Quantifying Flavin mononucleotide: an internationally validated methodological approach for enhanced decision making in organ transplantation

Keyue Sun,[a] Chunbao Jiao,[a] Rebecca Panconesi,[a] Sangeeta Satish,[a,b] Omer F. Karakaya,[a] Femke H. C. De Goeij,[c] Tobias Diwan,[a] Khaled Ali,[b] Lorenzo A. Cadinu,[d] Beatrice Cazzaniga,[b] Qiang Liu,[b] Yuki Miyazaki,[b] Alejandro Pita,[b] Mazhar Khalil,[b] JaeKeun Kim,[b] Ahmed Hussein,[e] Philipp C. Müller,[f] Federico Aucejo,[b] David H. C. Kwon,[b] Eduardo Fernandes,[e] Jamak Modaresi Esfeh,[g] Jacek Cywinski,[h] Masato Fujiki,[b] Lidong Sun,[i] Antonio Pinna,[e] Philipp Dutkowski,[f] Chase J. Wehrle,[b] Robert L. Fairchild,[a] David Meierhofer,[j] Jeroen De Jonge,[c] Charles Miller,[b] Koji Hashimoto,[a,b] and Andrea Schlegel[a,b,*]

[a]Department of Inflammation & Immunity, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA
[b]Transplantation Center, Cleveland Clinic, Cleveland, OH, USA
[c]Department of Surgery, Division of Hepato-pancreato-biliary and Transplant Surgery, Erasmus MC Transplant Institute, University Medical Center Rotterdam, Rotterdam, the Netherlands
[d]Department of Electric and Electronic Engineering, University of Cagliari, Via Marengo 2, 09123, Cagliari, Italy
[e]Department of Liver Transplantation, Cleveland Clinic Weston Hospital, Weston, FL, USA
[f]Department of Visceral Surgery, University Hospital Basel, Switzerland
[g]Department of Gastroenterology, Hepatology & Nutrition, Cleveland Clinic, Digestive Diseases and Surgery Institute, Cleveland, OH, USA
[h]Anesthesia Institute, Cleveland Clinic, Cleveland, OH, USA
[i]Institute of Experimental Physics, Johannes Kepler University Linz, Austria
[j]Max Planck Institute for Molecular Genetics, Mass Spectrometry Facility, 14195, Berlin, Germany

eBioMedicine
2025;116: 105761
Published Online xxx
https://doi.org/10.1016/j.ebiom.2025.105761

Summary

Background Increasing donor risk, particularly in liver transplantation, where organs are often marginal, has made dynamic organ preservation techniques and viability assessment essential to safely improve organ quality and increase utilisation. However, existing viability parameters are based on routine clinical assessment in patients with acute liver failure, trauma, or liver resections. These parameters often do not correlate with clinically relevant post-transplant outcomes.

Methods This article presents a detailed protocol for the spectrophotometric quantification of Flavin mononucleotide (FMN), a marker of mitochondrial injury. FMN release from mitochondrial complex I was described many decades ago as the initial sign of ischaemia-reperfusion injury, i.e. when oxygen is reintroduced in ischaemic tissues during organ transplantation or machine perfusion. This study describes the detailed FMN quantification in donor plasma and various fluids obtained during machine perfusion, and discusses confounders, challenges, and the role of individual test components.

Findings FMN quantification was identified as an immediate organ assessment tool, demonstrating a strong correlation with graft survival and other relevant complications after human liver transplantation.

Interpretation The results highlight FMN quantification as a reliable and standardized method for assessing organ viability, offering significant potential for improving organ selection and better utilisation. This method could provide better a predictive value for transplant outcomes compared to existing parameters currently in use.

Funding This research received no external funding but was supported by the Catalyst grant No. CCG0280 at Cleveland Clinic Ohio, U.S. dedicated to A.S.

*Corresponding author. Transplantation Center, Department of Inflammation and Immunity, Lerner Research Institute Cleveland Clinic, OH, USA.
E-mail address: Schlega4@ccf.org (A. Schlegel).

Keywords: Machine perfusion; Ischaemia-reperfusion injury; Viability assessment; Flavin mononucleotide; Fluorescence spectroscopy; Organ transplantation

Research in context

Evidence before this study

Flavin mononucleotide (FMN), a marker of mitochondrial injury released from mitochondrial complex I, was described decades ago as an early indicator of ischaemia-reperfusion injury. While there is increasing evidence for the relevance of FMN as a biomarker to assess organ quality before transplantation, standardized protocols for its detection are lacking.

Added value of this study

This study provides a standardized and optimized protocol for FMN quantification in donor plasma and various organ preservation fluids, obtained during machine perfusion. The results are presented considering key confounders, methodological challenges, and the role of individual test components. FMN quantification has proven to be an attractive immediate organ assessment tool, strongly correlating with graft survival and post-transplant complications after liver transplantation. Additionally, this study supports the development of a standardized framework to facilitate the integration of FMN measurement programs in transplant centres. These findings lay the foundation for broader clinical adoption of a more consistent approach to organ viability assessment across institutions.

Implications of all the available evidence

These findings highlight the potential of FMN quantification as a standardized and reliable biomarker for assessing organ viability in liver transplantation. The validated fluorescence spectroscopy method allows for consistent implementation across transplant centres, enhancing organ evaluation and selection. Its reproducibility across various media, spectrometers, and international centres further reinforces its clinical utility. This non-invasive and efficient technique improves graft quality assessment, optimizing donor organ utilization and transplant success. Future research should refine threshold values, expand multi-centre validations, and explore FMN's role in other organs to establish it as a standard in transplantation medicine.

Introduction

Dynamic organ preservation techniques are increasingly important in solid organ transplantation due to their ability to reduce ischaemia-reperfusion injury (IRI) and evaluate organ quality, enhancing utilization while maintaining safety standards. Two main strategies, normothermic machine perfusion (NMP) and hypothermic oxygenated perfusion (HOPE), are used to improve liver preservation and assess organ viability.[1–8] Various markers like perfusate pH, lactate clearance, and transaminase release, often adapted from clinical liver surgery practices, have been proposed to assess organ condition during perfusion. However, these "biomarkers" lack robustness and validated thresholds for predicting clinically relevant outcomes.[9–15]

The identification of reliable biomarkers and their translation into wider clinical practice involves several key steps including initial discovery, the development of a reproducible assay, the calibration, and validation.[16,17] The discovery focuses on identifying biomarkers that correlate with clinically relevant outcome parameters, i.e. graft survival and complications in organ transplantation.[18–22] Although many biomarkers have been proposed, effective translation requires robust methods suitable for clinical use. Conventional techniques like enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS) are commonly used for biomarker detection and quantification. However, they require discrete samples, increasing the risk of contamination from wash or reagent buffers once they are opened. Additionally, sophisticated techniques and expensive culture media are required. These methods lack standardization for clinical use, limiting their role in routine clinical practice.[23,24] While spectrofluorometric methods offer continuous measurement potential, they have historically introduced lab-to-lab variability, complicating clinical standardization.[16]

Flavin-mononucleotide (FMN), also known as Riboflavin-5′-Phosphate, is an auto-fluorescent coenzyme. FMN and Flavin Adenine Dinucleotide (FAD), have both Riboflavin as precursor, serving as cofactors for various redox enzymes. While FAD is the more abundant form of Riboflavin in cells, FMN is non-covalently bound in its pocket in mitochondrial complex I.[25,26] Together with reactive oxygen species (ROS), FMN dissociates from this pocket as the first sign of IRI-related mitochondrial injury when oxygen is reintroduced in ischaemic tissue (Fig. 1a).[27–29] This dissociation was described as early as the 1960's for several tissue types including ischaemic brain after stroke and cardiomyocytes after myocardial infarct.[29–32]

FMN has been quantified in various fluids, including machine perfusates using fluorescence spectroscopy, with results confirmed by LC-MS.[33–35] Researchers, for example from Switzerland, proposed FMN as the optimal biomarker to assess organ viability and demonstrated that perfusate FMN levels during HOPE

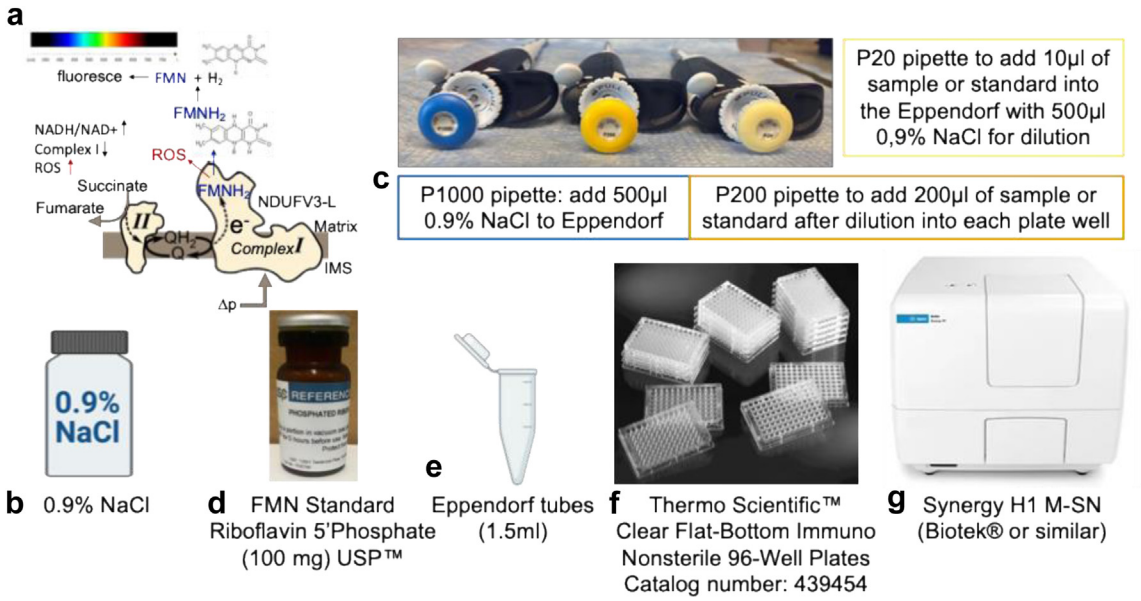


Fig. 1: **Required material for FMN quantification with fluorescence spectroscopy:** a) Succinate triggers uncoordinated electron flow and induces ROS and $FMNH_2$ release from complex I, that can be quantified by fluorescence spectroscopy when fully reduced to FMN. Required equipment for our technology: b) NaCl 0.9% (Saline for preparation of stock solutions and standard and sample dilution); c) set of pipettes (P1000, P200 and P20); d) FMN standard: Riboflavin 5′Phosphate (USP™, 100 mg), light protected; e) 1.5 ml Eppendorf tubes; f) Available 96 well plates (Thermo Scientific™); g) Synergy H1 M–SN (Spectrometer Biotek®).

