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# Direct Measurement of Hydrogen Peroxide ($H_2O_2$) or Nitric Oxide (NO) Release: A Powerful Tool to Assess Real-time Free Radical Production in Biological Models

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## Abstract

Vascular endothelial dysfunction is a common and early event occurring in many disease conditions, such as ischemia/reperfusion (I/R) injury, vascular complications of diabetes, and clinical procedures that injure blood vessels. The hallmark of endothelial dysfunction is reduced bioavailability of nitric oxide (NO) and consequent increased free radical production. These radicals trigger the inflammatory response, a major contributor to tissue injury. Measuring free radical release in biological models facilitates assessment of the biochemical mechanisms responsible for regulating the production of these biomolecules. This technical review focuses on the application of real-time measurement of hydrogen peroxide ($H_2O_2$) and NO release in models of I/R and blood vessel injury. The technique presents a valuable tool to study not only NO/$H_2O_2$ release, but also to identify novel pharmacologic agents modifying that release.

**Keywords:** hydrogen peroxide, nitric oxide, endothelial dysfunction, femoral ischemia-reperfusion, extracorporeal shock wave lithotripsy.

## Endothelial dysfunction

Endothelial dysfunction is defined as an increase in oxidative stress accompanied by decreased endothelial derived nitric oxide (NO) in blood (1). The condition may by chronic, which likely occurs in conditions such as type 2 diabetes (2), age-related hypertension (3), or the aging process itself (4). However, this paper deals with acute endothelial dysfunction, which can start within 5 min. of reperfusion and can be a key initiating event in ischemia/reperfusion (I/R) injury. Such dysfunction can occur during

  

reperfusion after an acute interruption of blood flow, as in certain surgeries (i.e. organ transplant, coronary angioplasty), ischemic events (i.e. stroke, myocardial infarction), and clinical procedures such as kidney lithotripsy (5). Even a brief loss of blood flow may be sufficient to trigger reduced endothelial NO synthase (eNOS) function, followed by rapid (seconds to minutes) expression of various cell adhesion molecules on the luminal face of the vascular endothelium (6-8).

Normally, eNOS produces NO from L-arginine in the presence of the essential cofactor, tetrahydrobiopterin ($BH_4$). However, when $BH_4$ is oxidized to dihydrobiopterin ($BH_2$) leading to an increased $BH_2/BH_4$ ratio, eNOS becomes uncoupled from L-arginine and shifts its product profile to superoxide by using molecular oxygen (9). Superoxide is short-lived (sec.) and converted to longer-lived hydrogen peroxide $H_2O_2$ (min.) by superoxide dismutase. Therefore, eNOS can become a source of oxidative stress during endothelial dysfunction (10, 11). These are the first events of an inflammatory response, which, even if checked, may result in permanent organ damage (7, 12, 13). Understanding the specific molecular mechanisms of endothelial dysfunction would allow identification of potential targets for therapeutic interventions.

Several methods for detecting NO, superoxide and $H_2O_2$ have been developed to measure these biomolecules by electrochemical, chemiluminescent and spectrophotometric procedures (14). However, the very rapid time course of endothelial dysfunction makes monitoring the biochemical changes electrochemically in real-time the most practical method of study. We describe here a recent technology which allows the real-time measurement of NO, $H_2O_2$, and other free radicals directly in the blood vessels. These methods will permit *in situ* evaluation of pharmacologic agents aimed at prevention of endothelial dysfunction and subsequent tissue damage.

## *In vivo* applications

Measuring free radicals in real time in blood vessels of anesthetized animals is a recent breakthrough technology facilitating study of oxidative stress in I/R injury. By using flexible $H_2O_2$ and NO sensors (World Precision Instruments (WPI), Sarasota, FL) inserted into catheterized veins, our lab has measured changes in free radical release under a number of different physiological and pathophysiological conditions (5, 15, 16). The sensors yield an immediate estimate of oxidative stress in a clinically relevant setting by real-time measurements in the intact animal. Such conditions are ideal for developing animal models aimed at *in situ* study of oxidative stress.

