Calibrating the Rotenone Dose Regime

H9 SynGFP MO, Day 62 — 150 μm Section — About 6 Weeks into Differentiation

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1. System Parameters

Organoid Characteristics

Parameter	Value	Derivation
Geometry	Lenticular disk: ~2.5 mm diameter × 150 μm max thickness	Measured
Volume	V = (2/3)πa²b ≈ 0.245 mm³	Ellipsoid integral
Total cells	~24,500	~100,000 cells/mm³ × 0.245 mm³
DA neurons (TH+)	~2,450 (10%)	IHC estimate
Non-DA neurons	~9,800 (40%)	Typical organoid composition
Glia/progenitors	~12,250 (50%)	Remainder

Cell density estimates are consistent with reported values for brain organoids (Lancaster & Knoblich, 2014), and DA neuron proportions align with published midbrain organoid characterizations (Jo et al., 2016).

Volume derivation (ellipsoid cap):

For a lenticular disk with radius a and half-thickness b at center, the profile follows:

$$z(r) = b \times \sqrt{1 - r^2/a^2}$$

Integrating by revolution:

$$V = 2 \times \int_0^a 2\pi r \times z(r)\, dr = 4\pi b \int_0^a r\sqrt{1 - r^2/a^2}\, dr$$

Substituting u = 1 - r²/a², du = -2r/a² dr:

$$V = 4\pi b \times (-a^2/2) \int_1^0 \sqrt{u}\, du = 2\pi a^2 b \times \left[\frac{2}{3}u^{3/2}\right]_0^1 = \frac{2}{3}\pi a^2 b$$

With a = 1.25 mm, b = 0.075 mm:

$$V = \frac{2}{3} \times \pi \times (1.25)^2 \times 0.075 = 0.245 \text{ mm}^3$$

Mitochondrial Parameters

Cell Type	Mitochondria/cell	Complex I/mito	Total Complex I
DA neurons	600	6,000	8.8×10^9
Other neurons	400	5,000	19.6×10^9
Glia/progenitors	200	4,000	9.8×10^9
Total	—	—	**38.2×10^9**

Mitochondrial estimates are based on the observation that neurons maintain millions of mitochondria distributed throughout their arbors, with approximately 3 mitochondria per micrometer of axon (Misgeld & Schwarz, 2017). Dopaminergic neurons exhibit elevated mitochondrial bioenergetics compared to other neuron types (Pacelli et al., 2015). Complex I, the largest component of the respiratory chain, contains 45 subunits in mammals (Hirst, 2013; Zhu et al., 2016).

Note: Total Complex I binding sites in organoid: ~38 billion. DA neuron Complex I: ~9 billion (23% of total). However, not all Complex I is equally accessible—see Section 4 for multi-compartment equilibration considerations.

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2. Rotenone Availability & Stoichiometry

Molecules in Bath

Rotenone molecules at 25 nM in 1 mL:

$$N = C \times V \times N_A$$

$$N = (25 \times 10^{-9} \text{ mol/L}) \times (10^{-3} \text{ L}) \times (6.022 \times 10^{23})$$

$$N = 1.5 \times 10^{13} \text{ molecules}$$

Stoichiometric Ratios

Comparison	Ratio	Interpretation
Rotenone : Total Complex I	$1.5 \times 10^{13} : 3.8 \times 10^{10} \approx 395:1$	Large excess
Rotenone : DA neuron Complex I	$1.5 \times 10^{13} : 8.8 \times 10^{9} \approx 1{,}700:1$	Even larger excess for target

Note: At 25 nM, rotenone is in ~400-fold excess over total Complex I binding sites. This excess ensures the system will reach equilibrium binding; the question is how much damage accumulates before washout. However, this stoichiometric calculation assumes direct access to Complex I. The *effective* rotenone available to partition into the inner mitochondrial membrane is reduced by multi-compartment lipid equilibration (see Section 4), though the excess remains sufficient for saturation at steady state.