are the best predictors of graft survival and post-transplant complications.[33,36,37] Despite its potential, the widespread adoption of FMN as a biomarker for decision making in organ transplantation has been delayed by inconsistent methodologies and non-standardized fluid concentration thresholds.

This article provides a detailed guide on using spectroscopy techniques for FMN quantification for decision making in organ transplantation, including step-by-step procedures and discussions on potential confounders, challenges and pitfalls.

Methods

Two optical spectroscopic techniques are widely used to quantify FMN: absorbance and fluorescence spectroscopy. Running an entire absorbance spectrum might have a benefit detecting sample contamination which is one reason why this is the preferred technique for Riboflavin quantification in food and supplements. While fluorescence spectroscopy has higher sensitivity, leading to greater signal/noise ratio and thus lower detection limits and wider concentration ranges (i.e. >3–6 log orders of molecule concentrations), this technology is also very specific because only molecules that fluoresce at the specific wavelength are detected. Based on this, fluorescence spectroscopy was preferred over absorbance to quantify FMN in perfusates when first presented by the Zurich group in 2019.[33] Since then, FMN quantification was successfully performed in various fluids, tissues, mitochondria and complex I, confirmed by LC-MS.[38,39] In the setting of organ transplantation, FMN levels can be measured in perfusate (circulating fluid on a machine during perfusion), bile, plasma and organ flush solution for decision making.[17,40,41] The next section presents the detailed method developed by our team (Fig. 1). Based on the different organ type, size and weight, perfusate composition, volume and perfusion temperature, diverse dilution factors for the FMN standard and samples were established. Various solutions and fluids are used during organ donation, transport, machine perfusion and implantation of human organs. This section highlights a few typical examples how such fluids are sampled, processed and stored for subsequent fluorescence spectroscopy.

Sample collection and processing

Hypothermic oxygenated perfusion (HOPE)

Belzer machine perfusion solution (Belzer MPS) is the most frequently used perfusate during HOPE. Perfusate is obtained from the perfusion circuit or the reservoir, i.e. bowl and is stored in 1.5 ml Eppendorf tubes. No further processing is needed. FMN levels remain stable for months and years given samples are protected from light and stored at temperatures between minus 20 °C and minus 80 °C. From procurement and organ packing at the donor site to the recipient centre, organs are

flushed and stored in preservation solutions, which can also be sampled to quantify molecules, including FMN. Routinely used solutions are University of Wisconsin solution (UW), Institute-George-Lopez 1 (IGL-1) and Histidine-tryptophan-ketoglutarate (HTK). During flushing in the donor or in a bowl, organs release molecules including FMN and even cells. Flushing and transport fluids can be sampled in the same 1.5 ml Eppendorf vials with direct freezing and light protection as described above for HOPE samples. This flush solution sampling method may be applied prior to any other ex-situ perfusion technique, including NMP or combined approaches.[42–44]

Normothermic machine perfusion (NMP)

Perfusate Sampling: At our centre the Organox® Metra device is routinely used to perfuse human livers with the end ischaemic normothermic technique. Perfusate samples are routinely collected both before liver connection to the machine, and during NMP. Standard yellow blood collection tubes are used (BD Vacutainer Ref. 367986). The perfusate during NMP is red-cell based and perfusate samples are centrifuged for 5 min at 3000 RMP (Avanti J-15 Centrifuge, Beckman Coulter). The supernatant is transferred into a new Eppendorf vial. Each sample can either be measured directly or stored frozen. FMN levels remain stable for months and years when protected from light and stored at temperatures between minus 20 °C and minus 80 °C.

Bile Sampling: Pure Bile released during NMP is collected from the bile duct tube or a reservoir into 1.5 ml Eppendorf Tubes. The fluorescence measurement can be done immediately without further sample processing, or the sample can directly be frozen in nitrogen and stored at minus 80 °C.

Blood samples (i.e. donors, recipients)

Samples of donor blood (serum or plasma) are also collected during organ donation surgery using yellow or red serum blood collection tubes (Vacutainer BD, Ref. 367986, includes gel that separates blood clot and serum with clot activator) with subsequent centrifugation for 10 min at 3000 RMP (Avanti J-15 Centrifuge, Beckman Coulter®). If samples are obtained in glass vacutainer (glass tube whole blood ACD tube), the process including centrifugation and storage is the same. The supernatant is transferred into a new Eppendorf tube. Each sample can either be measured directly or stored frozen. FMN levels remain stable for months and years given samples are protected from light and stored at temperatures between minus 20 °C and minus 80 °C.

FMN standard and sample preparation guide

The quantification of FMN is easily done while an organ is perfused. It appears therefore practical to have the standard and required dilutions ready whenever needed, because organ transplantation happens often outside office hours, at night or during weekends. At our centre, perfusate FMN levels are often measured early morning for decision making whether to proceed with a liver transplantation or not. The following section details the standard preparation, sample dilutions and measurement at our centre.

To establish standard stock solution A (FMN concentration: 1 mg/ml), 10 mg FMN standard powder (Riboflavin 5′-Phosphate, USP™ Reference Standard, France, purity ≥70%, stored at –20 °C) are added to a conical Eppendorf tube and mixed with 0.9% NaCl at 1:1 ratio (i.e. 10 mg FMN powder and 10 ml 0.9% NaCl). We use a digital scale to weigh the powder (Mettler Toledo AL54). This Stock Solution A is the basis for subsequent standard dilutions and measurements in various fluids as described in sections 2.2.1–2.2.3 (Fig. 2, step 1). Stock solutions, diluted standards and samples are stored at –80 °C.

Standard preparation to measure FMN from HOPE perfusates

For perfusates obtained during HOPE, the main FMN Stock Solution A (concentration 1 mg/ml), is further diluted with 0.9% NaCl at 1:2000 in a new 50 ml conical Eppendorf Tube (i.e. pipette 10 µl Stock Solution A and 19′990 µl 0.9% NaCl). This results in the HOPE Stock solution, which is also the standard 1 (highest FMN standard concentration: 0.5 µg/ml) (Fig. 2, step 2). To produce serial standard dilutions, for example dilute Standard 1 further and pipet 500 µl of Standard 1 into a new Eppendorf tube together with 500 µl of NaCl 0.9% (Fig. 2, step 4). Such dilution is repeated in a serial 1:1 fashion, i.e. 500 µl of the higher standard are pipetted into a new Eppendorf tube with 500 µl NaCl 0.9% to create the next lower ("diluted") standard concentration (standard 1–2; standard 2–3, etc.). For the HOPE perfusate analysis, there are seven standards, starting at concentrations of 0.5 µg FMN/ml NaCl 0.9% (standard 1) to Standard 6 at 0.015625 µg FMN/ml NaCl 0.9%. Standard 7 contains only NaCl 0.9% at an FMN concentration of 0 mg/ml (Fig. 2, step 4).

