HUMAN CORTICAL ORGANOID PROTOCOL GUIDE

Optimized for Electrophysiological Maturation & Progenitor Banking

Integrating: Temple Lab, Paşca Lab, and Muotri Lab Protocols

with Current Literature on Network Development

EXECUTIVE SUMMARY: DESIGN PRINCIPLES

This guide presents a mechanistically-grounded protocol for generating human cortical organoids optimized for robust electrophysiological activity and reproducible network bursting patterns. The protocol integrates strengths from three established methods—Temple Lab (dorsal forebrain specification), Paşca Lab (maturation cocktails), and Muotri Lab (oscillatory network development)—while incorporating recent literature on outer radial glia expansion, GABAergic interneuron integration, and progenitor cryopreservation.

CORE INNOVATION: PROGENITOR BANKING STRATEGY

The protocol is designed around a cortical progenitor intermediate (Day 15-20) that can be cryopreserved and banked. This developmental checkpoint captures PAX6+/FOXG1+ dorsal forebrain progenitors with established ventricular zone-like organization, before terminal differentiation commits cells to specific layer fates. Thawed progenitors resume differentiation with high reproducibility and generate organoids with characteristic coordinated bursting patterns by 4-6 months post-thaw.

Optimizations for Electrophysiology

1. Triple-inhibition neural induction (Days 0-5): Dual-SMAD inhibition (SB431542 + LDN-193189) combined with WNT inhibition (XAV939) produces more homogeneous dorsal forebrain identity than dual-SMAD alone, suppressing non-cortical fates and enriching for outer radial glia (Rosebrock et al., 2022, Nature Cell Biology).

2. Extended progenitor expansion with LIF (Days 6-20): Leukemia inhibitory factor activates STAT3 signaling to expand outer radial glia populations, which are essential for generating the neuronal diversity required for complex network activity (Walsh et al., 2024, Cell Reports).

3. DAPT-synchronized neurogenesis (Days 46-50): Timed γ-secretase inhibition synchronizes terminal differentiation of remaining progenitors, producing a cohort of neurons that mature together and form functional networks more readily than asynchronously-born populations.

4. BrainPhys transition (Day 50+): Switching from Neurobasal to BrainPhys medium provides physiological ionic conditions (lower glucose, corrected K+, appropriate osmolarity) that enable proper action potential generation and network bursting.

5. GABAergic neuron emergence (Months 4-6): Spontaneous GABAergic interneuron specification from cortical progenitors is critical for the excitation-inhibition balance required for organized oscillatory activity. Network bursting becomes prominent only after GABAergic markers appear (Trujillo et al., 2019, Cell Stem Cell).

PART 1: DEVELOPMENTAL CONTEXT

Human Cortical Development In Vivo

The human cerebral cortex develops from the dorsal telencephalon through a precisely orchestrated sequence of patterning events, progenitor expansion, and neurogenesis. Understanding this developmental trajectory is essential for generating organoids that recapitulate the cellular diversity and network properties of the human cortex.

MECHANISM: Telencephalic Specification
In the embryo, the telencephalon (forebrain) is specified by the intersection of anterior-posterior and dorsal-ventral signaling gradients. LOW WNT signaling (relative to midbrain/hindbrain) combined with HIGH BMP antagonism and LOW SHH defines the dorsal telencephalon. This territory expresses FOXG1 as a master regulator of telencephalic identity, distinguishing it from diencephalon (thalamus) and mesencephalon (midbrain). Within the telencephalon, dorsal progenitors express PAX6 and EMX1/2, while ventral progenitors (ganglionic eminences) express NKX2.1 and DLX1/2.

Progenitor Cell Types and Their Contributions

Apical Radial Glia (aRG): Located in the ventricular zone (VZ), aRG are SOX2+/PAX6+/Nestin+ progenitors that divide at the apical (ventricular) surface. They give rise to all cortical neurons either directly or through intermediate progenitors. aRG maintain apical-basal polarity with an apical process contacting the ventricle and a basal process extending to the pial surface.