Other investigators have used similar sensors to record NO or $H_2O_2$ changes in smooth muscle, extracellular fluid and blood *in vivo* in real-time. After hindlimb ischemia in rabbits, Huk et al. (17) showed increases in NO release in femoral artery smooth muscle during reperfusion in the range of 100 – 200 nM in quercetin-treated group. These are similar to our own observations in rats measuring the effect of test compounds designed to improve NO release derived from eNOS (10). These measurements have helped to confirm that increased NO release during reperfusion attenuates reperfusion-associated inflammation (17).

By using NO and $H_2O_2$ sensors in the stomach of *Helocobacter pylori*-infected animals, Elfvin et al. (18) demonstrated that some strains of *H. pylori* can increase $H_2O_2$ release in the millimolar range, without affecting NO release. Other labs have conducted clinical studies in humans using selective NO sensors placed in the stomach. Iijima et. al. (19) found micromolar increases in gastric NO release after ingestion of nitrate-supplemented water. These changes are substantially larger than the nanomolar (NO) and micromolar ($H_2O_2$) ranges we have observed in our labs. These differences are most likely due to differences in the biological compartment in which the sensors were placed, i.e. stomach cavity versus blood vessels.

In another human study, Takarada et. al. (20) placed an NO sensor (700 μm diameter) in the great cardiac vein of dilated cardiomyopathy patients and found that acetylcholine dose-dependently increased NO release in the nanomolar range from the left anterior descending coronary artery. The response was blocked by $N^G$-

  

nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor. This group also validated the real-time NO measurement by comparison with the Greiss reaction, which showed an increase in serum nitrite/nitrate levels after acetylcholine administration. The human intravascular responses are similar to the range detected in our rat femoral I/R model, using a 100 μm diameter sensor (10).

Superoxide microsensors have been developed and used to measure this reactive oxygen species from the right atrium in humans (21). Unfortunately, these sensors are nearly three times the diameter of the microsensors used in our rat studies. Consequently, they are of limited utility in small animal studies. We believe the adoption of the smaller sensors (i.e. 100 μm) will allow not only for further adoption in the vasculature of small animals, but also for more direct application in humans as a low-invasive method to monitor free radical generation and oxidative stress. Below we explain our technique for the measurement of free radicals in real-time in blood vessels (*In Vivo Methods)*.

### *In vitro* applications

Real-time measurement of $H_2O_2$ and NO can also be performed on isolated tissue bathed in physiologic buffers or on cultured cells in tissue culture medium (15, 22-27). Previous studies have even utilized electrodes in organ chambers to record NO concentration (28). It should be emphasized that this technology measures the relative changes in NO or $H_2O_2$, but not the absolute baseline concentration in solution. In studies utilizing the Griess reaction to estimate NO release, nitrates were found to accumulate in serum or tissue culture (29-31). The advantage of the Griess reaction is that it provides an estimate of baseline concentration in addition to relative changes after treatment. However, this technique is less sensitive than direct real-time measurement as NO reacts with hemoglobin producing a quenching effect (14). Intracellular levels of reactive oxygen species can be measured using fluorescent probes. This method is of limited utility, because it cannot distinguish between superoxide and $H_2O_2$ and, thus, lacks specificity (30).

The utility of this technology is limited only by the availability of sensors. At present, sensors are commercially available for NO, $H_2O_2$, and $H_2S$. As the technology develops, sensors for other free radicals will become available. *In vivo*, it is the only available means to measure free radical generation directly in blood in real-time. *In vitro,* it is an effective tool for development of high-throughput screening assays to discover new drugs to up or down regulate free radical generation that may be of clinical interest.

### *In Vivo Methods:*

Instrumentation A free radical gas analyzer (TBR 4100, WPI, Sarasota, FL) and installed LabTrax 2 software are used to record the NO and $H_2O_2$ electrical potential traces in real time in biological fluids as needed to conduct the experiments. Sensors to monitor NO, $H_2O_2$, $H_2S$, as well as oxygen, are available in a wide range of sizes (WPI, Sarasota, FL). These sensors are connected to the analyzer by a cable. The free radical of interest diffuses through a selective membrane covering the sensor and is oxidized at the working electrode, resulting in an electrical (oxidation/reduction) signal whose amplitude is proportional to the free radical concentration in the sample. Each type of sensor has a selective poise voltage that is important for obtaining reliable data. A high performance Faraday shield incorporated in the sensor minimizes environmental noise (32). The sensor is calibrated by constructing a standard curve using known concentrations of the free radical of interest, enabling conversion of the electrical signal to a molar concentration in a biological sample. For example, NO microsensors (100 μm diameter) are calibrated using S-nitroso-N-acetyl-D,L-penicillamine (SNAP). When exposed to $Cu^{2+}$ ion, SNAP quantitatively decomposes to NO and a disulfide product:

$$2\,SNAP \xrightarrow{Cu(II)} 2\,NO + RS - SR$$

In theory, the concentration of NO generated should be equal to the final concentration of