Standard preparation to measure FMN from NMP samples

For perfusates and bile obtained during NMP, the main FMN Stock Solution A (concentration 1 mg/ml), is further diluted with 0.9% NaCl at 1:20 pipetting 1 ml Stock Solution A and 19 ml NaCl 0.9% in a new conical Eppendorf tube (Fig. 2, step 2). The result of this step 2 is Stock Solution B (concentration 50 µg FMN/ml) used to quantify FMN levels during NMP and in plasma of organ donors (section 2.2.3). Stock Solution B is further diluted to receive NMP and Plasma/Serum stock solutions. This extra step of dilution is essential to avoid signal "overflow" due to the higher FMN levels released during normothermic perfusion compared to HOPE or cold flush solution secondary to the metabolic

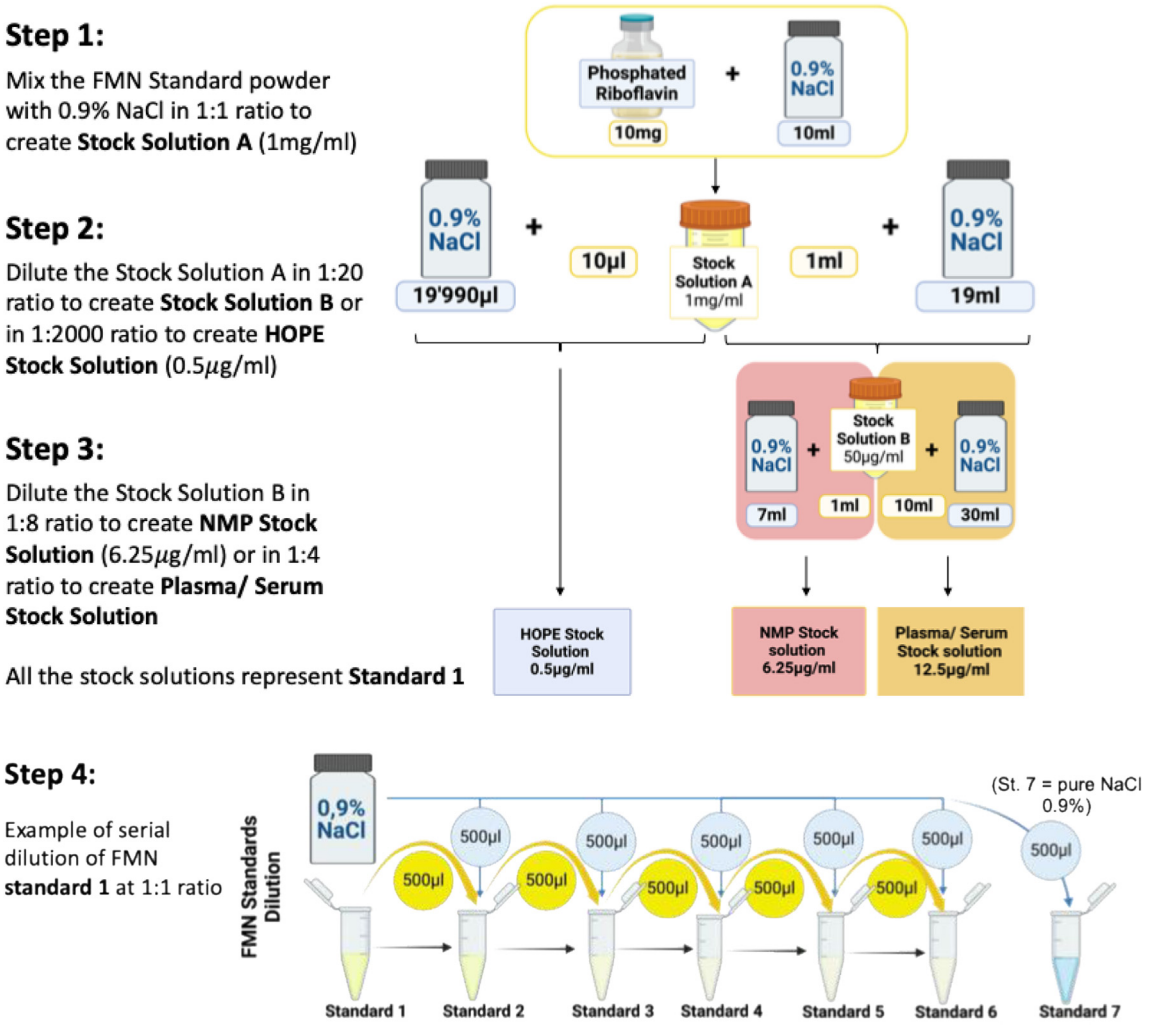


Fig. 2: **FMN stock solution and standard preparation**: Step 1: Create Stock Solution A (1 mg/ml)–Dilute and mix Riboflavin 5′-Phosphate powder with 0.9% NaCl at 1:1 ratio. FMN is light sensitive; the powder and the FMN standard solutions must be protected from light and kept cold (i.e. wrap the tubes with aluminium foil and put them on ice). Step 2 and 3: Prepare HOPE, NMP and Plasma/Serum stock solution: Step 2: For HOPE Standard 1 Solution, dilute Stock Solution A (1 mg/ml) directly in 0.9% NaCl at 1:2000 ratio (i.e. 10 µl Stock solution A with 19′990 µl 0.9% NaCl). For NMP and Plasma/Serum Stock Solution, a Stock Solution B (50 µg/ml) will be created by diluting Stock Solution A in 0.9% NaCl in a 1:20 ratio (i.e. 1 ml Stock solution A with 19 ml 0.9% NaCl). Step 3: prepares the final NMP and Plasma/Serum Standard 1 by diluting Stock Solution B (50 µg/ml). Dilute Stock Solution B with 0.9% NaCl at a 1:8 ratio for NMP Standard 1 (FMN concentration 6.25 µg/ml). Dilute Stock Solution B 1:4 with 0.9% NaCl to receive Plasma/Serum Standard 1 (FMN concentration of 25 µg/ml). Step 4 includes the serial dilution of HOPE, NMP, Plasma/Serum Standard 1 with 0.9% NaCl at 1:1 to receive Standard 2, 3, 4, 5 and 6. In general, the concentration of the next higher ("diluted") standard is always half of the previous standard (i.e. NMP St.1 = 6.25 µg/ml, St.2 = 6.25/2 µg/ml, St. 3 = 6.25/4 µg/ml etc.). Standard 7 is pure 0.9% NaCl (FMN concentration: 0 µg/ml) for all standards.

re-activation of the liver graft during NMP. For NMP measurements, Stock Solution B is further diluted mixing 1 ml Stock Solution B with 7 ml NaCl 0.9% to receive NMP Stock solution and the standard 1 (FMN concentration 6.25 µg/ml) (Fig. 2, step 3). To produce serial standard dilutions, NMP Standard 1 is diluted further pipetting 500 µl of Standard 1 into a new Eppendorf tube together with 500 µl of NaCl 0.9% (Fig. 2, step 4). Such dilution is repeated in a serial 1:1 fashion, i.e. 500 µl of the higher standard are pipetted into a new Eppendorf tube with 500 µl NaCl 0.9% to create the next lower standard concentration (i.e. standard 1–2; standard 2–3, etc).

Standard preparation to measure FMN in serum

As described above, for serum measurements, Stock Solution B (concentration 50 µg FMN/ml) is further diluted mixing 10 ml Stock Solution B with 30 ml NaCl

0.9% to receive Serum/Plasma Stock solution and standard 1 (FMN concentration 12.5 µg/ml) (Fig. 2, step 3). To produce serial standard dilutions, 500 µl of Standard 1 are further mixed with 500 µl of NaCl 0.9% in a new Eppendorf (Fig. 2, step 4). Such dilution is repeated in a serial 1:1 fashion to create the next lower standard concentration (standard 1–2; standard 2–3, etc). For Serum analysis, seven standards are required, starting with Standard 1 (concentrations of 12.5 g FMN/ml NaCl 0.9%) to Standard 6 (concentration of 0.1,953,125 µg FMN/ml NaCl 0.9%). Standard 7 contains only NaCl 0.9% at an FMN concentration of 0 mg/ml (Fig. 2, step 4).

To further standardize this FMN measurement for routine use and rapid quantification before or during organ perfusion, multiple standard replicates can be prediluted and stored at –80 °C. NMP perfusate sample testing can be prepared by dilution of 1 ml NMP standard 1 (6.25 µg/ml) in 50 ml 0.9% NaCl using a 50 ml tube (Fischer, disposable Centrifuge Tube cat. No. 06-443-18). To produce serial standard dilutions, this NMP Standard 1 is diluted further pipetting 25 ml into a new 50 ml tube together with 25 ml of NaCl 0.9% to create standard 2. Such dilution is repeated in a serial 1:1 fashion for standards 3–6. The same principle applies for HOPE and Serum samples. The standard series can be aliquoted for single use and stored at –80 °C for 3–4 weeks. Despite minimal differences observed, it is recommended that a new standard set is prepared at least every month with comparative testing of this new standard at first use together with the stored standard set.

FMN fluorescence spectroscopy

Additional dilutions are required based on the sample type before the measurement to ensure that all samples and standard signals fall within the spectrometer's detectable range. For HOPE measurement, prepare the final dilutions (1:4) of HOPE standards[1–6] and samples and add them in duplicates to a 96-well plate (Thermo-Scientific®) (Fig. 3, procedure steps 6 & 7). For NMP samples, create the final dilution of standards[1–6] and