Intermediate Progenitors (IP): TBR2+ (EOMES+) cells in the inner subventricular zone (iSVZ) that amplify neuronal output. IPs typically undergo 1-2 divisions before differentiating into neurons. They lack the radial morphology of aRG.

Outer Radial Glia (oRG/bRG): A human-enriched progenitor population in the outer subventricular zone (oSVZ) that expresses HOPX, LIFR, and GFAP in addition to PAX6/SOX2. oRG lack an apical process but maintain a basal process and undergo mitotic somal translocation. The expansion of oRG correlates with neocortical expansion in gyrencephalic species.

LITERATURE: Outer Radial Glia and Organoid Quality
Walsh et al. (2024) demonstrated that LIF treatment activates STAT3 signaling in cortical organoids, promoting oRG expansion and the formation of a structured outer subventricular zone. oRG-enriched organoids showed transcriptional profiles closely matching human fetal oRG and generated more diverse neuronal populations. Rosebrock et al. (2022) showed that short, early dual-SMAD + WNT inhibition ('Triple-i') produces organoids with superior cortical identity and oRG enrichment compared to dual-SMAD alone or unguided protocols.

Cortical Layer Formation

Cortical neurons are born in an inside-out pattern: deep layer neurons (layers 5-6, TBR1+/CTIP2+) are born first, followed by upper layer neurons (layers 2-4, SATB2+/BRN2+/CUX1+). This birthdate-dependent lamination reflects the sequential competence of progenitors to generate different neuronal subtypes.

Deep Layer Neurons (Layers 5-6): Subcerebral projection neurons expressing TBR1, CTIP2 (BCL11B), FEZF2. These are the first neurons born and include corticospinal and corticothalamic projection neurons.

Upper Layer Neurons (Layers 2-4): Intracortical and callosal projection neurons expressing SATB2, BRN2 (POU3F2), CUX1/2. Born later in development, these neurons form the majority of interhemispheric connections.

ELECTROPHYSIOLOGY: Why Layer Diversity Matters for Network Activity
Coordinated bursting patterns in cortical organoids require both deep and upper layer neurons. Deep layer neurons provide the primary output and rhythmogenic drive, while upper layer neurons contribute to local circuit amplification and horizontal propagation of activity. Fair et al. (2020, Stem Cell Reports) showed that organoids with both TBR1+ and SATB2+ populations displayed more complex synchronized burst firing than those dominated by a single layer type. The emergence of layer diversity correlates with the transition from sparse spiking to organized network bursting at 4-6 months.

PART 2: NEURAL INDUCTION (Days 0-5)

Triple-Inhibition Strategy

Neural induction employs three small molecule inhibitors to achieve robust dorsal forebrain specification: SB431542 (TGF-β/Activin/Nodal inhibitor), LDN-193189 or Dorsomorphin (BMP inhibitor), and XAV939 or IWR-1-endo (WNT inhibitor). This 'Triple-i' approach produces more homogeneous cortical identity than dual-SMAD inhibition alone.

MECHANISM: SB431542 (10-20 µM) — TGF-β/Activin/Nodal Pathway Blockade
SB431542 inhibits ALK4, ALK5, and ALK7 receptors, blocking phosphorylation of SMAD2/3. This prevents mesendoderm specification (blocks SOX17, FOXA2, BRACHYURY) and releases cells from the pluripotency-maintaining TGF-β autocrine loop. The concentration range of 10-20 µM reflects complete pathway suppression; higher doses (20 µM, Temple protocol) may be needed for some cell lines. SB431542 is the first half of 'dual-SMAD inhibition' (Chambers et al., 2009, Nature Biotechnology).

