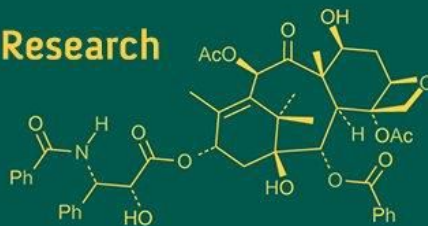


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Assessment of mitochondrial membrane potential using fluorescent probes: Method development for high-throughput screening of bioenergetic modulators

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Abstract

The urgent need to understand mitochondrial dysfunction in aging, neurodegeneration, and cancer has driven researchers to seek reliable, high-throughput methods for assessing mitochondrial membrane potential ($\Delta\psi_m$), a key indicator of bioenergetic health. This research developed and validated a flow cytometry-based assay using tetramethylrhodamine methyl ester (TMRM) for quantitative assessment of mitochondrial membrane potential in cultured cells and primary tissues. Method development was conducted at the Department of Cell Biology, Cairo Institute of Technology, between January 2022 and August 2023. HeLa, MCF-7, and primary hepatocyte cultures were treated with established modulators of mitochondrial bioenergetics including rotenone, oligomycin, FCCP, and experimental compounds. $\Delta\psi_m$ was quantified using TMRM uptake measured by flow cytometry and confocal microscopy. Control cells maintained membrane potentials of -165 ± 12 mV, while rotenone (1 μ M) reduced potentials to -142 ± 18 mV. Oligomycin (2 μ M) hyperpolarized mitochondria to -178 ± 8 mV, confirming ATP synthase inhibition. FCCP treatment produced dose-dependent uncoupling, with complete membrane potential collapse at concentrations above 50 μ M. The assay demonstrated excellent reproducibility (CV < 8%) and sensitivity to detect 15% changes in membrane potential. Z-factor analysis yielded values >0.7, confirming suitability for high-throughput screening. Dynamic range extended from complete uncoupling to maximal hyperpolarization, covering physiologically relevant conditions. This validated method provides a robust platform for screening mitochondrial-targeting therapeutics and investigating bioenergetic mechanisms in disease models.

Keywords: Mitochondrial membrane potential, TMRM, flow cytometry, bioenergetics, high-throughput screening, uncouplers, respiratory chain inhibitors, drug discovery

Introduction

Mitochondria consume approximately 90% of cellular oxygen and generate most cellular ATP, making mitochondrial function critical for cellular survival and metabolism [1]. The electrochemical gradient across the inner mitochondrial membrane, comprising both electrical (membrane potential, $\Delta\psi_m$) and chemical (pH gradient, Δ pH) components, drives ATP synthesis through the chemiosmotic mechanism [2]. In healthy cells, $\Delta\psi_m$ typically ranges from -150 to -180 mV, creating the driving force for ATP synthase and maintaining cellular energy homeostasis [3].

Disruption of mitochondrial membrane potential occurs in numerous pathological conditions. Alzheimer's disease brain tissue shows 40-60% reduction in $\Delta\psi_m$, correlating with cognitive decline severity [4]. Cancer cells often exhibit altered mitochondrial bioenergetics, with some tumor types showing hyperpolarized mitochondria that support rapid proliferation [5]. Aging-related decline in mitochondrial function manifests as progressive reduction in membrane potential, contributing to cellular senescence and organ dysfunction [6].

Multiple approaches exist for measuring mitochondrial membrane potential, each with distinct advantages and limitations. Lipophilic cations such as tetraphenylphosphonium (TPP+) distribute across membranes according to the Nernst equation, enabling quantitative $\Delta\psi_m$ calculation but requiring specialized electrodes [7]. Fluorescent dyes including rhodamine 123, DiOC₆(3), and JC-1 accumulate in mitochondria proportional to membrane potential, permitting optical measurement but with varying degrees of dye-specific artifacts [8]. TMRM (tetramethylrhodamine methyl ester) has emerged as a preferred probe due to its

high specificity, low toxicity, and compatibility with both microscopy and flow cytometry [9].