  

SNAP in the copper sulfate solution. In practice, some NO is immediately oxidized to nitrite and not detected by the sensor. Furthermore, SNAP decomposition does not go to completion even in the presence of a catalyst [10]. Consequently, the detected NO is about 60% of the SNAP concentration. The calibration curve for NO is generally linear over a concentration range of 10 to 200 nM SNAP, and the sensitivity should be about 10 pA/nM. Sensors for other free radicals are calibrated in a similar manner, using suitable systems for generating the free radical of interest.

Animal Preparation As illustrated in Figures 1A and 1B, the animal (rat) is anesthetized and placed on a flat thermo neutral surface to minimize the rapid heat loss from the animal. Thereafter, 25 gauge infusion needles are placed into the rat tail vein to establish an intravenous route to administer saline or test compounds. For the femoral ischemic hind limb model, the femoral arteries and veins are exteriorized in both hind limbs, and silk suture is looped around each vessel (10, 15, 33). For the renal vein model, access to the left renal vein is gained via a midline incision to the peritoneal cavity (5, 16).

Electrode Placement For *in vivo* measurements, an intravenous catheter is utilized to help secure the free radical microsensor inside the interested blood vessel, as well as to decrease the background noise associated with blood flow, thus improving the accuracy of NO or $H_2O_2$ measurement. Blood seen inside the lumen of the catheter after insertion and before sensor placement indicates that the catheter has been placed correctly.

As the microsensors are available in several sizes, the catheter is placed such that both the working and reference electrodes are in full contact with blood flow. For placement in the femoral vein, a 24x3/4" catheter is precut to a length that is sufficient to include the working and reference electrodes such that the working electrode extends about 1 mm into the vessel beyond the end of the catheter. A needle-type sensor is used for renal vein placement. The entire sensor is inserted into a 22x1" catheter; the hub of the catheter fits securely onto the base this type of sensor.

Once placed securely, the sensors are connected to the cable which has been plugged into the free radical analyzer. Once placed, it is recommended that the sensor be allowed to rest on a stack of 2"x2" 12-ply cotton gauze; this helps to maintain the proper orientation in the catheterized vessel throughout the experiment. Figures 1A and 1B illustrate the experimental set up of both preparations.

After sensor placement, the program is opened up to monitor the electrical current generated by the redox reaction. Baseline readings are made until the traces are stable (i.e. sequential 5 minutes readings are within 300 pA of each other).

After a stable baseline is achieved in the femoral I/R model, one limb is subjected to ischemia by clamping the femoral artery and vein. The contralateral limb serves as a sham control. At the end of the ischemic period (e.g. 20 min.), the clamp is removed to allow the reperfusion of blood to the ischemic limb for 45 minutes. Test compounds can be administered via the tail vein before, during, or after termination of the ischemic period.

Below is a $H_2O_2$ sample trace obtained from the femoral I/R model (34). Compared to the sham limb, there was a significant and sustained increase in $H_2O_2$ release from the I/R limb in saline-treated control animals (Fig. 2A). By contrast, tail vein administration of a PKC epsilon peptide inhibitor at the beginning of reperfusion markedly attenuated post-ischemic $H_2O_2$ generation (Fig. 2B) (33).