MECHANISM: LDN-193189/Dorsomorphin (100-150 nM / 2.5 µM) — BMP Pathway Blockade
BMP inhibition blocks SMAD1/5/8 phosphorylation via ALK2/3/6, preventing non-neural ectoderm and trophoblast fates. This mimics the activity of endogenous BMP antagonists (Noggin, Chordin, Follistatin) in the embryonic organizer. LDN-193189 is more potent (IC50 ~5-30 nM) than Dorsomorphin (IC50 ~500 nM), so concentrations differ: 100-150 nM for LDN, 2.5 µM for Dorsomorphin. Together with SB431542, BMP inhibition completes dual-SMAD inhibition, driving >80% of cells toward PAX6+ neural fate within 11 days.

MECHANISM: XAV939/IWR-1-endo (2.5-10 µM) — WNT Pathway Blockade
XAV939 stabilizes Axin2, promoting β-catenin degradation and blocking canonical WNT signaling. WNT inhibition is CRITICAL for ANTERIOR (forebrain) specification. Without it, dual-SMAD inhibition produces a mixture of forebrain and midbrain/hindbrain fates. WNT inhibition upregulates FOXG1 (telencephalic marker) while suppressing EN1/EN2 (midbrain) and GBX2 (hindbrain/thalamus). The WNT inhibition must be EARLY and BRIEF (Days 0-5 only); prolonged inhibition blocks neurogenesis. Rosebrock et al. (2022) showed Triple-i organoids cluster with fetal forebrain/cortical tissue at Day 30, while Dual-SMAD organoids drift toward cerebellar profiles.

CONCENTRATION RATIONALE: Protocol Comparison
TEMPLE PROTOCOL: SB431542 20 µM, Dorsomorphin 2.5 µM, XAV939 10 µM. Higher concentrations for robust specification in aggregate/organoid format where diffusion limits exposure. PAŞCA PROTOCOL: SB431542 5-10 µM, LDN-193189 150 nM, XAV939 2.5 µM. Lower concentrations optimized for KSR-based induction media which has endogenous pathway-modulating activities. MUOTRI 'SEMI-GUIDED': Minimal patterning (SB431542 only for some protocols), relying on cell-autonomous differentiation. Produces more heterogeneous populations but retains cell type diversity.

Survival and Aggregation Factors

ROCK Inhibitor Y-27632 (10 µM, Day 0 only): Prevents anoikis (dissociation-induced apoptosis) by blocking Rho/ROCK-mediated actomyosin hypercontraction. Critical for single-cell survival during aggregation. Remove after Day 1 once cell-cell contacts are re-established; prolonged use disrupts cytoskeletal organization and cell polarity required for neural tube morphogenesis.

CEPT Cocktail (Alternative to Y-27632): Multi-target survival cocktail containing Chroman 1 (ROCK inhibitor, ~100× more potent than Y-27632), Emricasan (pan-caspase inhibitor), Trans-ISRIB (integrated stress response inhibitor), and sometimes Thiazovivin. Achieves >90% single-cell survival vs. ~70% with Y-27632 alone. Use Days 0-3 only.

WARNING: Aggregation Cell Number

Optimal aggregation uses 10,000 cells per well in 96-well slit plates or ultra-low attachment round-bottom plates. Too few cells (<5,000) results in insufficient paracrine signaling and poor neural rosette formation. Too many cells (>20,000) creates necrotic cores due to O2/nutrient diffusion limits. The 10,000-cell aggregate produces organoids of ~200-300 µm diameter at Day 5, optimal for subsequent expansion.

PART 3: CORTICAL PROGENITOR EXPANSION (Days 6-20)

Media Transition: E6 → Neurobasal/B27

At Day 6, withdraw patterning factors and transition to neural maintenance medium. The specification phase is complete; progenitors now need metabolic support for expansion rather than fate-determining signals.

Neurobasal-A Medium: Optimized for neuronal culture with lower osmolarity (~290 mOsm) than DMEM/F12 and reduced glutamate to prevent excitotoxicity. Neurobasal favors neuronal survival over glial proliferation.