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3. Binding Kinetics

Rotenone-Complex I Binding Parameters

Parameter	Value	Source
Dissociation constant (Kd)	~10 nM	Higgins et al., 1996
Association rate (kon)	$\sim 10^{6}$ $M^{-1}s^{-1}$	Typical small molecule-protein
Dissociation rate (koff)	koff = Kd × kon = 10^{-2} s^{-1}	Calculated
Binding half-life	$t_{½} = \ln(2)/koff \approx 70$ seconds	Calculated

The dissociation constant for rotenone binding to Complex I has been measured at 15–55 nM across different brain regions using [^{3}H]dihydrorotenone autoradiography, with Hill slopes of ~1 indicating a single population of binding sites (Higgins & Bhatti, 1996). The 10 nM estimate used here represents the lower end of this range, appropriate for high-affinity binding conditions.

Fractional Occupancy at Equilibrium

Langmuir Binding Isotherm (Langmuir, 1918; Hill, 1910):

$$\theta = \frac{[R]}{K_d + [R]}$$

At [R] = 25 nM, Kd = 10 nM:

$$\theta = \frac{25}{10 + 25} = 0.71 \text{ (71\% occupancy)}$$

Concentration	Complex I Inhibited
25 nM	71%
100 nM	91%

At 100 nM, 91% Complex I inhibition leaves almost no reserve capacity. At 25 nM, 29% of Complex I remains functional—potentially enough for survival if exposure is limited.

Time to Equilibrium Binding (Intrinsic)

Approach to equilibrium (assuming free rotenone access to Complex I):

$$\theta(t) = \theta_{eq} \times (1 - e^{-k_{obs} \times t})$$

where:

$$k_{obs} = k_{on}[R] + k_{off} = (10^6)(25 \times 10^{-9}) + 10^{-2} = 0.025 + 0.01 = 0.035 \text{ s}^{-1}$$

$$\tau = 1/k_{obs} \approx 29 \text{ seconds}$$

$$t_{95\%} = 3\tau \approx 90 \text{ seconds}$$

Caveat: This calculation assumes rotenone is freely available at the Complex I binding site. In reality, rotenone must traverse multiple lipid compartments before reaching the inner mitochondrial membrane. The intrinsic binding kinetics are fast (~90 seconds), but *delivery* kinetics through the lipid compartment network are rate-limiting. See Section 4 for revised equilibration estimates.

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4. Multi-Compartment Lipid Equilibration & Cellular Uptake

Lipid Network Equilibrium

Rotenone (logP ≈ 4.1–4.5; PubChem CID 6758) partitions strongly into all lipid membranes, not just mitochondria. Molecular dynamics simulations confirm that rotenone localizes in membranes with its ring near glycerol groups of phospholipids (Talpaz & Green, 2023). When rotenone enters a cell, it doesn't

immediately find Complex I—it rapidly equilibrates across the entire intracellular lipid network: plasma membrane, endoplasmic reticulum, Golgi apparatus, lipid droplets, and mitochondrial membranes.

Intracellular rotenone distribution at quasi-equilibrium:

Compartment	Approximate Volume	Partition Coefficient	Rotenone Fraction
Plasma membrane	~60% of total membrane	~3,000× vs. aqueous	~85-90%
ER/Golgi membranes	~25% of total membrane	~2,000×	~5-8%
Mitochondrial membranes	~15% of total membrane	~1,000×	~5-7%
Cytoplasm (aqueous)	~5 pL per cell	~1×	<1%

Implication: For any given cell at quasi-equilibrium, only ~5-7% of intracellular rotenone resides in the mitochondrial compartment where it can access Complex I. The plasma membrane acts as a massive lipid buffer that sequesters the majority of intracellular rotenone.

