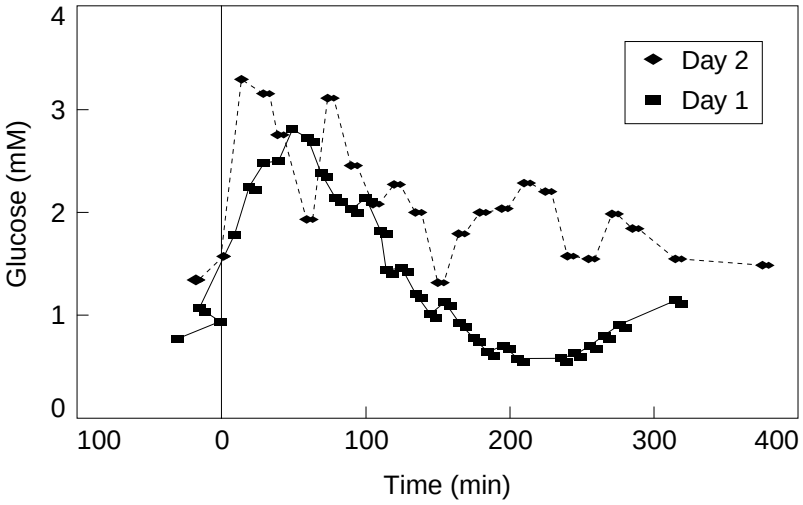
**F3**

*Microdialysate samples in fasted rats obtained at 5 minute intervals using subcutaneously implanted DL-5 probes with a flow rate of 5 µL/min. Both the lean and obese 2-month-old rats show normal glucose concentrations. The 6-month-old obese rat is diabetic.*




**F4**

*Subcutaneous microdialysis sampling during an oral glucose tolerance in a 2-month-old obese rat.*




ing on glucose concentration of the sample.

## Results and Discussion

### Determination of in vitro recovery

It has previously been determined that ultrafiltration probe recovery for glucose is 100%. Therefore, by comparing the ultrafiltrate sample glucose concentration with the concentration of glucose in the simultaneously obtained microdialysate sample, one can determine the glucose recovery of the microdialysate probe. Utilizing this technique for UF and DL probe implantation in the 6-month-old obese rat, the glucose recovery of the DL-5 probe was 32% at a flow rate of 5 µL/min. and 69% at 2 µL/min. in this rat. For the 2-month-old lean rat the recovery was 42% at 5 µL/min. These recovery rates can be used to calculate the actual subcutaneous interstitial glucose levels in this rat.

### In Vivo Studies

*Fasting glucose levels:* Microdialysis samples were obtained at 5 minute intervals under fasting conditions using a 5 µL/min. flow rate in three different rats over a 7 hour period. The average microdialysate glucose in a 2-month-old lean rat was 2.1 mM. The average glucose in the obese littermate of this rat at the same age was 1.7 mM. An obese rat at 6 months of age had an average fasting glucose of 9.6 mM. Since the recovery was previously determined for the probe in the 6-month-old fatty rat, the actual average subcutaneous glucose levels can be calculated to be 30.1 mM. This rat is severely diabetic. **F3** shows the degree of variation in short interval glucose samples.

*Glucose Tolerance Test:* Two glucose tolerance tests were done on the 2-month-old obese rat 4 days apart. Since these tests were done before a feeding needle was obtained, the dosing was only approximate. The rat was fasted overnight before the test. The glucose was dissolved in water and fed to the rats via a pasteur pipette.

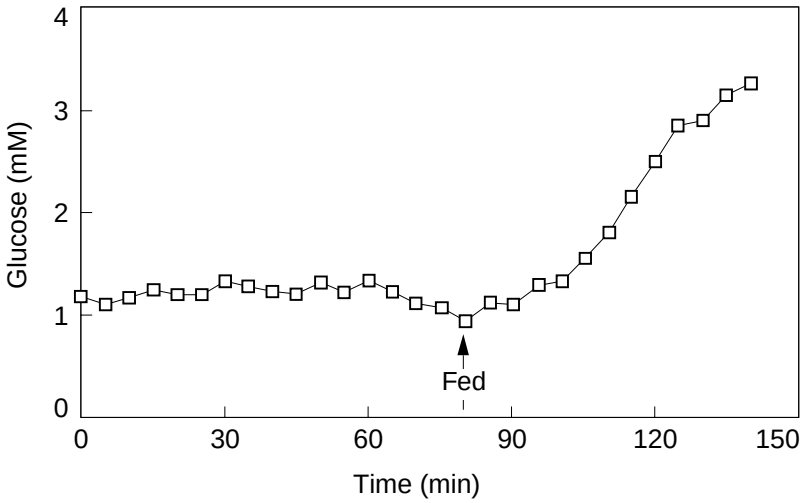
Overnight fasting in this rat resulted in a low glucose concentration. The exact glucose level was not determined, because the rat did not have a UF probe for *in vivo* calibration. An estimation of *in vivo* glucose concentration can be made by assuming that the recovery was between 32% and 42% obtained for the other rats. This would mean that the fasting glucose concentrations are between 3 and 4 mM. The first test had a classic glucose tolerance curve pattern. The glucose rose to a peak at 50 minutes, fell to a minimum level at 210 minutes (which was below the fasting level), and then returned to the fasting level by 300 minutes. This pattern is similar to the glucose tolerance pattern demonstrated by Rohner-Jeanrenaud (15) in 13-15 week old *fa/fa* rats.