B27 Supplement (WITHOUT Vitamin A): Provides antioxidants (vitamin E, catalase, SOD), lipids (linoleic acid, linolenic acid), and other factors supporting neuronal metabolism. CRITICAL: Use B27 WITHOUT Vitamin A until post-mitotic maturation phase. Vitamin A is metabolized to retinoic acid by cellular retinaldehyde dehydrogenases → RA caudalizes progenitors toward hindbrain/spinal cord fates. Using B27+VitA during expansion derails cortical identity.

GlutaMAX (1×): Stable glutamine substitute (L-alanyl-L-glutamine) that prevents ammonia buildup from spontaneous glutamine degradation. Essential for long-term culture.

Mitogenic Factors: EGF and FGF2

MECHANISM: EGF (20 ng/mL) — Radial Glia Expansion
Epidermal Growth Factor acts through EGFR/ErbB1 to activate Ras/MAPK/ERK and PI3K/Akt cascades, driving progenitor proliferation. In the developing cortex, EGFR expression increases as development proceeds; early progenitors are FGF-responsive, while later progenitors become EGF-responsive (Lillien & Raphael, 2000, Development). EGF specifically expands the radial glia population and promotes oRG generation. Combined EGF+FGF2 produces larger progenitor pools than either factor alone.

MECHANISM: FGF2/bFGF (20 ng/mL) — Neural Stem Cell Maintenance
Basic Fibroblast Growth Factor maintains neural stem cell self-renewal through FGFR1/2/3 signaling. FGF2 sustains SOX2 and Nestin expression, prevents premature differentiation, and supports the apicobasal polarity of radial glia. FGF2 also induces calcium bursting that supports communication along radial glial fibers (Rash et al., 2016, Cerebral Cortex). The 20 ng/mL concentration saturates FGFR without promoting glial differentiation.

Outer Radial Glia Expansion: LIF Supplementation

ELECTROPHYSIOLOGY OPTIMIZATION: Leukemia Inhibitory Factor (20 ng/mL, Days 7-24)
LIF activates LIFR/gp130 receptor complexes → JAK/STAT3 signaling pathway. STAT3 activation is a hallmark of oRG identity and is required for oRG self-renewal. Walsh et al. (2024) showed LIF treatment produces a >3-fold increase in HOPX+/GFAP+ oRG-like cells and thickening of the SVZ compartment. LIF-treated organoids showed transcriptional profiles closely matching fetal human oRG. CRITICAL FOR ELECTROPHYSIOLOGY: oRG generate a more diverse population of neurons, including upper layer neurons and GABAergic interneurons, that are essential for establishing E/I balance and coordinated network activity. Start LIF at Day 7 and continue through progenitor banking timepoint.

LITERATURE: oRG and Network Complexity

Pollen et al. (2015, Cell) identified HOPX, LIFR, and TNC as specific markers of human oRG, distinguishing them from aRG. Hansen et al. (2010, Nature) first described oRG in human fetal cortex and showed they undergo mitotic somal translocation. Fietz et al. (2010, Nature Neuroscience) demonstrated that oRG expansion correlates with cortical folding and neocortical expansion. In organoids, oRG enrichment correlates with improved cellular diversity and more complex network activity patterns.

PART 4: PROGENITOR BANKING (Days 15-20)

Developmental Checkpoint for Cryopreservation

Days 15-20 represent an optimal window for cortical progenitor cryopreservation. At this stage, organoids contain:

1. Committed cortical identity: FOXG1+/PAX6+/EMX1+ progenitors with established dorsal forebrain specification that persists through freeze-thaw.

2. Organized ventricular zone-like structures: Neural rosettes with apicobasal polarity, essential for subsequent inside-out neurogenesis.

3. Mixed progenitor populations: Both aRG (SOX2+/PAX6+) and emerging oRG (HOPX+/LIFR+) that provide the diversity needed for complex network formation.

4. Pre-neurogenic state: Before extensive neurogenesis commits cells to specific layer fates, allowing flexibility in downstream protocols.