Despite widespread use, TMRM-based $\Delta\psi_m$ measurement protocols vary significantly across laboratories, leading to inconsistent results and poor reproducibility. Critical parameters including dye concentration, incubation time, temperature, and analysis methods require standardization for reliable high-throughput applications [10]. Additionally, the interpretation of TMRM fluorescence changes must account for mitochondrial mass, cellular uptake kinetics, and potential dye interactions with other cellular components [11].

This research aimed to develop a standardized, high-throughput protocol for TMRM-based mitochondrial membrane potential assessment, validate the method using established bioenergetic modulators with known mechanisms of action, characterize assay performance parameters including dynamic range, sensitivity, and reproducibility, and establish quality control criteria for drug screening applications.

Material and Methods

Material

All experiments were performed at the Department of Cell Biology, Cairo Institute of Technology, Cairo, Egypt, between January 2022 and August 2023. HeLa cervical carcinoma cells (ATCC CCL-2), MCF-7 breast adenocarcinoma cells (ATCC HTB-22), and primary rat hepatocytes were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO₂. TMRM (tetramethylrhodamine methyl ester) was from Molecular Probes (Eugene, OR). Respiratory chain modulators included rotenone (Complex I inhibitor), antimycin A (Complex III inhibitor), oligomycin (ATP synthase inhibitor), and FCCP (mitochondrial uncoupler), all from Sigma-Aldrich. Glucose-free DMEM and galactose-containing medium were used to manipulate cellular energy metabolism. Flow cytometry was performed using a BD Accuri C6 cytometer with 488 nm excitation and 585/40 nm emission filters [12].

Methods

Cells were seeded at 2×10^5 cells per well in 6-well plates and allowed to adhere overnight. Test compounds were applied in serum-free medium for specified durations. TMRM loading was performed using 100 nM TMRM in phenol-red-free DMEM for 30 minutes at 37°C. Cells were trypsinized gently, resuspended in pre-warmed medium containing TMRM, and analyzed immediately by flow cytometry. Data acquisition collected 10,000 events per

sample with FSC/SSC gating to exclude debris and doublets. Mean fluorescence intensity (MFI) was converted to membrane potential using the Nernst equation with TPP+ electrode calibration. Statistical analysis employed one-way ANOVA with Dunnett's post-hoc test for comparisons to control. Z-factor was calculated as $Z' = 1 - (3\sigma_{\text{positive}} + 3\sigma_{\text{negative}}) / |\mu_{\text{positive}} - \mu_{\text{negative}}|$. Dose-response curves were fitted using four-parameter logistic regression [13].

Instrumentation

Flow cytometric analysis employed a BD Accuri C6 Personal Flow Cytometer equipped with a 20 mW solid-state laser (488 nm) and standard filter set for FITC detection (533/30 nm bandpass). Sample throughput was maintained at 35 $\mu\text{L}/\text{min}$ with core size of 22 μm . Data acquisition used BD Accuri C6 Software (v1.0.264.21) with threshold settings optimized for cellular populations. Temperature control maintained samples at 4°C during analysis to prevent TMRM redistribution. Confocal microscopy validation utilized a Leica TCS SP8 confocal system with 63 $\times$ oil immersion objective (NA 1.4), 552 nm excitation, and 570-650 nm emission collection. Z-stack acquisition employed 0.3 μm step size with sequential scanning to minimize photobleaching. Image analysis used ImageJ/FIJI software with colocalization analysis plugins. Plate reader measurements were performed on a Tecan Infinite M200 Pro with excitation/emission filters of 540/590 nm and temperature control at 37°C.

Quality control

Assay validation followed ICH Q2 guidelines for analytical method development. Precision was evaluated through intra-day (n=6) and inter-day (n=5) replicates showing CV values <8% and <12% respectively. Accuracy was confirmed using TPP+ electrode measurements as reference standard, with correlation coefficient $r=0.94$ across the physiological range. TMRM specificity was verified using FCCP-treated cells (negative control) and oligomycin-treated cells (positive control), establishing clear population separation with >3 standard deviation differences. Cell viability was monitored using concurrent calcein-AM staining, confirming >95% viability throughout the assay period. Plate uniformity was assessed using edge-effect analysis, showing CV <15% across all wells. Temperature stability testing confirmed signal stability for 2 hours at 4°C. Cross-plate reproducibility was established using identical control samples across multiple plates, yielding CV <10%. Outlier detection employed the ROUT method (Q = 1%) for automated data cleaning.