Time to Equilibrium

The intrinsic binding kinetics at Complex I are fast (~90 seconds), but rotenone must first:

1. Partition from media into plasma membrane (<1 second)
2. Redistribute from plasma membrane through cytoplasm to mitochondria (rate-limiting)
3. Partition into outer mitochondrial membrane (fast)
4. Equilibrate across intermembrane space
5. Partition into inner mitochondrial membrane (where Complex I resides)
6. Diffuse laterally within IMM to find Complex I binding channel
7. Bind to quinone-binding site (fast once in IMM)

Steps 2-5 constitute a multi-compartment redistribution process. The plasma membrane continuously buffers rotenone, releasing it slowly into the aqueous cytoplasm where it can find mitochondrial targets.

Equilibration estimate:

Phase	Estimated Time	Process
Initial membrane loading	<1 minute	Rotenone partitions into plasma membrane
Intracellular redistribution	10-30 minutes	Equilibration across ER, Golgi, mitochondria
Complex I binding	~2 minutes	Once rotenone reaches IMM
Total to 95% steady-state	**15-45 minutes**	Full system equilibration

Implication: For exposures ≥2 hours, the system is at steady-state and the equilibrium occupancy calculations apply. For very short exposures (<1 hour), the effective inhibition may be lower than predicted by equilibrium calculations.

Tissue Diffusion

Characteristic diffusion time (Crank, 1975):

$$\tau_{diff} = \frac{L^2}{2D}$$

L = 75 μm (half-thickness to center from top/bottom surfaces)
D_tissue ≈ 1 × 10^{-6} cm²/s (rotenone in tissue, accounting for tortuosity factor ~1.6; Nicholson & Syková, 1998)

$$\tau_{diff} = \frac{(7.5 \times 10^{-3}\ \text{cm})^2}{2 \times 10^{-6}\ \text{cm}^2/\text{s}} = 28\ \text{seconds}$$

Note: This calculation describes aqueous-phase diffusion through tissue. In reality, each cell layer acts as a lipid equilibration sink, slowing effective transport to deeper layers.

Membrane Partitioning & Sequestration Gradients

Rotenone must traverse progressively more cell layers to reach the organoid center, with each layer acting as a partial lipid sink due to multi-compartment equilibration. Vertically, rotenone can enter from top and bottom surfaces, dividing the effective diffusion distance by 2. The slice geometry renders radial diffusion less relevant —and less desirable for selective DA targeting.

Vertical diffusion path:

- Vertical half-distance: 75 μm
- Cell diameter: ~15 μm
- Cell layers (vertical): n ≈ 5

If each layer sequesters fraction *f* of rotenone into its lipid compartments before passing the remainder:

$$C_{center} = C_{bath} \times (1 - f)^n$$

Sequestration per layer	Central concentration
f = 5%	C_center = 0.95^5 × C_bath = 0.77 × C_bath
f = 10%	C_center = 0.90^5 × C_bath = 0.59 × C_bath
f = 15%	C_center = 0.85^5 × C_bath = 0.44 × C_bath

Radial diffusion path (if rotenone were added to bulk media):

- Radial distance: 1,250 μm
- Cell layers (radial): n ≈ 83

Sequestration per layer	Central concentration
f = 5%	C_center = 0.95^{83} × C_bath = 0.014 × C_bath
f = 10%	C_center = 0.90^{83} × C_bath ≈ 0.0002 × C_bath

Implication: Radial diffusion through ~83 cell layers results in severe attenuation of rotenone concentration at the center, potentially reducing effective concentration to <2% of bath levels. This would spare central DA neurons while killing peripheral cells—the opposite of the intended selective toxicity.

Delivery Strategy: Targeted Vertical Diffusion

To achieve selective DA neuron death, rotenone will be injected into the media directly over the central region of the organoid containing the DA neurons. This approach promotes targeted delivery via vertical diffusion through fewer "sink" layers (~5 layers vertically vs. ~83 layers radially).