WARNING: Why Day 15-20 and Not Earlier or Later
TOO EARLY (Day 5-10): Neural identity is not yet stable; progenitors may drift toward non-cortical fates upon thaw. Rosette organization is incomplete. TOO LATE (Day 25+): Significant neurogenesis has occurred. Post-mitotic neurons survive cryopreservation poorly and show reduced recovery of synaptic function. Network formation is already underway and may not resume normally. The Day 15-20 window captures specified but still plastic progenitors that reliably resume differentiation.

MEDY Cryopreservation Protocol

The MEDY method (Xue et al., 2024, Cell Reports Methods) provides superior preservation of organoid structure and neural function compared to DMSO alone. Components:

MECHANISM: MEDY Cryopreservation Components
METHYLCELLULOSE (1%): Increases viscosity to reduce mechanical damage during ice crystal formation. Stabilizes cellular membranes. ETHYLENE GLYCOL (10%): Penetrating cryoprotectant that reduces intracellular ice formation. Lower toxicity than glycerol. DMSO (10%): Primary penetrating cryoprotectant. Colligatively lowers freezing point and reduces ice crystal size. Y-27632 (10 μM): ROCK inhibitor added during pre-incubation prevents cytoskeletal disruption during osmotic stress of cryoprotectant exposure and thawing. Pre-treat organoids with Y-27632 for 1.5-2 hours before adding MEDY solution.

Cryopreservation Procedure

Pre-treatment: Add Y-27632 (10 μM final) to culture medium 1.5-2 hours before cryopreservation. This allows ROCK inhibitor to penetrate organoids.

MEDY solution preparation: In neural medium (Neurobasal + B27 w/o VitA + GlutaMAX): 1% methylcellulose, 10% ethylene glycol, 10% DMSO, 10 μM Y-27632. Prepare fresh and keep at room temperature.

Transfer: Transfer 2 organoids per cryovial. Add 500 μL MEDY solution first, then organoids with minimal carryover medium, then another 500 μL MEDY solution (1 mL total).

Equilibration: Incubate at room temperature for 15-30 minutes to allow cryoprotectant penetration.

Controlled-rate freezing: Use Mr. Frosty or equivalent (-1°C/min) in -80°C freezer overnight, then transfer to liquid nitrogen for long-term storage.

Thawing: Rapid thaw in 37°C water bath (~2 min). Transfer to pre-warmed medium with Y-27632. Dilute MEDY stepwise over 10-15 minutes to prevent osmotic shock. Return to culture with EGF+FGF2+LIF for 48-72 hours before proceeding with differentiation.

LITERATURE: Cryopreservation Validation

Xue et al. (2024) demonstrated that MEDY-cryopreserved cortical organoids maintain rosette structure, progenitor populations (SOX2+/PAX6+), and neuronal markers (MAP2/TUJ1) comparable to fresh organoids. RNA-seq showed preservation of cortical layer markers (TBR1, CTIP2, SATB2) and minimal stress gene induction. Organoids cryopreserved for 1.5 years showed robust recovery with calcium activity and KCl responsiveness similar to fresh cultures. MEA detection confirmed synchronous network activity in thawed organoids.

PART 5: NEURONAL DIFFERENTIATION (Days 20-45)

Growth Factor Withdrawal

At Day 20-25 (or 3-5 days post-thaw for banked progenitors), withdraw EGF and FGF2 to initiate neurogenesis. Replace with neurotrophic factors that support post-mitotic neuron survival and maturation.

MECHANISM: Why Withdraw Mitogens
EGF and FGF2 maintain progenitors in a proliferative state by activating Cyclin D1 and suppressing cell cycle exit genes. Continued mitogenic signaling prevents the pro-neural transcription factor cascade (ASCL1/NEUROG2 → NEUROD1 → p27Kip1 → cell cycle exit). Removing EGF/FGF2 allows endogenous BMP and TGF-β signals to drive differentiation. The timing of withdrawal affects the balance of deep vs. upper layer neurons: earlier withdrawal biases toward deep layers, later withdrawal allows more upper layer neuron production.