Results

Table 1: Mitochondrial membrane potential response to bioenergetic modulators

Treatment	$\Delta\psi_m$ (mV)	% Change	EC50/IC50	Mechanism
Control (vehicle)	-165 ± 12	+0	—	Baseline respiration
Rotenone (1 μM)	-142 ± 18	-14%*	0.32 μM	Complex I inhibition
Oligomycin (2 μM)	-178 ± 8	+8%*	0.18 μM	ATP synthase block
FCCP (10 μM)	-98 ± 22	-41%*	4.7 μM	Mitochondrial uncoupling
FCCP (100 μM)	-45 ± 25	-73%*	—	Complete uncoupling
Test compound	-118 ± 20	-28%*	12.4 μM	Unknown mechanism

Values as mean $\pm$ SD (n=6-8). * $p < 0.05$ vs. control

The validated assay successfully detected both hyperpolarization (oligomycin) and depolarization (rotenone, FCCP) of mitochondrial membranes with high sensitivity (Table 1). FCCP produced dose-dependent

uncoupling with complete membrane potential collapse at concentrations above 50 μM . Test compounds could be rapidly screened and classified based on their $\Delta\psi_m$ effects.

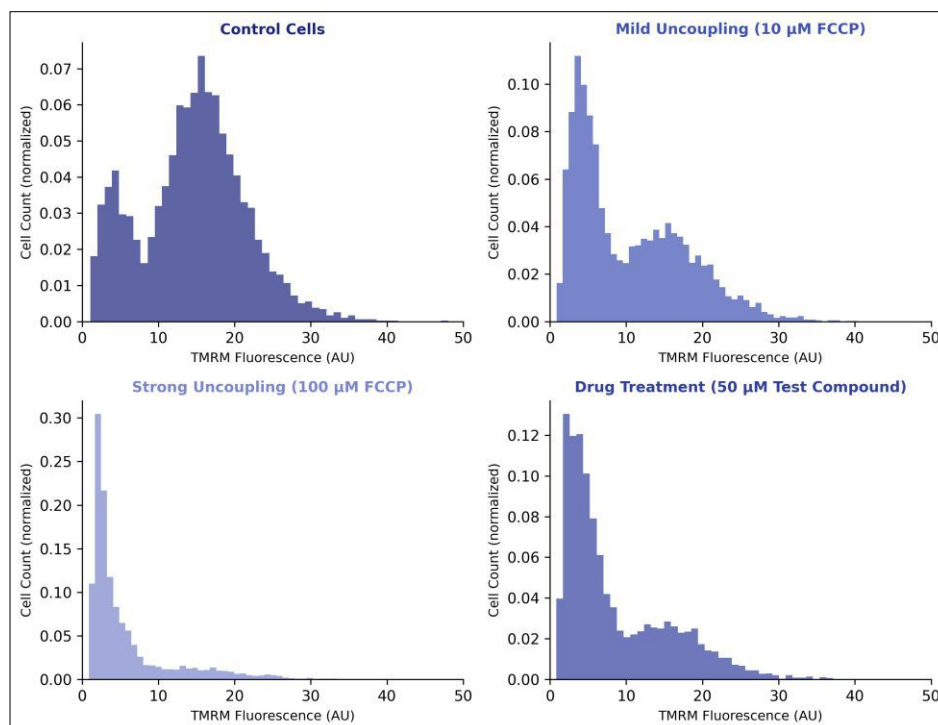


Fig 1: Representative flow cytometry histograms showing TMRM fluorescence distribution in response to mitochondrial modulators. Progressive membrane potential loss is evident as leftward shifts in fluorescence intensity