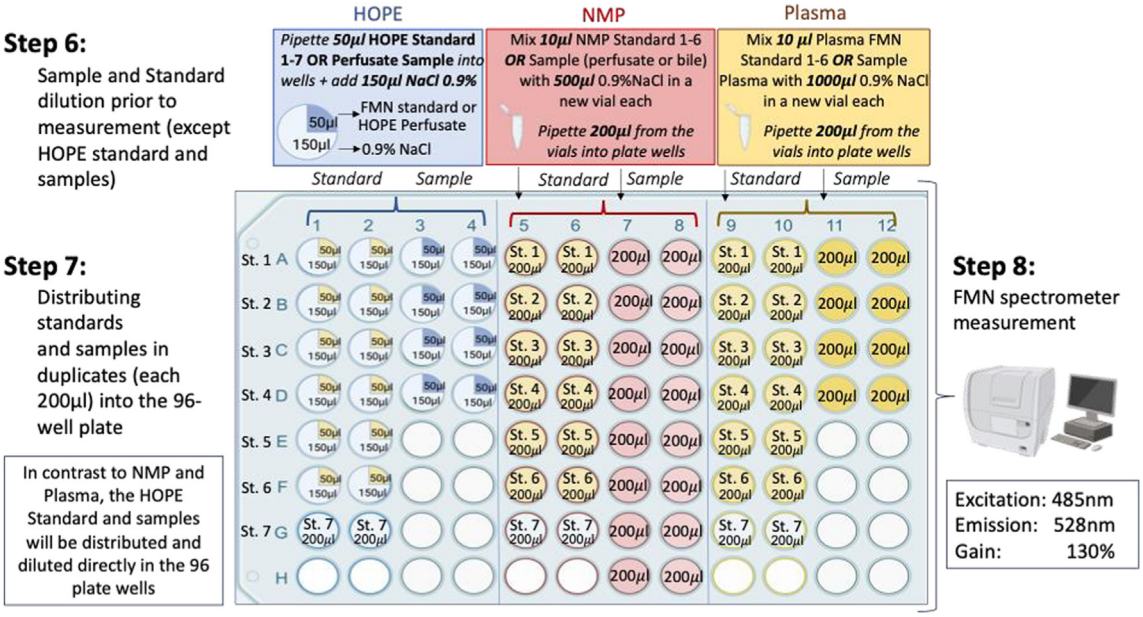


***Fig. 3:* Standard and sample distribution into the 96-well plate for FMN quantification.** A clear, flat bottom 96-well plate (Thermo-Scientific®) is used with a conventional fluorescence spectrometer. The total fluid volume added to each well for the measurement is 200 µl. Steps 6 and 7 describe the required additional dilutions for standard and samples based on the sample origin. Any number of samples can be aliquoted onto the plate in duplicates or triplicates, depending on the experimental settings. For HOPE perfusates, there is no need for additional sample dilutions prior to distribution in the plate. Fill the subsequent plate wells with 150 µl 0.9% NaCl and distribute 50 µl of HOPE standards and HOPE samples to those wells. For NMP, dilute the sample and NMP standards in a separate Eppendorf tube with 0.9% NaCl. NMP samples (perfusate and bile) and NMP standard 1–6 will be diluted in a 1:51 ratio with 0.9% NaCl in a new empty 1.5 ml Eppendorf tube (i.e. mix 500 µl 0.9% NaCl and 10 µl of sample). Vortex and distribute 200 µl from those Eppendorf vials in empty plate wells. For Plasma/Serum analyses, dilute the Sample and Standard, required to avoid overflow (the signal has exceeded the maximal capacity of the system used to detect it). Serum/Plasma FMN standards and samples are diluted with 0.9% NaCl in a ratio of 1:101 in an empty 1.5 ml Eppendorf tube (mix 1000 µl 0.9% NaCl and 10 µl of sample). Vortex the mixture well before distributing 200 µl in the 96-well plate. Standard 7 equals 200 µl NaCl 0.9% without FMN, which will be added to the corresponding plate wells for all three fluid types. Spectrometer settings are shown for FMN quantification (Step 8). Settings for Fluorescence Spectroscopy include excitation wavelength: 485 nm; emission wavelength: 528 nm; Gain: 130%. FMN, Flavin mononucleotide; HOPE, Hypothermic oxygenated perfusion; NMP, normothermic machine perfusion.

sample at a ratio of 1:51 in a 1.5 ml Eppendorf tube. After vortexing, 200 µL diluted standards and samples are directly loaded into the 96 well plate (Fig. 3, procedure steps 6 & 7). For FMN in Plasma/Serum, predilute 10 µL sample or corresponding standard in 1000 µL NaCl (1:101). Load 200 µL of this solution into each well (Fig. 3, steps 6 & 7). In all groups, Standard 7 represents 200 µL NaCl in each well. All standards and samples are measured in duplicates or triplicates. Protect the plate from light until reading with the Synergy H1M-SN® (Biotek) or similar spectrometer. Further details on spectrometer settings are provided in the Supplementary Material (Supplementary Figure S1).

Factors affecting FMN concentrations

Once our standard method was established, the following tests were done to assess the impact of well-known confounders. In addition to the spectrometer type and the settings for wavelength and gain, the 96-well plate, the fluid used for standard and sample dilution and the temperature are some of the confounders explored here.

First, the impact of different 96-well plates was tested. For this purpose, three transparent (ThermoScientific®, Costar®, Brandtech®) and one black plate (ThermoScientific®) were used for FMN quantification using our established methodology.

Standard and samples are diluted in 0.9% NaCl as per our routine. In a next set of experiments, different fluids (i.e. distilled water, Ringer, Belzer MPS) were used to dilute FMN standard and samples in comparison to our established technique with 0.9% NaCl. The spectrometer settings were the same and our usual transparent 96-well plate (ThermoScientific®) was used for this set of tests.

Third, FMN concentrations were measured using standards and samples diluted in four different fluids (0.9% NaCl, Belzer MPS, distilled water and Ringer) with different spectrometer settings. While our standard settings include an excitation wavelength: 485 nm; emission wavelength: 528 nm and Gain: 130%, such numbers were slightly modified by others. These modified wavelength and gains are now tested here and compared to our standard procedure. For example, Wang et al.[35] and the team from Vienna uses an excitation and emission wavelength of 450 nm and 525 nm, respectively with a gain of 100%. The group from Italy quantified FMN using a fluorescence excitation and emission wavelength of 445 nm and 538 nm, respectively with a gain of 100%. Three different wavelength settings were tested with gain 100 and 130%, resulting in 6 different measurements. Standard and sample dilutions were done in the different fluids, and the same transparent 96-well plate was used (ThermoScientific®). Next, FMN NMP standards were read using 3 different spectrometers. The standard (established) Synergy H1 reader was compared to a new Synergy H1 reader and a Cytation 5 reader available in an adjacent laboratory. To explore the role of the perfusate where the samples are obtained from, 5 different spectrometers were used to read the HOPE FMN standard. Standard (established) and a new Synergy H1 reader were compared with three devices at the Erasmus Medical Centre in Rotterdam, The Netherlands. The team there produced the same FMN HOPE standard 1–7 dilutions based on our described technique and used their spectrometers to read the A.U. signals with subsequent FMN concentration calculation (i.e. Spectramax ID3 with autogain, Clariostar with a gain of 1000 and 1500; Cytofluor with a gain of 50 and 90). In a final step, standard dilutions using Riboflavin 5′-phosphate powder from three different providers were compared. To explore the bleaching effect, the same NMP FMN standard was measured up to 15 times using the same transparent plate and spectrometer settings.

Ethics

The analysis of included samples was approved on December 21st, 2023, by Cleveland Clinic Institutional Review Board (IRB) with the protocol number 23-1348.

Statistical analysis

Serial FMN standard dilutions were done as per established techniques. Pearson's product moment correlation coefficient was used to measure the linear relationship between two variables (i.e. spectrometer signal intensity in A.U. and FMN concentrations in µg/ml, or concentrations determined by different spectrometers or settings). GraphPad prism version 8.0 was used for analysis and Figure conceptualization.

Role of funders

This study was supported by an internal Catalyst grant No. CCG0280 at Cleveland Clinic Ohio, US. The grant committee had no role in study design, data collection, data analysis, interpretation or writing of the manuscript. No other funding body was involved.

Results

Fluorescence signal based FMN–calibration curves in different media

Fluorescence intensities in arbitrary units (A.U.) were recorded as generated by the spectrometer. The experiment was conducted in duplicates, and the average fluorescence readings of the FMN standard solutions were plotted against their known concentrations to create a standard curve for each of the different sample fluids. Fluorescence spectrometer signals were read for standards 1–7 and samples for FMN HOPE, NMP and Serum/Plasma (Fig. 4a–c). The FMN concentration of the standard series prior to dilution was used to quantify the FMN concentration.

For all fluid types the 96-well plate and spectrometer settings were identical. An example fluorescence