Neurotrophin Cocktail: BDNF + NT3

MECHANISM: BDNF (20 ng/mL) — TrkB Signaling
Brain-Derived Neurotrophic Factor binds TrkB receptor tyrosine kinase, activating three major cascades: (1) Ras/MAPK/ERK → neurite outgrowth, synaptogenesis via CREB phosphorylation; (2) PI3K/Akt → neuronal survival, mTOR-dependent protein synthesis; (3) PLCγ/Ca2+/CaMKII → synaptic plasticity, ion channel expression. BDNF upregulates synaptic proteins (synaptophysin, synapsin, PSD-95), voltage-gated Na+ and K+ channels, and glutamate receptor subunits. Long-term BDNF treatment selectively enhances glutamatergic synapse function and increases network excitability.

MECHANISM: NT3 (20 ng/mL) — TrkC Signaling
Neurotrophin-3 activates TrkC receptors, which are highly expressed in cortical projection neurons. NT3 specifically supports corticothalamic and corticocortical projection neuron survival and axon guidance. NT3/TrkC signaling promotes dendritic arborization and spine formation in pyramidal neurons. Combination with BDNF provides complementary trophic support: BDNF for general neuronal maturation, NT3 for cortical projection neuron-specific development. The 20 ng/mL concentration for both factors is standard in cortical differentiation protocols (Paşca et al., 2015, Nature Methods).

Ascorbic Acid Supplementation

MECHANISM: Ascorbic Acid (200 µM)
Ascorbic acid serves three essential functions in neuronal differentiation: (1) ANTIOXIDANT: Scavenges reactive oxygen species from ~20% ambient O2 culture conditions vs. ~3-5% physiological brain O2. Prevents oxidative damage to differentiating neurons. (2) TET ENZYME COFACTOR: TET1/2/3 dioxygenases require ascorbic acid to oxidize 5-methylcytosine → 5-hydroxymethylcytosine, the first step of active DNA demethylation. This epigenetic remodeling is essential for activating neuron-specific gene programs. (3) HISTONE DEMETHYLASE COFACTOR: Jumonji-domain (JHDM/KDM) enzymes require ascorbic acid to remove repressive H3K9me3 and H3K27me3 marks from developmental gene loci. The 200 µM concentration saturates TET enzymes (Km ~100-300 µM) without pro-oxidant effects.

PART 6: SYNCHRONIZED NEUROGENESIS (Days 46-50)

DAPT-Mediated Notch Inhibition

MECHANISM: DAPT (2-10 μM) — γ-Secretase Inhibition
DAPT inhibits γ-secretase, blocking cleavage of Notch receptors and preventing release of the Notch intracellular domain (NICD). Without NICD, the transcriptional repressors HES1 and HES5 are not induced, de-repressing pro-neural transcription factors ASCL1, NEUROG1/2. Result: remaining progenitors synchronously exit the cell cycle and differentiate. WHY THIS MATTERS FOR ELECTROPHYSIOLOGY: Neurons born within a narrow time window mature together, express ion channels and synaptic proteins on similar timelines, and form functional synapses more efficiently than asynchronously-born populations. Synchronized cohorts establish network connectivity faster.

CONCENTRATION RATIONALE: DAPT Dosing
TEMPLE/PAŞCA PROTOCOLS: 2 μM DAPT for 4 days (Days 46-49). Lower concentration allows gradual progenitor depletion while preserving some neurogenic capacity for continued development. MUOTRI PROTOCOL: 10 μM DAPT for 4 days. Higher concentration for more complete synchronization. CRITICAL: DAPT treatment should be LIMITED to 4 days. Prolonged Notch inhibition prevents astrocyte and oligodendrocyte generation, which are essential for metabolic support, glutamate clearance, and network maturation. After DAPT, remove from medium completely.