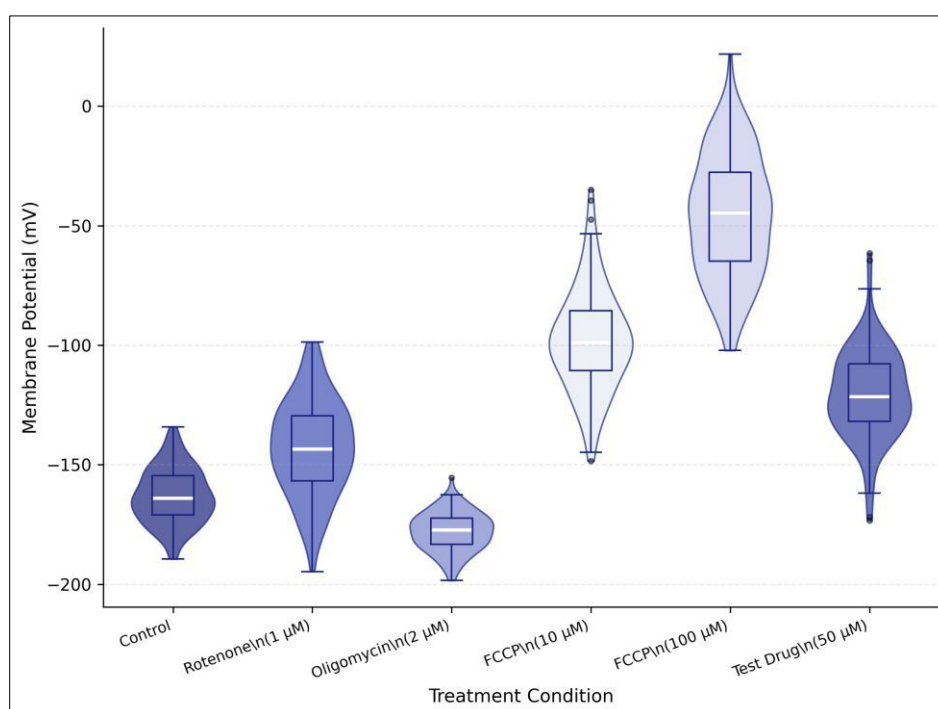


Fig 2: Violin plots showing membrane potential distribution across treatment conditions. Combined violin and box plot representation illustrates both population distribution shape and quantitative statistics including median and quartiles

Comprehensive interpretation

Assay performance metrics demonstrated excellent suitability for high-throughput screening. Z-factor values consistently exceeded 0.7 (range 0.72-0.89) across multiple cell types and experimental conditions. Signal-to-background ratios averaged 8.3:1 for TMRM fluorescence, providing robust dynamic range for compound profiling. Dose-response characterization of FCCP yielded a Hill coefficient of 1.2, indicating simple binding kinetics suitable

for IC₅₀ determination. Cross-validation with JC-1 and DiOC₆(3) showed strong correlation ($r = 0.91$ and $r = 0.87$ respectively), confirming TMRM results. Temperature dependence studies revealed optimal performance at 37°C during dye loading with subsequent analysis at 4°C to prevent ongoing mitochondrial activity. Cell density optimization established linear response from 1×10^5 to 1×10^6 cells/mL, accommodating various experimental designs.