**Figure 1A**

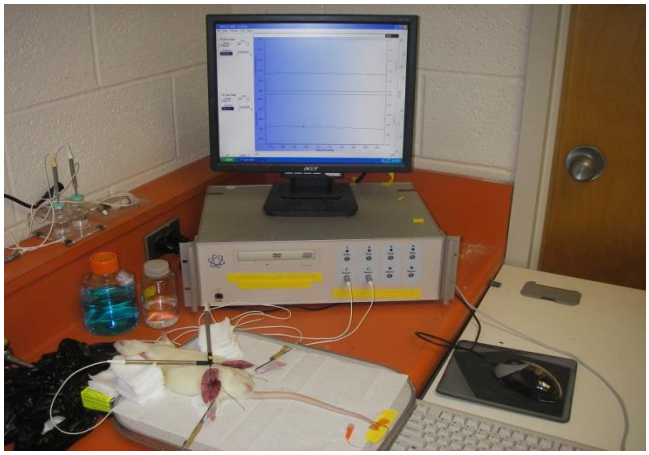

  

**Figure 1B**

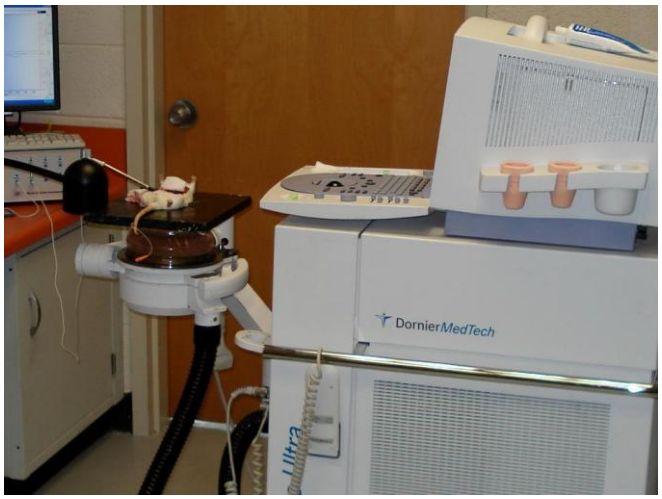


Fig. 1 (A) An illustration of the experimental setup of rat femoral I/R model and (B) rat renal vein model subjected to extracorporeal shockwave lithotripsy.

The renal vein model is used to evaluate free radical generation consequent to a traumatic intervention, such as extracorporeal shockwave lithotripsy (ESWL). The animal is instrumented as described, while continuously recording free radical generation. As with the femoral I/R model, test substances can be administered before, during or after treatment, and the free radical generation followed for up to 30 minutes post-trauma.

Our ESWL rat model indicates that there is a significant increase in $H_2O_2$ release accompanied by a significant decrease in endothelial-derived NO release in blood in left renal vein during the 30 minute post-ESWL period compared to non-ESWL rats (5, 16). These results suggest that tissue injury, and specifically endothelial dysfunction, are induced by ESWL. Intravenous administration of a PKC beta II peptide inhibitor significantly attenuates the development of $H_2O_2$ in blood post-ESWL (5, 16).