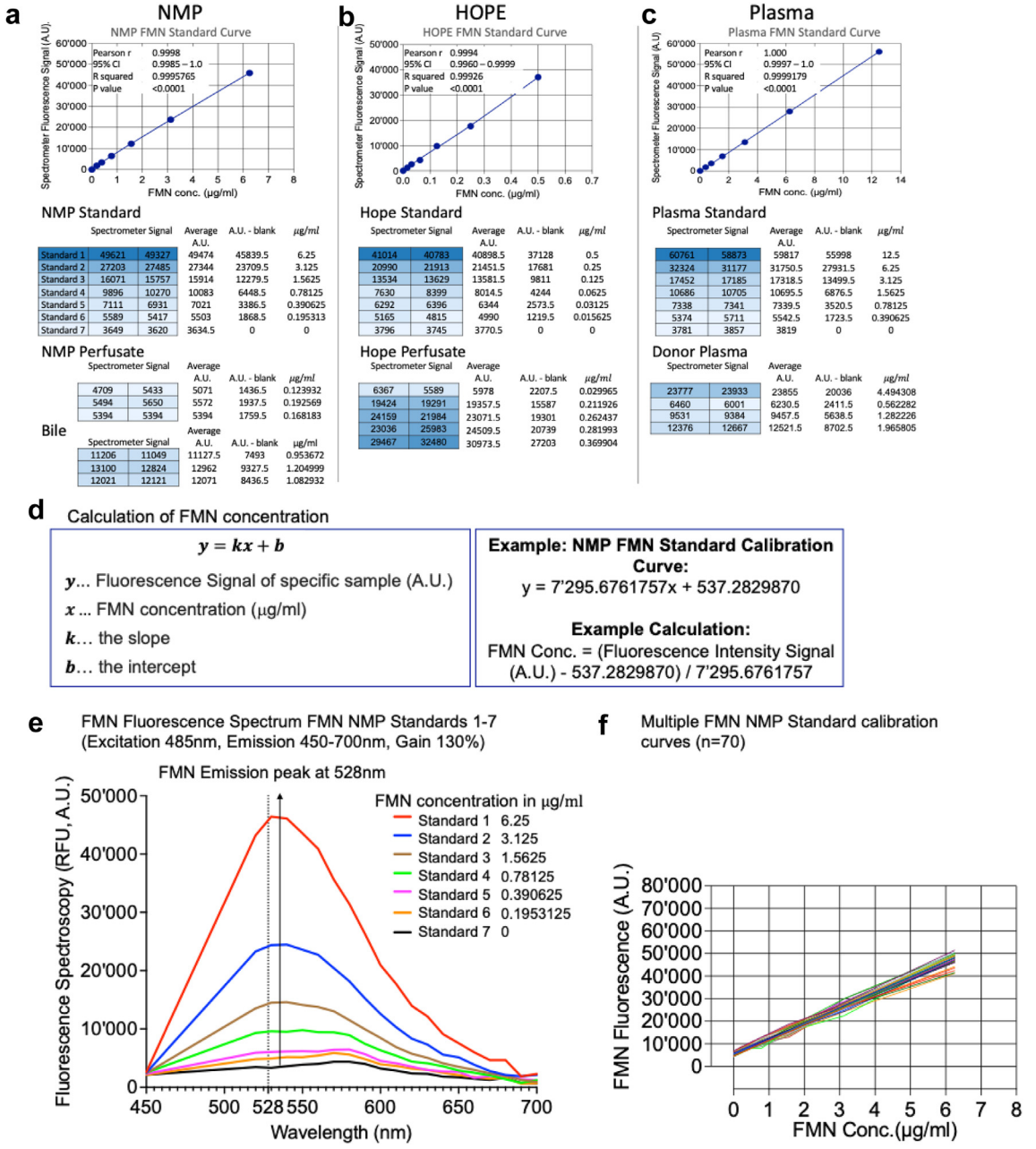


Fig. 4: **Calculation of FMN concentrations in different media obtained with fluorescence spectroscopy** (Excitation: 485 nm; Emission: 528 nm; Gain: 130%). The measured fluorescence signal (A.U.) of FMN standards is plotted with the corresponding FMN concentration (µg/ml) in a linear function a) r = 0.994; b) r = 0.997; c) r = 1.0). Each A.U. value corresponds to a specific FMN concentration in µg/ml. Seven digits are considered for calculation. Sample FMN concentrations are listed for each of the different fluids (a, b &c). Based on the same dilution ratio of all standards and samples for each fluid type (i.e. HOPE perfusate, NMP perfusate, Serum/Plasma) additional dilution factors do not need to be considered for value calculation. The FMN concentration prior to final standard and sample dilution (HOPE 1:4; NMP 1:51; Plasma/Serum 1:101) can be considered for the calculation instead. The increasing FMN concentration from NMP Standard 7–1 can be observed in the fluorescence spectrum (450 nm–700 nm) by steadily increasing the peak A.U. The lowest detected FMN concentration in this series was 0.0034905910 µg/ml; d) Example formula and calculation of FMN concentration. Based on the calibration curves in a, b and c, the corresponding sample concentrations were determined using this formula. e) The spectrum peak is seen at an excitation wavelength of 485 nm within an enlarged emission range of 300–700 nm showing that our settings result in the ideal dynamic range of FMN, which means the FMN signal can be adequately detected. f) Multiple calibration curves were produced by 6 different operators with our method with identical signal intensity, as shown with the example of the NMP standard (example month June 2024, n = 70). Large volume stock solution preparation with stock solution aliquot and standard freezing contributes further to the high reproducibility.

spectrum for FMN standards 1–7 is shown in Fig. 4d together with 70 standard calibration curves developed by 6 different operators within only 1 month (Fig. 4e and f).

What is the impact of different 96-well plates?

To quantify FMN with standard Fluorescence spectroscopy, a 96-well plate is needed. Fluorescence readings of FMN solutions were plotted against known concentrations to create standard curves for each of the different sample fluids. Fluorescence readings for FMN were repeated with 4 different plates commercially available to evaluate the impact of different materials and plate colours on FMN concentration. If the same standard concentration protocol and spectroscope settings were applied, the effect of the 96-well plate is minimal. However, while the use of a black plate is standard and suggested by many, we observed that the type of transparent plate may impact the measurement results, emphasizing that both its material and manufacturing process are contributing factors. Using for example the transparent Costar® Assay plate, specific sample FMN concentrations appear lower and may be even negative compared to the other two transparent plates (Fig. 5a–e). Next, the repeat measurement of the same standard samples resulted in minimal standard curve samples fluorescence differences showing minimal photobleaching effect (Fig. 5f).

What is the impact of different standard and sample dilution solutions and spectrometer settings?

Different signal behaviours of FMN are seen when the same FMN standards are diluted in different solutions. This implies that the fluid used to dilute FMN standard, and samples should be identical to avoid noise and achieve highly accurate results. Ringer solution and distilled water dilutions triggered the most significant differences of the linear curve incline compared to 0.9% NaCl and Belzer MPS. FMN standard and NMP samples, which are diluted in Belzer MPS and 0.9% NaCl, have been found to have similar fluorescence signals (Fig. 6a). The previously described role of ringers and distilled water for standard and sample dilution becomes even more relevant when spectrometer settings are changed. We used different settings, which have been proposed by different centres worldwide (Fig. 6b–g).

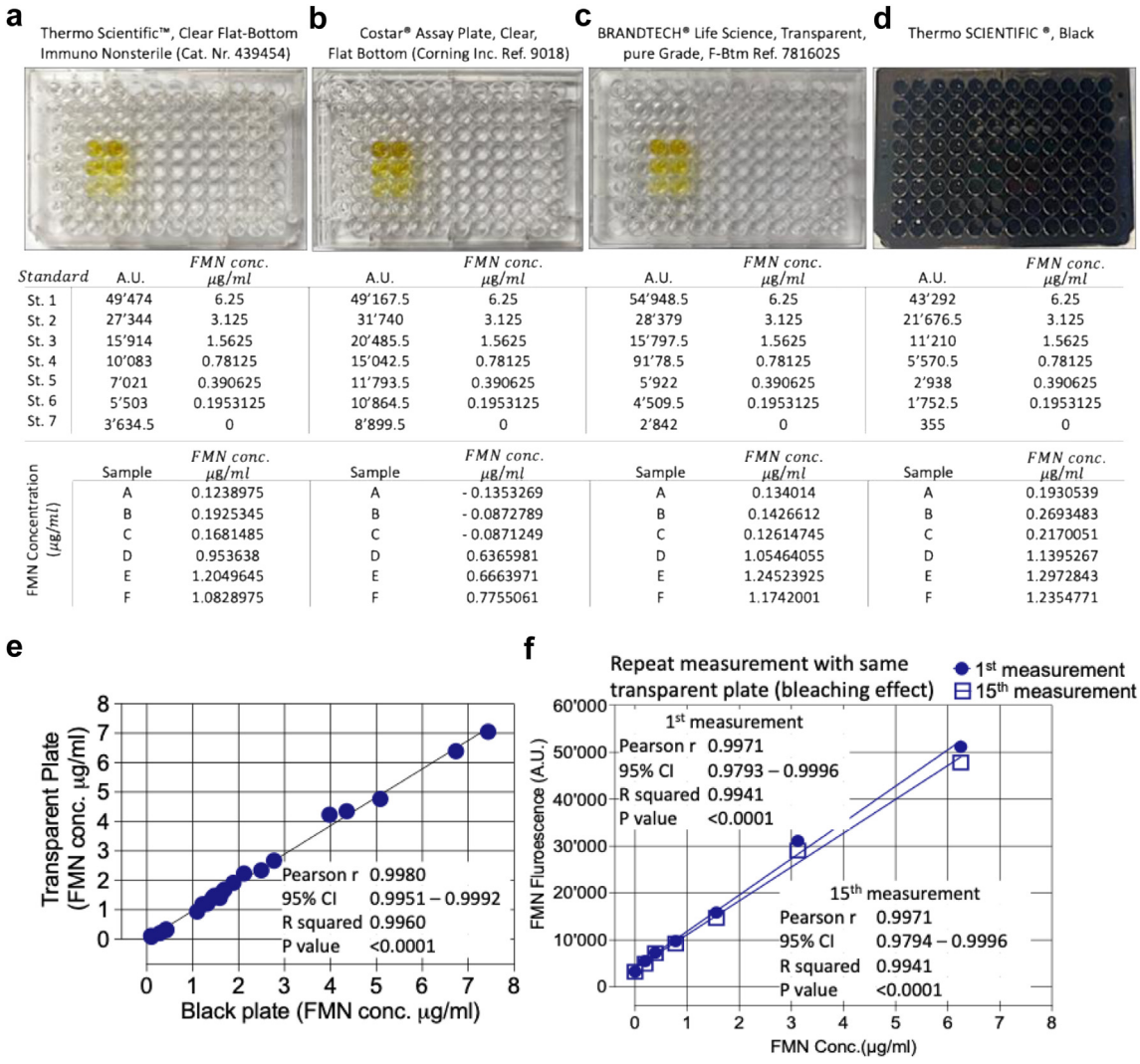
Given that samples and standard are diluted in the same fluid, FMN concentrations are only minimally affected by the wavelength. Different excitation and emission wavelengths can be reliably used, given the standard is read together with the samples. The gain can be used to enhance low signals. Our results show that adjusted gains enhance the A.U. but do not affect the FMN concentration if samples are measured with the standard. Higher A.U. are seen in Fig. 6b, d and f compared to templates Fig. 6c, e and g due to amplification by the higher gain of 130%. Excellent correlations were seen comparing different gains and wavelength used in different centres, i.e. comparing our institution ("Cleveland settings") and "Wang et al. settings" (Fig. 6h and i).[35] Most spectrometers offer primarily an autogain adjustment function to for example prevent "overflow" (i.e. too high signal) in high concentration samples (Fig. 6j).