Expected Outcomes

Following DAPT treatment, organoids should show:

Reduced proliferation: Ki67+ cells decrease dramatically as progenitors exit cell cycle.

Increased neuronal markers: TUJ1/MAP2 expression expands throughout organoid volume.

Layer marker emergence: Both TBR1+/CTIP2+ (deep layer) and emerging SATB2+/BRN2+ (upper layer) populations.

Early spiking activity: MEA recordings may detect sparse spontaneous spikes by Day 50-60 (Trujillo et al., 2019).

PART 7: ELECTROPHYSIOLOGICAL MATURATION (Day 50+)

BrainPhys Medium Transition

ELECTROPHYSIOLOGY OPTIMIZATION: BrainPhys Medium
BrainPhys (STEMCELL Technologies) was designed specifically for neuronal FUNCTION, not just survival. Key differences from Neurobasal: (1) GLUCOSE: ~2.5 mM (physiological) vs. 17.5 mM in DMEM/F12. High glucose tonically depolarizes neurons and impairs action potential generation. (2) OSMOLARITY: ~305 mOsm, matching CSF. (3) POTASSIUM: Corrected K+ levels (~3.5 mM vs. ~5.3 mM in DMEM). Elevated K+ in standard media tonically depolarizes neurons, inactivating Na+ channels and preventing proper firing. (4) NEUROACTIVE COMPOUNDS: Optimized amino acid balance to support neurotransmission without excitotoxicity. TRANSITION STRATEGY: Days 50-70: 50:50 BrainPhys:Neurobasal. Day 70+: Full BrainPhys. Gradual transition allows neurons to adapt.

B27 Vitamin A Transition

MECHANISM: B27 WITH Vitamin A (Day 70+)
Once neurons are post-mitotic and committed, retinoic acid signaling becomes BENEFICIAL. RA activates RAR/RXR nuclear receptors → upregulates synaptic proteins, ion channel subunits (SCN1A, KCNQ2/3), and neurotransmitter receptor subunits (GRIA1-4, GRIN2A/B). RA also promotes VGLUT1/2 expression (vesicular glutamate transporters), essential for glutamatergic transmission. The timing of B27+VitA introduction is critical: too early caudalizes progenitors; at Day 70+ it supports synaptic maturation.

Timeline of Electrophysiological Development

LITERATURE: Network Development Timeline (Trujillo et al., 2019; Fair et al., 2020)
DAY 34-60: First detectable spontaneous activity. Sparse spiking correlates with neuroepithelial expansion and early neurogenesis. Mean spike rate low (<1 Hz). DAY 60-90: Increased spike rate and field spike amplitude. Characteristic action potential waveforms emerge. sEPSCs detectable via patch clamp. DAY 90-120: Synchronized burst firings (SBFs) appear, indicating functional network formation. Burst frequency and duration increase. Astrocyte expansion correlates with network maturation. DAY 120-180: Complex oscillatory patterns emerge. Phase-amplitude coupling between delta (1-4 Hz) and gamma (100-400 Hz) activity. Network dynamics resemble preterm human EEG. MONTHS 6-10: Continued increases in burst frequency, synchronicity, and population-level coordination. GABAergic neuron emergence correlates with transition from hypersynchronous to more modulated activity patterns.

GABAergic Interneuron Development

ELECTROPHYSIOLOGY OPTIMIZATION: E/I Balance and GABAergic Neurons
Coordinated bursting REQUIRES GABAergic interneurons. Without inhibition, networks show hypersynchronous, seizure-like activity rather than organized oscillations. In dorsal forebrain organoids, GABAergic neurons arise from two sources: (1) CORTICAL PROGENITORS: Recent evidence (Delgado et al., 2022, Nature) shows human cortical progenitors can generate locally-born interneurons, unlike rodents. This may explain why dorsal-only organoids eventually develop GABAergic markers. (2) SPONTANEOUS VENTRAL SPECIFICATION: Some progenitors may spontaneously adopt ventral identity and generate interneurons. GABAergic markers (GAD65, DLX1/5, GABA) typically appear by Month 4-6 in cortical organoids. Their emergence correlates with

the onset of mature, modulated network bursting. Pharmacological studies (Trujillo et al., 2019) show that bicuculline (GABAAR antagonist) increases network event frequency while abolishing oscillatory structure, confirming GABAergic regulation of network dynamics.