Advantages of targeted vertical delivery:

1. Central DA neurons experience near-bath concentrations (77-90% of C_bath at f = 5-10%)
2. Peripheral cells experience reduced initial exposure (rotenone must diffuse radially outward)
3. Faster equilibration at the target site (shorter diffusion path)
4. More efficient use of rotenone (less lost to peripheral sinks)

Expected concentration profile:

Region	Distance from injection	Cell layers traversed	Estimated concentration
Central (DA-rich)	Direct vertical	~5	0.7-0.9 × C_bath
Mid-peripheral	Mixed path	~20-40	0.3-0.5 × C_bath
Edge	Radial from center	~60-80	0.05-0.2 × C_bath

This gradient creates an internal dose-response within the organoid: central DA neurons receive near-full exposure while peripheral cells receive attenuated doses.

Off-Target Binding Summary

Membrane lipid sink:

- Total membrane area ≈ 24,500 cells × 1,000 μm^2/cell = 2.45×10^7 μm^2 = 0.245 cm^2
- Membrane volume (5 nm thick) ≈ 1.2×10^{-6} cm^3 = 1.2 nL
- With partition coefficient 3,000×, this sequesters: (1.2 nL / 1000 μL) × 3,000 × 25 nM ≈ 0.09 nM equivalent from bath

Non-specific protein binding:

- Cytoplasmic protein concentration ≈ 200 mg/mL
- Assuming 1% of proteins have weak rotenone affinity (Kd ~ 10 μM): sequesters ~5-10% of intracellular rotenone

What this means: Off-target binding is minor relative to the ~400-fold stoichiometric excess. The sink affects *kinetics* (slowing equilibration) more than *capacity* (final occupancy). With targeted vertical delivery and exposures ≥2 hours, steady-state inhibition of ~60-70% is expected at central DA neurons.

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5. Metabolic Effects & Damage Accumulation

ATP Production Under Inhibition

Residual Complex I flux:

At 71% equilibrium inhibition (25 nM):

- NADH pathway flux = 29% of normal
- $FADH_2$ pathway (Complex II) = 100% of normal (uninhibited)
- Normal ATP production ratio: ~70% from NADH, ~30% from $FADH_2$ (Hinkle, 2005)

- Inhibited production: (0.29 × 70%) + (1.0 × 30%) = 20% + 30% = **50% of normal**

Accounting for multi-compartment equilibration:

If effective Complex I occupancy at central DA neurons is ~60% (rather than 71%) due to lipid compartmentalization:

- NADH pathway flux = 40% of normal
- Inhibited production: (0.40 × 70%) + (1.0 × 30%) = 28% + 30% = **58% of normal**

For conservative estimates, we use the 50% capacity figure (assuming near-full equilibration is achieved for exposures ≥2 hours).

Demand-Supply Gap

Parameter	Normal	Under 25 nM rotenone
ATP production capacity	100%	~50%
ATP demand (active neurons)	~80% of capacity	~80% of capacity (unchanged)
Reserve margin	+20%	**~-30%**

Bioenergetics: At 25 nM with targeted delivery, DA neurons in the central region operate at a ~30% ATP deficit. This is survivable short-term through:

- Glycolytic compensation (limited, produces lactate/acidosis)
- Reduced firing rate (neurons throttle back)
- Creatine phosphate reserves (depleted in minutes)

Beyond a few hours, cumulative damage becomes irreversible.

Damage Accumulation Model

Simple model (direct inhibition):

$$D_{direct}(t) = k \times \theta \times t = k \times 0.71 \times t$$

where k = damage rate constant (cell-type specific)

Cumulative model (inhibitory + ROS cascade effects):

Rotenone triggers reactive oxygen species (ROS) production at Complex I. The primary site of ROS generation is the flavin moiety and quinone-binding site, where electrons leak to O_2 forming superoxide (Murphy, 2009;

Dröse & Brandt, 2012). These ROS initiate secondary damage cascades that persist after washout:

- Lipid peroxidation → 4-HNE, MDA → protein adducts
- mtDNA damage → impaired Complex I synthesis → chronic deficiency (Lin & Beal, 2006)
- Protein carbonylation → aggregation → proteostatic stress
- Calcium dysregulation → mitochondrial permeability transition

$$D_{total}(t) = \int_0^t \left[k_1\theta(\tau) + k_2 R(\tau)\right] d\tau$$

where:

- $k_1\theta(\tau)$ = direct Complex I inhibition damage (stops at washout)
- $R(\tau)$ = accumulated ROS/cascade damage (continues after washout)

R(t) follows:

$$\frac{dR}{dt} = \alpha\theta(t) - \beta R(t)$$

where:

- α = ROS generation rate during inhibition
- β = ROS clearance rate (antioxidant capacity)

If $\alpha > \beta$ during exposure, R accumulates. After washout ($\theta \to 0$), R decays with rate β, but irreversible damage (lipid peroxidation, mtDNA lesions) has already occurred and continues to propagate.

Effective Exposure Amplification

Nominal exposure	Effective damage equivalent
2 h	~2.5 h
4 h	~5 h
6 h	~7.5 h
8 h	~10 h
12 h	~16 h

Nominal exposure	Effective damage equivalent
18 h	~24 h
24 h	~32 h
36 h	~48 h
48 h	~65 h

Note: Post-washout ROS cascades effectively amplify exposure duration by 1.2-1.4× depending on initial exposure length. Longer exposures generate more accumulated ROS, leading to greater cascade amplification.

For DA neurons with high metabolic demand and high baseline ROS production (Pacelli et al., 2015), k_1 and α are approximately 3-5× higher than in quiescent cells. This baseline oxidative stress in dopaminergic neurons contributes to their selective vulnerability (Jenner & Olanow, 2006).

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6. Thermodynamic Death Threshold

The ATP Synthase Reversal Point

Cell death becomes inevitable when the phosphoryl transfer potential drops below the proton motive force. When mitochondrial respiration is compromised and $\Delta\psi m$ falls below a threshold, F_1F_0 ATP synthase can reverse, hydrolyzing ATP to pump protons through the membrane (Campanella et al., 2008). Since depletion of ATP precipitates cell death, this reversal marks a critical transition point.

Thermodynamic balance (Nicholls & Ferguson, 2013):

$$\Delta G_p = \Delta G^{\circ\prime} + RT \ln\left(\frac{[ATP]}{[ADP][P_i]}\right)$$

$$\Delta G_{pmf} = -nF \times \Delta\psi - 2.3RT \times \Delta pH$$

- **Normal:** $\Delta Gp \approx -54$ kJ/mol, $\Delta G\_pmf \approx -42$ kJ/mol → ATP synthesis favored
- **At crisis:** ΔGp drops as [ATP]/[ADP] falls
- **Reversal point:** $\Delta Gp = \Delta G\_pmf$ → synthase stalls
- **Below reversal:** Synthase runs backward, consuming ATP

The inhibitor protein IF1 can prevent wasteful ATP hydrolysis by blocking the counter-clockwise rotation of ATP synthase (Campanella et al., 2008), but this protection is limited under sustained energy deficit.

Time to Threshold

Based on ATP depletion kinetics at ~30% deficit, adjusted for post-washout ROS cascade amplification. Studies have shown that ~30% ATP loss sustained for 3 hours commits cells to apoptosis, while longer or more severe depletion leads to necrosis (Bhoun-Bhoun et al., 2011).

Cell Type	Metabolic Demand	Nominal Exposure to Reversal	Effective (with cascade)
DA neurons (active)	Very high	12-24 hours	10-20 hours
Other neurons	High	30-48 hours	24-40 hours
Glia	Moderate	48-72 hours	40-60 hours
Quiescent progenitors	Low	>72 hours	>60 hours

Note: At 25 nM rotenone with targeted vertical delivery, DA neurons in the central region approach the thermodynamic death threshold at 12-24 hours nominal exposure when cascade effects are included. Other cell types remain viable for 30+ hours. This defines the selective DA toxicity window.