What is the impact of different spectrometers?

Spectrometers provided by different companies are often programmed using various scales resulting in different A.U. ranges. Even new and old (established) spectrometers of the same type and provided by the same company can show different detection ranges with different A.U. levels for the same molecule concentrations. Fluorescence signals might be affected by the age of the detectors and calibration dates. Some devices also have different scales for amplification, i.e. gain and voltage. However, despite the different spectrometer signals, FMN concentrations were not affected by using the described standard dilutions and spectrometer wavelength. Excellent correlations were found for FMN concentrations measured by different spectrometers using identical wavelength (Fig. 7a and b). Five different spectrometers in two institutions were used to compare HOPE FMN standard readings. Only the A.U. was affected by the adjusted gain and voltage (amplification), while specific FMN concentrations remained the same. For example, the highest FMN standard 1 had an A.U. of >90,000,000 with the Spectramax ID3 device and less than 10,000 A.U. using the Cytofluor with gain 50 or 90 (Fig. 7c). For consistent and reproducible results, the excitation and emission wavelengths must be either the same or comparable across different devices. Additionally, standard and sample dilutions should be performed the same way.

What is the impact of different FMN standards?

Three providers for FMN powder (Riboflavin 5′-Phosphate) were identified and their products were tested in establishing the standard dilutions based on our protocol. Riboflavin 5′- Phosphate provided by USP™ or Sigma Aldrich® led to identical results. Both providers guarantee a purity of ≥70%. The alternative provided by PFL achieved half the intensity (A.U. signal) suggesting a lower purity of the powder (Fig. 7d and e). Spectrometers are often shared among different research groups and the devices suffer long hours of analyses with device overheating. The first spectrometer used at our centre exposed our group to such challenges. We therefore analysed the role of device temperature on FMN concentration results. Fluorescence signals were identical when measured with the established Synergy H1 spectrometer at room temperature (i.e. 22–25 °C) compared to a measurement after a long spectrometer use leading to high temperatures of 37 °C



Standard	A.U.	FMN conc. µg/ml	A.U.	FMN conc. µg/ml	A.U.	FMN conc. µg/ml	A.U.	FMN conc. µg/ml
St. 1	49'474	6.25	49'167.5	6.25	54'948.5	6.25	43'292	6.25
St. 2	27'344	3.125	31'740	3.125	28'379	3.125	21'676.5	3.125
St. 3	15'914	1.5625	20'485.5	1.5625	15'797.5	1.5625	11'210	1.5625
St. 4	10'083	0.78125	15'042.5	0.78125	91'78.5	0.78125	5'570.5	0.78125
St. 5	7'021	0.390625	11'793.5	0.390625	5'922	0.390625	2'938	0.390625
St. 6	5'503	0.1953125	10'864.5	0.1953125	4'509.5	0.1953125	1'752.5	0.1953125
St. 7	3'634.5	0	8'899.5	0	2'842	0	355	0

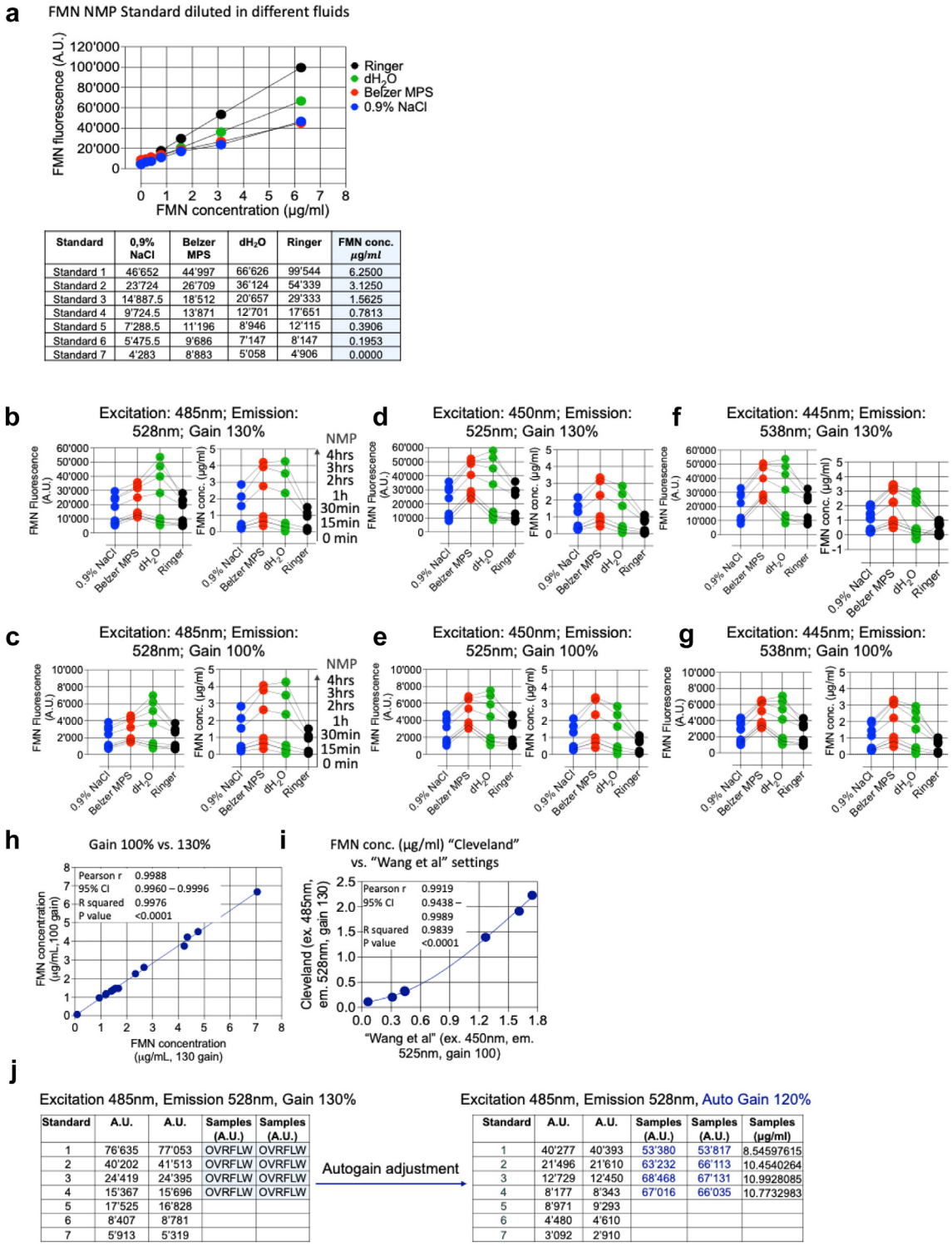
FMN Concentration (µg/ml)	Sample	FMN conc. µg/ml	Sample	FMN conc. µg/ml	Sample	FMN conc. µg/ml	Sample	FMN conc. µg/ml
	A	0.1238975	A	- 0.1353269	A	0.134014	A	0.1930539
	B	0.1925345	B	- 0.0872789	B	0.1426612	B	0.2693483
	C	0.1681485	C	- 0.0871249	C	0.12614745	C	0.2170051
	D	0.953638	D	0.6365981	D	1.05464055	D	1.1395267
	E	1.2049645	E	0.6663971	E	1.24523925	E	1.2972843
	F	1.0828975	F	0.7755061	F	1.1742001	F	1.2354771

Fig. 5: **Fluorescence spectroscopy comparing FMN NMP standard and sample readings using different 96 well plates** (Excitation: 485 nm; Emission: 528 nm; Gain: 130%). a–d) Black and most of the transparent plates (a, c & d) do not affect the FMN concentration because they are calibrated with the standard despite the different fluorescence intensities and absolute A.U. values. However, among the different transparent plates, material differences may affect the FMN results. Plate b seems to reduce the absolute A.U. and sample FMN concentration. Black plate compared with clear plate (Excitation: 485 nm; Emission: 528 nm; Gain: 130%). e) Concentrations read using the ThermoScientific® clear plate (as used in all tests and in a) correlated well with the available ThermoScientific® black plate (as used in test d, r = 0.9980). f) Using our standard powder with standard dilutions and spectrometer settings as described we experienced only a very minimal photobleaching effect. Repeat measurements of the same standards in the same plate (1st–15th measurement) showed almost identical results for FMN concentrations.

(Fig. 7f). Such tests can also easily be repeated because most spectrometers have an option to adjust the temperature during measurement.

