Optimization of rodent brain slice cultures for intermittent live-imaging of identical cells over many weeks. Applications to Alzheimer's disease and stroke

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Why don’t the *$#%*)*%^%#%^ cultures work today
People Who Actually Did the Work Being Discussed Today

Laurie Minamide
O’Neil Wiggan
Alisa Shaw
Isaac Babcock
Ben Fixman
Outline:

1. Normal Cellular Functions of Cofilin, the major neuronal ADF/cofilin
2. Classical AD pathology - is cofilin pathology important?
3. Brain slice cultures- pros and cons of membrane vs roller tube
4. Current status and issues with regard to brain slice culture
   Medium issues for survival and reduction of spontaneous rods
4. Development of spine labeling method for live slices
5. Current status and applications to neurodegenerative diseases
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Cofilin

Severing/dynamizing F-actin

Competing with myosin II

Nuclear architecture transcription

G-actin nuclear transport

Phospho-cofilin

Phosphatidic acid (PA)

Mitochondrial Fission

Sequestered in cofilin-actin rods

Life or death

Mitochondrial-dependent apoptosis

Sequestration in life or death

Energy/cell death

Lipid metabolism

Signal transduction

Cell motility/morphology/synaptic plasticity

Many Functions of ADF/Cofilin
Cofilin regulates actin turnover directing growth cone pathfinding, polarized cell migration and dendritic spine remodeling.

Turning in response to active cofilin gradient

Cofilin (S3A)-GFP in Migrating Fibroblast

LifeAct before and 10 min after CALI of Cofilin KillerRed

Kymograph of F-actin intensity

Marsick et al., 2010.

Cramer et al., 2005

Vitriol et al., 2013
Silencing ADF and coflin causes massive blebbing that is myo II dependent. During aborted cytokinesis massive nuclear movement occurs between cells.
Cofilin signaling in dendritic spines regulates actin cytoskeletal changes associated with LTP and LTD.

Peptide regulators of LIMK and SSH can be used to modulate spine changes that occur in disorders.
Role of cofilin in spine dynamics and function

Spine basal state (B). Stimulation of Long term depression (LTD) (A) leads to cofilin activation, actin disassembly and decrease in spine size and dynamics. Stimulation of Long term potentiation (LTP) (C) leads to transient cofilin activation for channel insertion (glutamate receptors) and nucleation of actin assembly for spine enlargement. Cofilin inactivation leads to consolidation (D) in which pCofilin accumulates in the spine neck.

Based on: J. Gu et al., Nat Neurosci. 2010 (LTP)
B. Calabrese et al., PLoS One 2014 (LTD)
Modified from Rust MB. Cell Mol Life Sci. 2015
Outline:

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   1. Human AD brain
   2. Mammalian neurons in culture- rod signaling pathways
   3. AD Mouse model
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Classical Pathology of Alzheimer Disease

Alzheimer Disease Pathology
(stain for amyloid)

Aβ Pathology
Plaque

Neuropil
Threads

Tau Pathology

Neurofibrillary Tangle
(but threads are more abundant)

Minamide unpublished

Boyle TEM image
Alzheimer Disease Pathology
(stain for amyloid)

Aβ Pathology

Plaque

Neuropil Threads

Neurofibrillary Tangle
(but threads are more abundant)

Tau Pathology

Cofilin Pathology
(immunostain)

Age match control

Human AD

Microglia

Human AD

10 µm

10 µm

Minamide unpublished


Boyle TEM image
ADF/Cofilin-actin (1:1) rods are another AD pathology.

ADF/Cofilin-stained rods in early AD human brain.

Minamide et al, in preparation
Entorhinal Cortex from Human AD Subject Stained for pTau and Cofilin
Comparison between cofilin-actin rod pathology and neuropil thread (phospho-tau) pathology in human entorhinal cortex (ERC). Variability in area of 10 fields, Quartiles 2-3 (boxed) and ave. (bar).

Subjects are from a longitudinal study at Sanders and Brown Center on Aging at Univ. of Kentucky. Longest postmortem interval for fixation is 4 h (ave is 2.6 h). Subject age distribution in each cohort is similar (ave. of 86, 90, 90 and 88 for different cohorts).