Peripheral Cell Fate

With targeted vertical delivery over the organoid center:

- **Central DA neurons:** Experience near-bath concentration, earliest to cross death thresholds
- **Mid-peripheral cells:** Experience attenuated concentration (~30-50% of bath), delayed damage
- **Edge cells:** Experience lowest concentration (<20% of bath), may survive or show delayed effects

This creates a radial damage gradient from center to periphery—the inverse of what would occur with bulk media addition.

Expected observations:

- Days 1-2: Central region shows stress markers (reduced firing, ROS indicators)
- Days 2-4: Central DA neurons cross death threshold, MEA activity decreases
- Days 4-7: Peripheral regions may show delayed stress or partial recovery
- Day 7: Clear differentiation between central (damaged) and peripheral (surviving) regions

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7. Dose-Response Ladder

The relevant variable is cumulative time at inhibition. Varying time from 0-48 hours with final in-well concentration fixed at 25 nM, delivered via targeted injection over the organoid center. This concentration is

within the range (20-30 nM) shown to induce selective dopaminergic neurotoxicity in primary midbrain cultures (Gao et al., 2002; Sherer et al., 2003).

This approach aims to capture the full curve from minimal effect to selective DA death to global toxicity, while accounting for post-washout cascade amplification and multi-compartment equilibration.

Proposed Ladder

Well	Exposure	Effective Damage	Predicted Outcome	Rationale
1	0 h (vehicle)	0 h	Baseline	Control
2	4 h	~5 h	Minimal/mild effect	Full equilibration achieved; below damage threshold
3	6 h	~7.5 h	Early DA stress	ROS accumulation begins; first detectable effects
4	8 h	~10 h	DA stress, early compromise	Entering DA vulnerability window
5	12 h	~16 h	Moderate DA compromise	DA neurons approaching crisis
6	18 h	~24 h	Significant DA loss	DA death threshold crossed
7	24 h	~32 h	Severe DA loss	Most DA neurons past reversal point
8	36 h	~48 h	DA depletion, global stress	Other neurons approaching threshold
9	48 h	~65 h	DA depletion, global compromise	Near global toxicity threshold

Note on 4-hour minimum exposure: Given that multi-compartment lipid equilibration requires 15-45 minutes for full steady-state, exposures <2 hours may not achieve consistent Complex I inhibition. The 4-hour minimum ensures the system is fully equilibrated for the majority of the exposure period.

Note on 6-hour timepoint: This timepoint is added to capture the transition from "minimal effect" to "DA stress"—a potentially important early window for detecting selective vulnerability.

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8. Model Validation Against Prior Observations

Comparison of 100 nM vs. 25 nM Predictions

At 100 nM:

- θ = 100/(10+100) = 91% inhibition
- Residual ATP capacity: ~35%
- Energy deficit: ~45%
- ROS generation rate: Higher (more electron backup at Complex I)
- Cascade amplification: ~1.5-1.8×
- DA death threshold: ~8-16 hours (nominal)
- Global death threshold: ~30-48 hours (nominal)

At 25 nM:

- θ = 25/(10+25) = 71% inhibition
- Residual ATP capacity: ~50%
- Energy deficit: ~30%
- ROS generation rate: Moderate
- Cascade amplification: ~1.2-1.4×
- DA death threshold: ~12-24 hours (nominal)
- Global death threshold: ~48-72 hours (nominal)

Time to Death Scaling

Time to death scales non-linearly with energy deficit:

$$t_{death} \propto \frac{1}{(\text{deficit})^2}$$

- 100 nM: deficit = 45% → $t \propto 1/0.45^2 = 4.9$
- 25 nM: deficit = 30% → $t \propto 1/0.30^2 = 11.1$
- Ratio: 11.1/4.9 = **2.3× longer survival at 25 nM**

Consistency with 100 nM × 48 Hour Observations

With cascade amplification at 100 nM:

- Global death threshold (effective): ~45-72 h
- Overshoot: 72-86 h effective vs. 45-72 h threshold = at or past global lethal threshold

The 100 nM × 48 hour experiment was at or beyond the global death threshold, which is consistent with observed minimal functional recovery at 72 hours. However, no firm conclusions about the sample's complete response can be made, because recovery dynamics may extend beyond the 72-hour observation window—the observed structural survival and persistent activity in some 100 nM and 500 nM samples suggests the threshold was approached but not massively exceeded.