Discussion

Based on the high clinical relevance of quantifying ischaemia-reperfusion injury (IRI) in correlation with post-transplant outcomes, there is increasing interest in using Flavin mononucleotide (FMN) as a viability marker for decision making in liver transplantation. This work provides a detailed methodology for immediate florescence spectroscopy to quantify FMN in different fluids of relevance in organ transplantation. A detailed guide how to produce stock solutions, serial dilutions of standards and sample preparation for fluorescence spectroscopy is provided first. Second, the calculation of FMN concentrations based on spectrometer intensity signals is demonstrated with a linear correlation curve for the different source fluids,



Standard	0,9% NaCl	Belzer MPS	dH2O	Ringer	FMN conc. µg/ml
Standard 1	46'652	44'997	66'626	99'544	6.2500
Standard 2	23'724	26'709	36'124	54'339	3.1250
Standard 3	14'887.5	18'512	20'657	29'333	1.5625
Standard 4	9'724.5	13'871	12'701	17'651	0.7813
Standard 5	7'288.5	11'196	8'946	12'115	0.3906
Standard 6	5'475.5	9'686	7'147	8'147	0.1953
Standard 7	4'283	8'883	5'058	4'906	0.0000

Standard	A.U.	A.U.	Samples (A.U.)	Samples (A.U.)
1	76'635	77'053	OVRFLW	OVRFLW
2	40'202	41'513	OVRFLW	OVRFLW
3	24'419	24'395	OVRFLW	OVRFLW
4	15'367	15'696	OVRFLW	OVRFLW
5	17'525	16'828		
6	8'407	8'781		
7	5'913	5'319		

Standard	A.U.	A.U.	Samples (A.U.)	Samples (A.U.)	Samples (µg/ml)
1	40'277	40'393	53'380	53'817	8.54597615
2	21'496	21'610	63'232	66'113	10.4540264
3	12'729	12'450	68'468	67'131	10.9928085
4	8'177	8'343	67'016	66'035	10.7732983
5	8'971	9'293			
6	4'480	4'610			
7	3'092	2'910			

Fig. 6: **FMN quantification in Standard and NMP perfusate using different solutions for dilution, measured with different spectrometer settings** (Excitation: 485 nm; Emission: 528 nm; Gain: 130%; n = 2 each measurement). The different slope of the FMN standard line shows that different solutions chosen for dilution can affect the signal intensity of the FMN. However, the correlation between the fluorescence signal of FMN Standards and their concentration remains linear. The most common solutions, such as 0.9% NaCl and Belzer MPS, behave similarly (a). Next, NMP perfusate samples were diluted in different fluids including our routine 0.9% NaCl, Belzer MPS, distilled water and Ringers. Such

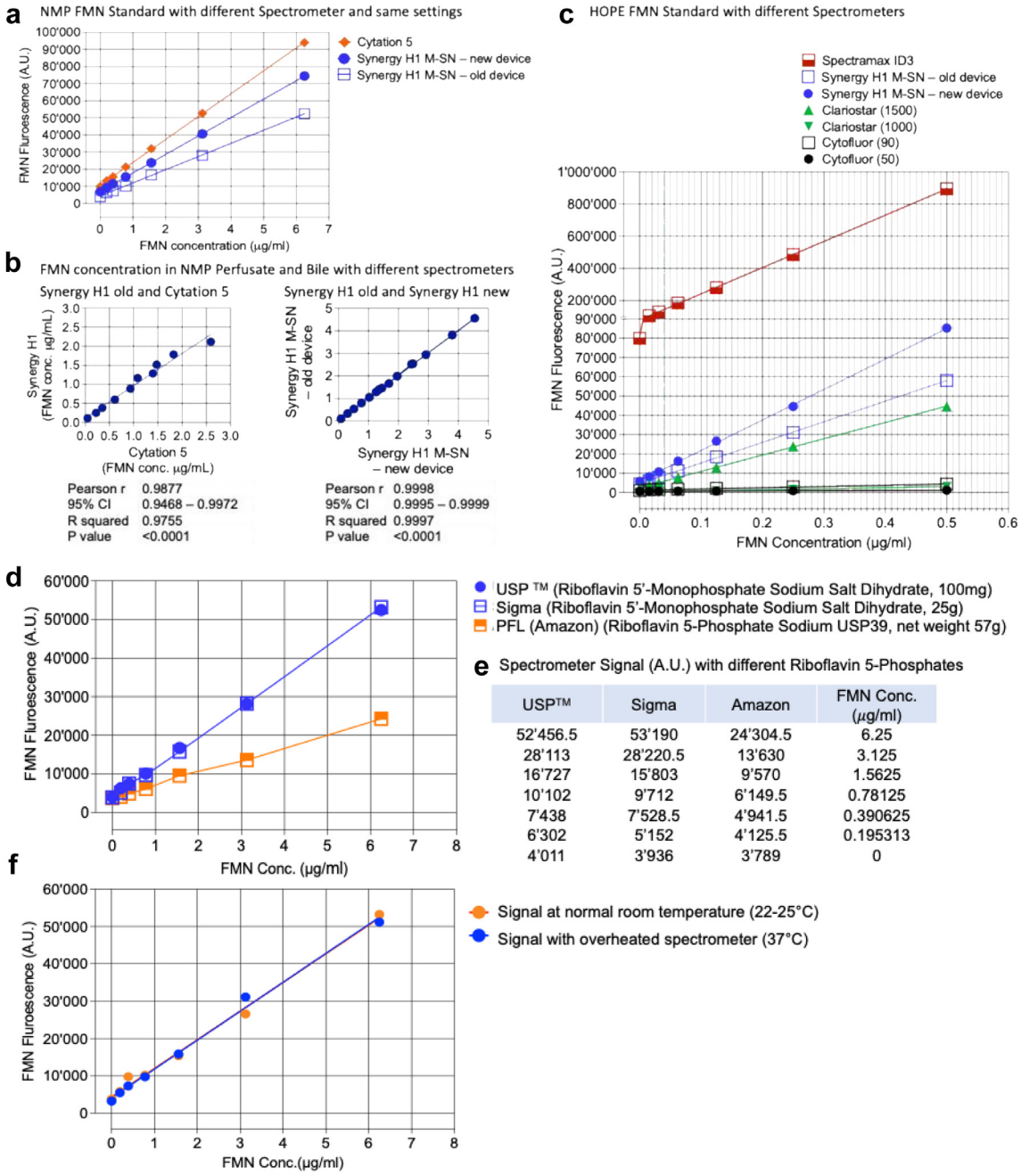
including HOPE and NMP perfusates. Third, the impact of various confounders including available 96-well plates, the dilution medium, spectrometer type and settings and the temperature during analysis is assessed. The results show excellent correlations between spectrometer signal intensity and FMN concentration with various 96-well plates, spectrometer types and settings, provided that precise methodology is followed for serial diluted standards. Most consistent results were found when 0.9% NaCl or Belzer MPS were used for standard and sample dilution. Fluorescence spectroscopy serves as reliable method to quantify FMN independently of the sample origin. While our methodology requires specific spectrometer settings, the process can be successfully applied to other portable or stationary spectrometers provided that the same methodology is used.

The key role of mitochondria in cellular respiration, ATP production and as IRI–instigators is known for decades.[28,45–47] Mitochondrial complex I initiates electron transfer in the respiratory chain, containing 8 iron-sulphur clusters and non-covalently bound FMN.[48,49] It exclusively oxidizes NADH produced during catabolism (i.e. glycolysis, TCA cycle) and is a primary contributor to mitochondrial ATP synthesis. FMN acts as versatile electron carrier, coupling electron transfer with proton transfer,[50–52] initiating the series of redox reactions that generate the proton-motive force necessary for ATP synthesis.[26] While the overall structure and catalytic properties of the FMN site are well understood, several aspects are currently explored further including the molecular details of flavin binding to the N-module and the NADH-oxidizing part of complex I. In contrast, when the noncovalent bond between reduced FMN and complex I weakens during ischaemia, the dissociation of FMN from its pocket in complex I (NDUFV1) and emerging as a significant regulatory mechanism for complex I activity and ROS production, relevant for all tissues that undergo a form of ischaemia, either in context of transplantation or when a stroke or myocardial infarction occurs.[26,29,53–56] High levels of accumulated succinate support an undirected (and retrograde) electron transfer triggering FMN dissociation from complex I and ROS production.[57–59] Impaired complex I activity and mitochondrial dysfunction are the immediate consequences of FMN loss.[25,26,29,32,35] Livers with high FMN release have a significantly impaired Complex I function with subsequent lack of ATP.[37,38]

The release of FMN represents the level of mitochondrial injury during machine perfusion and prior to accepting an organ, which in turn predicts graft survival and complications after liver transplantation.[33,37,39] The release of FMN is temperature, complex I activity- and oxygen dependent.[36] Reoxygenation of ischaemic tissues at higher temperatures, i.e. under normothermic conditions, triggers more FMN release compared to a hypothermic re-oxygenation.[33,38] Independently from the temperature, FMN release into perfusates correlates with posttransplant outcomes, enabling the proposal of perfusate FMN thresholds to avoid severe complications and maintain safe organ transplantation despite increasing donor risk and prolonged ischaemia.[33,60] In addition to the demonstrated predictive value of FMN during HOPE in human livers, authors from the United Kingdom (UK) have assessed the role of FMN during normothermic perfusion, both in livers and kidneys.[35] Wang et al. presented in their proof-of-concept study evidence that perfusate FMN levels at 60 min of kidney NMP predict post-transplant renal function. The authors also measured FMN during the first 30 min of normothermic regional perfusion (NRP) at donor organ procurement and showed the role of FMN identifying livers suitable for transplantation.[35] Sousa Da Silva et al. supported previous work linking ischaemia and FMN release during reperfusion, reinforcing its potential as a biomarker for quantifying IRI. The study showed that as kidney ischaemia increased from 0 to 30 and 60 min, there was a corresponding rise in perfusate FMN levels during hypothermic kidney re-perfusion.[34]

While all molecules absorb photons, relatively few exhibits fluorescence. Flavins are one of two most important classes of molecules, that do fluoresce and have proven valuable in analytical procedures. The Fluorescence of FMN is well documented between 500 and 600 nm.[61,62] FMN is not only more stable, but also the most common biological form of flavin with high quantum yields (QY of 0.27) due to reduced quenching from the adenine ring, compared to flavin adenine dinucleotide (FAD) and other forms. While the exact absorbance maxima for protein-bound flavins depend on the nature of the flavin-binding site, free flavins, such as FMN released from mitochondrial complex I, exhibit specific wavelengths characteristic of pure FMN, supporting spectroscopic quantification methods. Fluorescence spectroscopy was proposed superior to absorbance due to higher specificity in detection of

samples were analysed for FMN concentrations using our standard spectrometer settings (i.e. Excitation 485 nm; Emission 528 nm; Gain 130%) (b) and with reduced Gain of 100% (c). Given the spectrometer settings and standard preparations are kept as per routine and as described (dilution in 0.9% NaCl) (d–g), gain modifications from 130% to 100% lead to the same FMN concentrations (r = 0.9988) (h). Similarly, applying our standard spectrometer settings in comparison with the settings presented by Wang et al.[35] and used by the team from Vienna (Austria) leads to comparable FMN concentrations (r = 0.9919) (i). A gain reduction to 120% (using the autogain option, see also Fig. 4, step 9, template 6b) results in the same FMN concentration but prevents "sample overflow" with too high A.U. and resulting in no FMN concentration. This might be relevant in fluids with very high FMN levels, such as bile released during NMP from livers with high risk (j).