Fig 2A

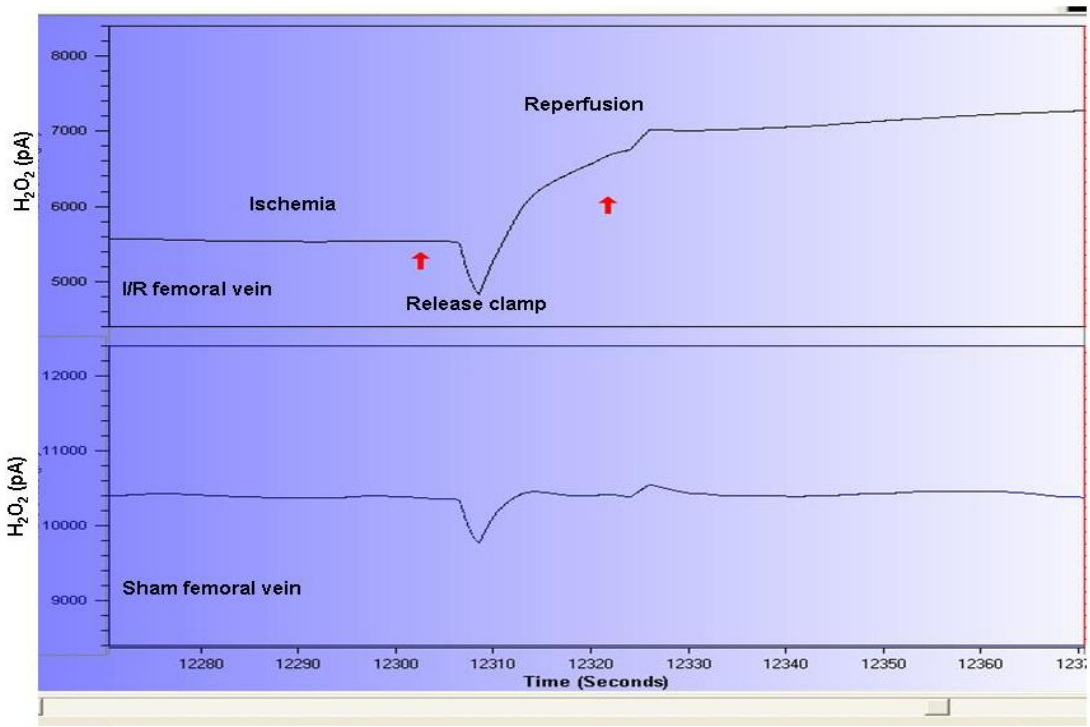


Fig. 2 (A) Representative $H_2O_2$ recording sample traces obtained from saline control group

  

Figure 2B

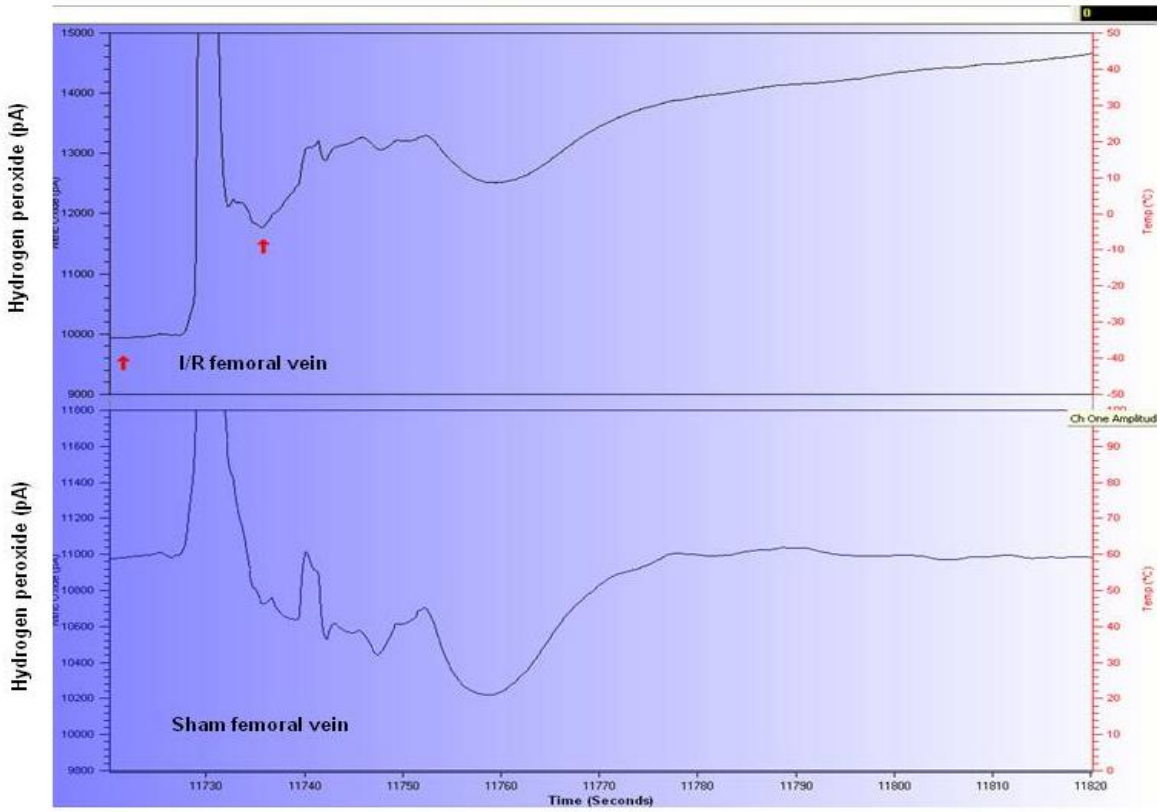


Fig. 2 (B) PKC epsilon inhibitor treated group in the femoral I/R model.