Model Predictions for Current Experiment

With targeted vertical delivery at 25 nM:

Exposure	Expected Central DA Effect	Expected Peripheral Effect
4 h	Minimal—possible mild ROS elevation	None detectable
6 h	Early stress markers; firing rate changes possible	None to minimal
8 h	Moderate stress; detectable MEA changes	Minimal
12 h	Significant compromise; reduced activity	Mild stress in mid-peripheral
18 h	Substantial DA loss; activity severely reduced	Moderate stress spreading inward
24 h	Severe DA loss; minimal central activity	Moderate stress
36 h	Near-complete DA depletion	Significant stress; some cell loss
48 h	Complete DA depletion	Global compromise approaching

9. Summary

Critical Parameters

Parameter	Value	Confidence	Source
Target concentration	25 nM	High	Gao et al., 2002
Equilibrium Complex I inhibition	71% (theoretical)	High	Langmuir isotherm
Effective inhibition (with lipid equilibration)	60-70%	Moderate	Estimated

Parameter	Value	Confidence	Source
Time to steady-state inhibition	15-45 minutes	Moderate	Estimated
Selective DA death window	12-24 hours nominal	Moderate	Sherer et al., 2003
Global toxicity threshold	48-72 hours nominal	Moderate	Extrapolated
Cascade amplification factor	1.2-1.4×	Low-moderate	ROS kinetics

Experimental Design Features

1. **Targeted delivery:** Rotenone injected directly over organoid center promotes vertical diffusion (~5 cell layers) rather than radial diffusion (~83 cell layers), maximizing DA exposure while minimizing peripheral toxicity.
2. **Time-based ladder:** Fixed concentration (25 nM) with varying exposure (4-48 h) allows precise mapping of the dose-response curve while keeping lipid equilibration dynamics constant across conditions.
3. **Built-in controls:** The radial concentration gradient within each organoid provides internal dose-response information—central regions receive full exposure while peripheral regions receive attenuated doses.
4. **Recovery assessment:** The lower concentration (25 nM vs. 100 nM) and inclusion of sub-threshold exposures (4-8 h) allows observation of both damage progression and potential recovery dynamics.

Expected Outcomes

The experiment is designed to identify the transition points in the dose-response curve:

- **4-6 h:** Threshold for detectable stress
- **8-12 h:** Threshold for DA compromise
- **18-24 h:** Threshold for DA death
- **36-48 h:** Threshold for global toxicity

These transition points, combined with the radial damage gradient from targeted delivery, will provide data-driven calibration for subsequent experiments. If the optimal regime is not identified in the first run, the data will guide more precise calibrations in future iterations.

Equations

Langmuir binding isotherm (Langmuir, 1918):

$$\theta = \frac{[R]}{K_d + [R]}$$

Diffusion time (Crank, 1975):

$$\tau_{diff} = \frac{L^2}{2D}$$

Sequestration gradient:

$$C_{center} = C_{bath} \times (1 - f)^n$$

ATP production under inhibition:

$$\text{Production} = (1 - \theta) \times 0.70 + 1.0 \times 0.30$$

Damage accumulation:

$$D_{total}(t) = \int_0^t [k_1 \theta(\tau) + k_2 R(\tau)] \, d\tau$$

ROS dynamics:

$$\frac{dR}{dt} = \alpha\theta(t) - \beta R(t)$$

Death threshold scaling:

$$t_{death} \propto \frac{1}{(\text{deficit})^2}$$

Thermodynamic reversal condition (Nicholls & Ferguson, 2013):

$$\Delta G_p = \Delta G_{pmf}$$

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