USP™	Sigma	Amazon	FMN Conc. (µg/ml)
52'456.5	53'190	24'304.5	6.25
28'113	28'220.5	13'630	3.125
16'727	15'803	9'570	1.5625
10'102	9'712	6'149.5	0.78125
7'438	7'528.5	4'941.5	0.390625
6'302	5'152	4'125.5	0.195313
4'011	3'936	3'789	0

***Fig. 7*: Fluorescence spectrum signal of NMP and HOPE standards measured with different spectrometers and the role of FMN standard powders and temperature effects** (Excitation 485 nm; Emission 528 nm; Gain 130%; duplicates for each measurement). Despite the use of different Spectrometers, NMP FMN standards behave linearly to the FMN concentration (a). FMN concentrations measured with the Synergy H1 (our own established reader) and the Cytation 5 were comparable and correlated well (r = 0.9877). Old (established) and new Synergy H1 readers quantified FMN with exact the same concentrations (r = 0.9998) (b). The absolute FMN concentration of HOPE standard remains the same despite using five different Spectrometers in two different centres (US and the Netherlands) (c), given the same wavelength was used for excitation and emission with all devices. The parameter gain had to be either adjusted or accepted as not adjustable. Despite the different devices and the various gain (i.e. Spectromax ID3 with autogain, Clariostar with 1000 or 1500, Cytofluor with 50 and 90), FMN concentrations were identical. Such analyses confirm the high relevance and reproducibility of our results with various spectrometers and settings using the same settings and standard dilutions. d) Repeat fluorescence spectroscopy with the same spectrometer and standard settings (Excitation: 485 nm; Emission: 528 nm; Gain: 130%) were done using standard FMN powder (Riboflavin 5′-phosphate, USPTM, 1535700, CAS: 6184-17-4) compared to Riboflavin 5′-phosphate provided by Sigma (Sigma Aldrich Fine Chemicals Biosciences (Riboflavin 5′-phosphate sodium slat

molecules that fluoresce with high quantum yield, which offer normally a higher signal noise/ratio over absorbance.[26,50–52,62] Free FMN, as released from complex I during reoxygenation emits light between 500 and 600 nm with a shoulder at 525 nm. Based on earlier studies, a monochrome light with excitation wavelength of 485 nm was used and the fluorescence emitted by FMN at a wavelength of 528 nm was recorded with a gain of 130%.[9,34,38] Our study shows further identical results when using transparent and black 96-well plates and different spectrometer settings, such as different gains or voltages, provided that the serial diluted standard is used for detection. Multiple calibration curves were produced with our method (average >70 per month) with identical signal intensity. Our described methodology largely follows established ELISA testing principles, emphasizing the high relevance in using a standardized procedure which is replicable with various spectrometers in every centre worldwide. Various dilution factors for standards and samples with 0.9% NaCl were tested at the beginning to develop a reproducible serial dilution method for the standard with high accuracy and a wide range to avoid sample overflow due to very high FMN concentrations outside the range. The distribution of the standard diluted with 0.9% NaCl was normal throughout several hundred tests and with excellent correlation in related calibration curves. Such results were also seen when dilutions were done with other media, including Belzer MPS, distilled water and Ringer, although the 0.9% NaCl and Belzer MPS were the two best solutions with the least interference and the highest accuracy. The impact of pH on FMN molecules and fluorescence signal intensity was previously assessed. FMN fluorescence was stable in both, fluids with low and high pH, values beyond 10 did however reduce the intensity.[36,61,63,64] Solutions used for organs during donation, transport and transplantation, assessed here, are within physiological pH range with no impact on results.

The importance of sample handling and light protection was repeatedly emphasized by many and strictly followed at our centre to avoid FMN conversion or loss in samples or stock solutions and prepared standards. While LC-MS data repeatedly confirmed spectroscopy results by many, others were not able to consistently measure FMN with both methods, which we suspect is due to suboptimal sample handling resulting in false low or undetectable FMN levels in kidney perfusates.[65] Light exposure catalyses FMN conversion to Riboflavin, which requires adjusted settings for LC-MS analysis.[61,63,66] In contrast, FMN levels in kidney perfusate were quantified using both LC-MS and the fluorescence method presented here, showing consistent results with increasing metabolic kidney injury.[34] Wang et al. further tested the stability of FMN under different temperatures.[35] Fluorescence readings of FMN solutions stored at 4 °C for 1 and 7 days, and at –80 °C for 28 days were plotted against known FMN concentrations for such solutions, to evaluate the impact of storage duration and temperature. Despite the different storage conditions and temperatures, FMN concentrations were identical.[35] Our analyses confirmed these findings, also showing almost identical FMN concentrations with different temperatures between 22 and 37 °C during spectrometer reading.

While our method is robust and reproducible, it may still be subject to the limitations common to fluorescence spectroscopy techniques. Precise calibration is essential to prevent errors and avoid contamination, which can lead to false results and reduced sensitivity. Despite these challenges, our methodology has demonstrated high reproducibility, with minimal deviation across different standard calibration curves. Consistent serial dilution of standards and samples is crucial for developing reliable results. Additionally, although photobleaching can reduce the fluorescence capacity of fluorophores under prolonged illumination, our measurements consistently produced identical calibrations, even after 15 or more repeat readings of the same samples.

In summary, this study provides a detailed methodology for FMN quantification with fluorescence spectroscopy showing reproducible results with valid linearity of calibration curves provided FMN powder is serially diluted in standards 1–7 and run with the samples. Our study confirms this with different media, wavelength, gain and spectrometers and application in different centres across two countries. Spectroscopy serves as excellent method to reliably assess organs prior to transplantation to safely increase donor organ utilization. While perfusate FMN thresholds during HOPE are in clinical use and internationally validated, such cutoffs are currently established and validated for NMP in livers.[37] This FMN quantification methodology is currently tested with organs other than the liver in clinical practice to further refine FMN thresholds.

hydrate, MFCD00150992, R777425G). Such two providers guarantee a >70% purity. "Prescribed For Life" (PFL) provides Riboflavin 5′-phosphate Sodium USP39 through Amazon (Lot—V702-2204001-R, net weight 57 g). Riboflavin 5′- phosphate provided by USP and Sigma has high purity and subsequently higher and identical A.U. signals when tested with our routine standard concentrations 1–7. e) The powder provided by PFL however seems to have a lower purity, triggering reduced signals as seen with an A.U. of 24′304.5 for the highest standard 1 with 6.25 µg FMN/ml. f) Our standard method was applied reading NMP FMN standards at different temperatures. Within a range of 22–37 °C results were identical.

Contributors

Conceptualization, K.S. and A.S.; methodology, K.S., C.J., R.P. and A.S.; software, K.S. and A.S.; validation, R.P., F.D.G. and J.D.J.; formal analysis, K.S., C.J., S.S., O.K. and T.D.; investigation, S.S. and O.K.; data curation, K.S., C.J., R.P., S.S., O.K., K.A., F.D.G., T.D., K.A., L.A.C., B.C., Q.L., M.Y., A.P., M.K., J.K., A.H., P.D., P.C.M, F.A., D.H.K., E.F., J.C., J.M.E., M.F., A.P., C.J.W., R.L.F., J.D.J. C.M., K.H. and A.S.; writing—original draft preparation, K.S. and A.S.; writing—review and editing, all coauthors; visualization, K.S. and A.S.; project administration and funding: A.S.; advisory and support: L.S., D.M., P.D.; All authors read and approved the final version of the manuscript.

Data sharing statement

Data supporting the figures and tables of this manuscript (analysis data) are available from the corresponding author upon reasonable request.

Declaration of interests

A.S. is a consultant at Bridge to life Ltd. and Organox Ltd, and has received a grant from Organox Ltd. The other authors have no conflicts of interest to disclose.

Acknowledgements

The experiments and analysis in the study were supported by the Catalyst grant No. CCG0280 at Cleveland Clinic Ohio, US, awarded to A.S.

The authors are grateful for the daily support by the entire team at Cleveland Clinic Ohio and Weston Florida, including the M19 preservation team, the research fellows and clinical ASTS transplant surgery fellows, the team of transplant donor and recipient coordinators, the physician assistants, members of adjacent specialties, operating theatre and nursing staff. The authors are also very grateful for the support by the transplant centre and Inflammation and Immunity administrators. The authors thank Bridge to life ltd. for their supply with Belzer MPS solution.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2025.105761.

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