6,200 pixel=1% of field area

Minamide, Scheff et al., MS in preparation, 2015
Cofilin-actin rods in 6 DIV cultured E18 rat hippocampal neurons. Amount of cofilin (0.2 mol/mol actin) is rod limiting.

Rods are induced by agents that cause energy stress and oxidative stress. These include mitochondrial inhibitors, excitotoxic glutamate, hypoxia/ischemia, proinflammatory cytokines, HIV gp120 envelope protein and amyloid-β peptides.

Endogenous Protein  2x Cofilin Expression

(Left Image is overexposed to show neurites)

Minamide et al., Nat Cell Biol 2000
Rods can grow to completely occlude the neurite and block transport.

Minamide et al., Nat Cell Biol 2000
Rods have been isolated and contain 1:1 ADF/cofilin (active):actin

Some potential pathways for cofilin-actin rod formation.

Rods isolated from mechanically disrupted neurons by optiprep gradients.
(Minamide et al., JBC 2010)
**Hypothesis:** proinflammatory cytokines, Aβd/t and gp120 work via coalescence of membrane microdomains to generate bursts of ROS to oxidize cofilin and generate rods.

Aβ-induced memory and learning defects in AD mice depend upon PrPc.

Sphingolipid and PrPc Rich Rod-Signaling Complex
Overexpression of PrP<sup>c</sup> is Sufficient to Induce Rods in Hippocampal Neurons. PrP<sup>c</sup>-induced rod formation is dependent on NADPH Oxidase activity.

Untagged PrP<sup>c</sup> also induces rods

*p<0.01; ** p<0.05

Walsh et al, 2014 and data from 2015
RODS ARE FOUND IN THE AD MOUSE LINE ($\text{APP}_{sw}/\text{PS1}\Delta E9$) IN BOTH THE HIPPOCAMPUS AND CORTEX.

Woo et al, Cell Death Dis, 2015- collaboration with Kang lab.
AD mice that are hemizygous for a cofilin activator (A) or cofilin itself (B) do not show cognitive deficits in contextual fear response and have a normal LTP.

Reducing activation of cofilin or total cofilin levels by 50% restores normal LTP response and cognition in AD mouse.
Brain Slice Culture: a system between whole animal and dissociated neurons.

Whole animals
  • Expensive
  • Unknown composition of CSF
  • Testing therapeutics requires knowing BBB passage and pharmacokinetics

Brain Slices
  • Keep glia/neuron relationships intact
  • Can do live imaging
  • Can culture long term to observe pathological changes
  • Can control and sample the medium
  • Might allow ePhys on long-term slices
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Brain slice culture methods

A) Rat whole brain → Cut on a microslicer → Brain slice (300 µm thick)

B) Roller tube culture on coverslip

Air/liquid interface on 0.4 µm membrane
Roller tube culture on coverslip

Slices above 200 μm thick may have cell death from oxygen depletion.

Enclosed system, good for viral infection but not for live imaging.

Plasma clot for holding slice before it adheres needs removal for imaging

Can use fiducial markers for slice imaging but need thinner slices

Slices may thin due to cell migration Leading to loss of morphology

Air/liquid interface

Tolerates slices of 300 μm because aeration is from both sides

Open system usually using immersion objectives not virus friendly

Observations not limited by embedding in holding matrix

Can observe same regions but not identical cells unless prelabeled

No slice thinning but imaging is more challenging
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Development of a simple system for live intermittent imaging of slices in an enclosed system (Fixman et al., 2017).

- Flat-sided Culture Tube
- Secure-seal ring
- Coverslip

Use 0.6 to 0.8 ml medium per tube.

- 6x18=108 tubes in incubator
- 6 tubes per holder
Custom Tube Holder and Stage Incubator for Slice Imaging

Designed by Jeff Field, MIN Director: https://www.research.colostate.edu/min/custom-machining/
Identical Cells Are Easily Found and Imaged Over Several Days

Fixman et al, 2017
Troubleshooting The Many Tricky Steps (work in progress)

1. Adhering slice to coverslip without inducing cell migration and thinning.
   Modify glass with 3-aminopropyl-triethoxysilane. Use nitrocellulose or Vitrogel.

2. Adhering coverslip to tube with slice attached- non toxic adhesives.
   Secure Seal (double sided). Punched our own but now order prepunched.