The second glucose tolerance test, performed 3 days after the first, resulted in an unusual glucose pattern. There was a rapid rise in glucose followed by a rapid decline and then a rebound in glucose concentrations. This pattern of decline and rebound was repeated until glucose appeared to stabilize at the pre-test concentration of about 5 hours. One could postulate that the initial glucose exposure induced a more rapid response to glucose disposal on the second exposure and that the very rapid fall in glucose initiated counter regulatory measures.

*Effect of food after fasting:* There was no hyperglycemia in the 2-month-old obese rat under fasting

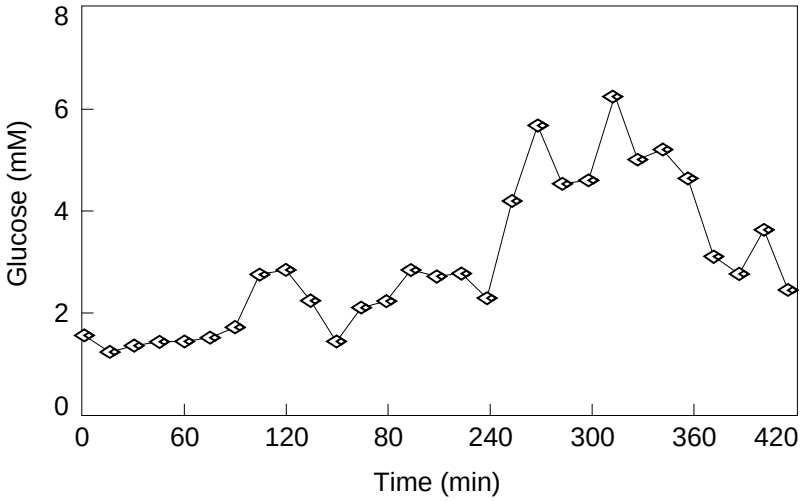
**F5**

*Microdialysis sampling. A 2-month-old obese rat was fasted and then fed at 80 minutes. Subcutaneous glucose rose sharply after feeding.*



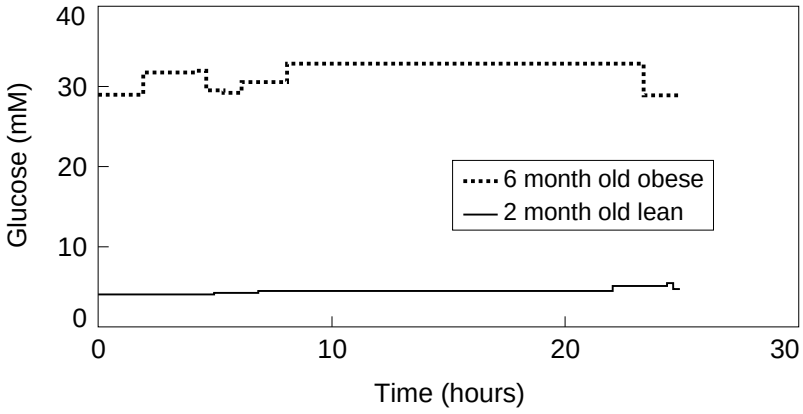

**F6**

*Microdialysate sampling during a normal day of a 2-month-old obese rat with food and water ad libitum.*




**F7**

*Ultrafiltrate sampling from lean and obese rats.*




conditions. But if this rat was given food after being fasted there was a rapid rise in glucose levels as seen in ***F5***. This indicates that the rat may have been at a transitional stage between non-diabetic and diabetic state.

All of the previous tests involved some type of experimental control: either fasting or dosing with glucose. It is also potentially useful to monitor the glucose dynamics and determine total glucose exposure if rats are allowed food *ad libitum* in an undisturbed condition. This type of monitoring could be useful in evaluating how well the animal model simulated the human type II diabetic condition.

***F6*** illustrates the normal fluctuation in the glucose of a rat during a normal day. Microdialysate samples were obtained at 15 minute intervals through out the day. Food placed in the cage the previous day had all been consumed. Food was placed in the cage after the third sample was taken. The rat ate some immediately and then buried the rest. Food was consumed *ad libitum* for the rest of the day.

Microdialysis provides for frequent sampling but does not give absolute concentrations because recovery is less than 100%. An alternative sampling technique, ultrafiltration does have recovery rates close to 100%; therefore, actual glucose concentrations can be observed directly. ***F7*** illustrates the glucose levels in a 2-month-old lean rat and a 6-month-old obese rat under conditions of food *ad libitum*. In this case the samples represent glucose averages over longer periods of time because the vacutainer system was used to obtain samples. It is also possible to use a peristaltic pump and fraction collector and obtain shorter duration samples.

The glucose concentrations observed in the obese rat are consistent with previous observations for rats of similar age and genotype (6).

### *Conclusions*

Using the fasting glucose levels, there is no indication of glucose intolerance in the 2-month-old lean rat. For the 2-month-old obese rat, even though fasting glucoses were normal, glucose levels in the fed condition and the rise in glucose levels when food was given after fasting indicates that glucose tolerance may have been decreasing at this time. The 6-month-old obese rat is diabetic.

These studies demonstrate that membrane probe sampling is an extremely useful technique for

studying glucose dynamics in animal models of diabetes. Sampling can be done at almost any interval desired down to at least 5 minutes. Because no blood is removed, sampling can be done at any desired frequency and for extended periods of time.

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