***In Vitro* Methods:**

Instrumentation

Essentially, this is the same as for the *in vivo* techniques. As it is not inserted into a blood vessel, a larger sensor (2 mm) may be used. These larger sensors are calibrated in a similar manner as the microsensors. The sensor is held in place using a micromanipulator, and the tissue or cell culture is kept warm on a water bath or similar device.

Methods

The thoracic aorta is isolated from rats under pentobarbital anesthesia (60 mg/kg). A 30 mm length is cleaned of adherent fat and connective tissue and then cut into 4 equal pieces. The pieces are cut longitudinally, pinned with the endothelial surface facing up, in 24-well culture dishes containing 1 ml of warm (i.e. 37 ℃), oxygenated (95% $O_2$/5% $CO_2$) Krebs Hensleit buffer (KHB; 10 mM dextrose, 119 mM NaCl, 12.5 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 4.8 mM LCl, 1.2 mM $KH_2PO_4$ and 1.2 mM $MgSO_4$). After equilibration, basal NO release is determined by placing the NO electrode in a well containing only KHB, then transferring to a well containing aortic tissue. The difference between the two readings is used to calculate the basal NO release for that particular aortic segment. After measuring basal release, the test compound is added, and the increase in current is used to calculate compound-stimulated NO release. That NO caused the increase in current is confirmed by repeating the test compound in the presence of 400-800 μM L-NAME (a NO synthase inhibitor). At the conclusion of the experiment, the aortic segments are blotted and weighed, and results are usually

  

reported as moles of NO released per gram of tissue (15, 23-27). Figure 3A is an illustration depicting the experimental setup for rat aortic segment tissue. Figure 3B is a trace from rat aortic segment response to acetylcholine.

Figure 3A

Figure 3B

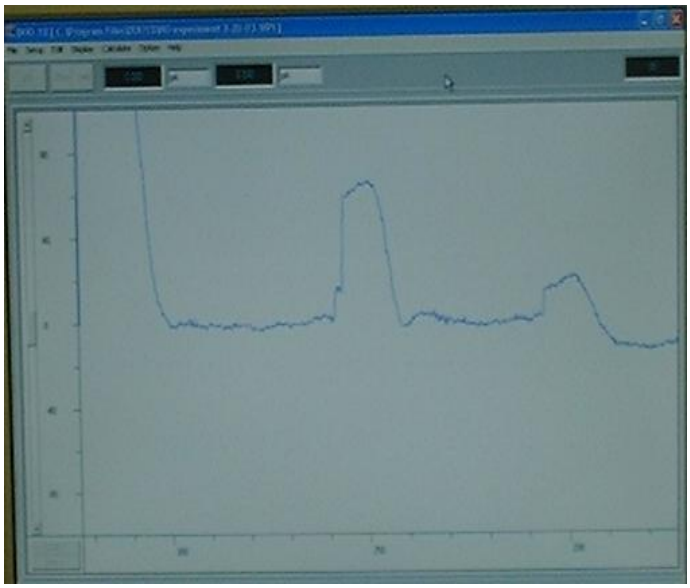

Fig. 3 (A) An illustration of the experimental setup for measurement of NO from the rat aortic segment tissue and (B) a sample trace of NO release from the rat aortic segment in the presence of acetylcholine (10 μM, first trace; 5 μM, second trace).

## Summary

Direct free radical measurement is a useful tool to screen for compounds that can reduce the oxidative stress associated with I/R injury, and will allow more precise definition of the role of free radicals in vascular function. These measurements cannot be obtained in real-time except with the instrumentation described here.

The application of free radical real-time measurement under live whole animal preparations (i.e. *in vivo*) may open the door to identify very rapid and discreet biochemical processes involved in regulating NO and $H_2O_2$ release under different disease conditions. The future of this technology holds enormous potential in that the microsensors can be subjected to ethylene oxide (gas) sterilization without affecting calibration, opening the door to direct measurement of free radicals *in situ* in humans.

The utility of the *in vitro* application is that one can use small amounts of test compounds to determine an effective dose range that will increase or decrease NO release. Doing so facilitates study of the process in the intact animal. Several publications have shown that the isolated rat aortic segment model is a prognostic indicator of whole organ function (15, 24-27). Overall, the experimental setup is cost effective in that small amount of reagents, tissues, or cells are needed to generate reliable data.

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