3. Preventing leakage- adequate pressure vs breaking coverslips
   Testing secondary sealant that can be applied after mounting.
4. Optimizing medium volume (min needed to cover slice during bottom of rotation)
   Slices can drown if not drained during rotation

5. Finding medium component responsible for slice death. Summer of 2017 we had 37 out of 40 slices survive for weeks. In late 2017 all slices were coming off coverslips (dead). Plasma, thrombin, and every reagent was replaced. After making our own homemade Neurobasal medium (39 components) we significantly reduced the problem, probably by using a high purity L-serine.

6. Optimizing home-made neurobasal (all 39 components). Laurie Minamide discovered that the high L-cysteine content of commercial neurobasal is the major inducer of “spontaneous” rods in dissociated cultures. Reduced this from 260 µM to 150-175 µM and got low spontaneous rods.
Troubleshooting The Many Tricky Steps (work in progress)

7. Optimizing slice thickness for maintaining viability

Original slices in plasma clots did OK when cut at a nominal 300 μm. When using a flatter adhesion to glass, slices 200 μm or less may be best.

Slices can withstand >30 min of imaging without rotation. But 2.5 hr (power outage on July 7) led to death of >60% of slices in 2 days.

<table>
<thead>
<tr>
<th>Cultures Started</th>
<th>6/17/19</th>
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7/3/19 7/9/19

percent slices with good morphology

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

preoutage postoutage

Good surviving slice
Hippocampus, NeuO (2x) NeuO (12 h) 60x

Alive

Going and gone.

Hippocampus (P10), 3 weeks in culture

Babcock unpublished
8. Visualizing dendritic spines. Internally expressed proteins, such as GFP or mRFP, or surface receptors (SEP-GluR1) did label spines but good images were only obtained on dissociated cultures in monolayer.

Troubleshooting The Many Tricky Steps (work in progress)
Troubleshooting The Many Tricky Steps (work in progress)

8. Visualizing dendritic spines.

Liquid DiI worked well to label spines in dissociated cultures but was internalized and signal was reduced.

Placement of large Dil crystal in slice using micromanipulator provided continuous labeling of neurons.

Isaac Babcock, unpublished
Attaching slice with VitroGel instead of plasma clot allows better visualization of Dil label.

Images obtained on spinning disc confocal two days after labeling. Same laser power and exposure time for capture. Z-stack, 5 µm thick and with step size of 0.17 µm. Identical post capture exposure and brightness settings.
Visualization of dendritic spines in Dil-labeled live hippocampal slice. Confocal imaging of 5 μm thick region using 100x objective and 0.17 μm steps on spinning disc confocal microscope.
Troubleshooting The Many Tricky Steps (work in progress)

9. Moving from postnatal to adult brain slices

New medium and mounting methods have been successful for cortex and hippocampus from adult mice (beyond breeding ages of 6 month)

L. Minamide, unpublished
Adenoviral-mediated mRFP expression in sub-population of pyramidal layer neurons

Mouse (6 mo) cortical slice 3 weeks in vitro, fixed and stained for NF-H to look at axons.

Nuclei are stained with DAPI
10. Imaging entire 3D volume of slices and quantifying rod pathology
   Keyence microscope allows for rapid imaging of complete slice in 3D. Isaac Babcock applied semi-automatic, unbiased Image J program for rod quantification.
Conclusions

Brain slice imaging on coverslips with fiduciary marks allows for repetitive imaging of same cells over long time period.

Cultures of adult slices show potential for long-term maintenance and study of age-related pathological changes. System is good for screening of drugs to reverse pathology in mouse models of human neurodegenerative diseases.
Other Applications of Slice Imaging System

Special adaptation for studying response of live brain slices to hypoxia.

CofilinR21Q-mRFP + IT Green

System for inducing hypoxia in slices while imaging (A,B)

Fixman, BS Thesis, 2019
Comparison of cofilin pathology in hypoxic mouse brain versus stroke.

Live Slice Data
Green = NeuO vital dye; Red= cofilinR21QmRFP rod reporter

A. Normoxic

B. 1 hr after N₂/CO₂ flush

C. Brain cortex from control and mice with photothrombic lesion
   Perfusion-fixed
   30 μm frozen
   Sections of cortex
   Blue= DAPI
   Red+ cofilin IHC


Fixman, B. Undergrad. Thesis, 2019