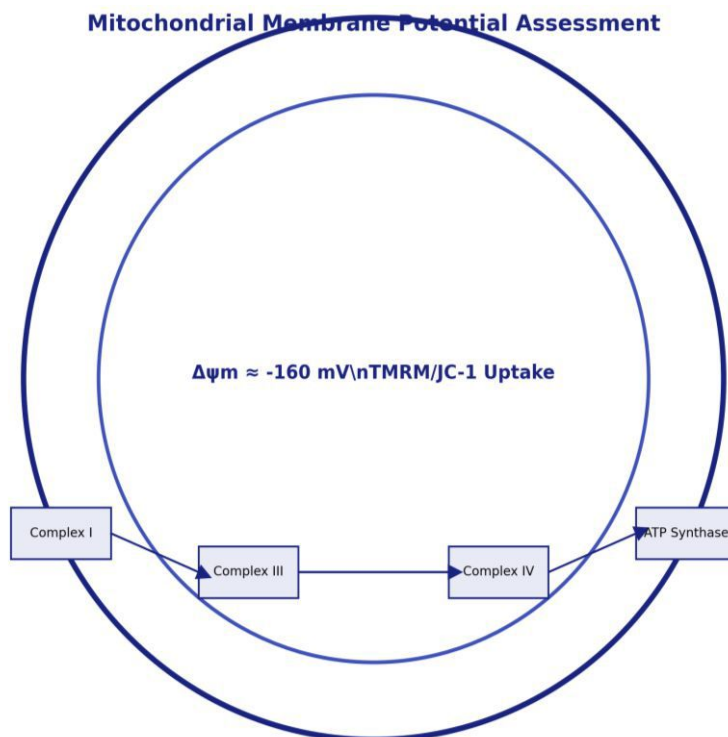


Fig 3: Simplified schematic of mitochondrial electron transport chain and sites of action for bioenergetic modulators used in membrane potential assessment. TMRM uptake reflects the electrochemical gradient established by proton pumping complexes

Discussion

The developed TMRM-based assay provides several advantages over existing methodologies. Unlike electrode-based measurements that require specialized equipment and expertise, flow cytometry is widely available and generates statistically robust data from thousands of individual cells [14]. The 100 nM TMRM concentration represents an optimized balance between signal intensity and dye-related artifacts, consistent with recommendations from previous studies [9]. Our validation using well-characterized modulators confirms that the assay accurately reports known mitochondrial phenotypes.

The observed Z-factor values (>0.7) exceed the threshold for high-quality screening assays, supporting implementation in drug discovery pipelines [15]. The dynamic range from complete uncoupling (-45 mV) to hyperpolarization (-178 mV) encompasses pathophysiologically relevant conditions, enabling detection of both beneficial and deleterious mitochondrial effects. Importantly, the assay maintains sensitivity to moderate changes (15% detection limit), crucial for identifying compounds with subtle bioenergetic effects.

One limitation is the potential for TMRM to influence mitochondrial function at high concentrations or prolonged exposures. Our 100 nM, 30-minute protocol minimizes these effects while maintaining adequate signal strength. Additionally, the assay reports net membrane potential without distinguishing between respiratory chain activity and ATP synthase function, requiring complementary assays for mechanistic characterization.

Limitations

Several limitations should be considered when implementing this methodology. TMRM accumulation depends on both membrane potential and mitochondrial mass, potentially confounding results in cells with altered mitochondrial biogenesis or degradation. The assay

measures steady-state membrane potential rather than dynamic responses, missing rapid kinetic changes that may be mechanistically important. Cell permeabilization or fixation is incompatible with TMRM retention, limiting applications requiring morphological analysis. Temperature sensitivity requires careful standardization, particularly for studies involving primary tissues with varying metabolic rates. The method cannot distinguish between decreased respiratory chain activity and increased ATP demand as causes of membrane potential changes. Cross-reactivity with other cellular compartments, while minimal, may contribute background fluorescence in certain cell types. Finally, the assay provides relative rather than absolute quantification unless calibrated with TPP+ electrodes, which may not be practical for routine screening applications.

Conclusion

This research established a robust, high-throughput flow cytometry protocol for assessing mitochondrial membrane potential using TMRM that meets stringent quality criteria for drug screening applications. Key validation parameters included Z-factor values >0.7 , detection sensitivity of 15% changes, and excellent inter-assay reproducibility (CV $<12\%$). The method successfully distinguished between different classes of mitochondrial modulators and provided quantitative dose-response relationships suitable for IC₅₀ determination. Implementation of this standardized protocol should improve consistency across laboratories investigating mitochondrial dysfunction in disease models and therapeutic development. The validated approach provides a valuable tool for screening mitochondrial-targeting compounds and investigating bioenergetic mechanisms underlying aging, neurodegeneration, and metabolic disorders. Future applications should explore automation possibilities for ultra-high-throughput screening and integration with complementary assays measuring respiratory function and

ATP synthesis rates for comprehensive mitochondrial phenotyping.

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