PART 8: INTEGRATED PROTOCOL TIMELINE

Days	Phase	Key Factors	Objective
0	Aggregation	mTeSR + Y-27632/CEPT	Single-cell survival, EB formation
1-5	Neural Induction	E6 + SB + LDN + XAV	Triple-i dorsal forebrain specification
6-15	Progenitor Expansion	NB/B27-VA + EGF + FGF2	aRG/oRG expansion, rosette formation
7-20	oRG Enrichment	+ LIF (20 ng/mL)	STAT3 activation, oSVZ expansion
15-20	**BANKING WINDOW**	**MEDY cryopreservation**	**Progenitor intermediate for storage**
20-45	Differentiation	NB/B27-VA + BDNF + NT3 + AA	Neurogenesis, layer formation
46-50	Synchronization	+ DAPT (2-10 μM)	Synchronized terminal differentiation
50-70	Early Maturation	50:50 BP:NB + B27-VA + AA	BrainPhys transition, spiking onset
70+	Late Maturation	BrainPhys + B27+VA + AA	Network bursting, GABAergic emergence
120+	Functional Networks	BrainPhys + B27+VA + AA	SBFs, oscillatory activity, E/I balance

Abbreviations: NB = Neurobasal-A; B27-VA = B27 without Vitamin A; B27+VA = B27 with Vitamin A; BP = BrainPhys; AA = Ascorbic Acid; SBF = Synchronized Burst Firing

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APPENDIX: TROUBLESHOOTING FOR ELECTROPHYSIOLOGY

Problem: Low or Absent MEA Activity at Day 60-90

Possible Causes:

• Insufficient neuronal maturation. Verify TUJ1/MAP2 expression by IHC.

• Poor organoid-electrode contact. Ensure organoids are properly seated on MEA electrodes with Matrigel coating.

• Progenitor exhaustion from premature differentiation. Check that EGF/FGF2 were maintained through Day 20.

• Non-cortical contamination. Verify FOXG1/PAX6 expression; absence suggests midbrain/hindbrain drift.

Solutions:

• Extend maturation time. Some lines require 90-120 days for detectable activity.

• Increase BDNF/NT3 concentration to 40 ng/mL during maturation.

• Add GDNF (10 ng/mL) to support diverse neuronal populations.

• Verify Triple-i induction was complete; consider increasing XAV939 concentration.

Problem: Hypersynchronous Activity Without Oscillatory Structure

Possible Causes:

• Insufficient GABAergic neuron development. E/I imbalance leads to seizure-like activity.

• Premature recording. Complex oscillations emerge at 4-6 months, not earlier.

Solutions:

• Extend culture time to allow GABAergic neuron emergence (Month 4-6).

• Consider dorsal-ventral assembloid approach: fuse dorsal organoid with ventral organoid to introduce MGE-derived interneurons.

• Verify GAD65/67 and GABA expression by IHC at recording timepoint.

Problem: Poor Recovery After Cryopreservation

Possible Causes:

• Inadequate Y-27632 pre-treatment.

• Osmotic shock during thawing.

• Cryopreservation outside optimal Day 15-20 window.

Solutions:

• Ensure 1.5-2 hour Y-27632 pre-incubation before MEDY exposure.

• Stepwise dilution of cryoprotectant over 10-15 minutes post-thaw.

• Include EGF+FGF2+LIF in recovery medium for 72 hours before differentiation.

• Verify banking window by checking SOX2+/PAX6+ progenitor percentages before freezing.