## Research Design and Methods

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| **Table XX:** Timeline for completion of proposed research. | **Year 2** | **Year 2** | **Year 3** |
| **Specific Aim 1.** Design and implement optogenetic population clamp |  |  |  |
|  | 1.  | Virally transduce optogenetic activators and silencers in glutamatergic and GABAergic subpopulations within DCNs. |  |  |  |
|  | 2. | Control design: optimize population clamp performance. |  |  |  |
| **Specific Aim 2.** Manipulate the homeostatic state of DNCs |  |  |  |
|  | 1. | Trigger homeostatic increases synaptic strength and cellular excitability using optogenetic population clamp. |  |  |  |
|  | 2. | Trigger homeostatic decreases synaptic strength and cellular excitability using optogenetic population clamp. |  |  |  |
|  | 3. | Induce bidirectional homeostatic changes within DCNs by clamping population activity over a range of firing rates. |  |  |  |
|  | 4. | Induce long-term plasticity in the homeostatic state of DCNs that eliminates the propensity for pathological bursting. |  |  |  |

**Rationale:** Our preliminary data has demonstrated that using a feedback controller and optogenetic excitation, the population firing rate of a heterogeneous cortical network can be clamped to specific set-points. This technique opens the door to new types of studies because the mean activity state of the network, typically regulated by homeostatic mechanisms, can be externally forced to different values. Voltage and current clamping of individual neurons analogously has provided many insights into the physiology of excitable membranes. Further, dynamic clamping (artificial current injection) has broadened our understanding of the role of a neuron in circuit function. A population clamp would provide a handle for shaping the homeostatic state of the network, potentially righting instabilities induced by a lack of external drive due to deafferentation.

*Our goal is to use the optogenetic population clamp to induce a known effect on the balance of synaptic input to pyramidal cells within DCNs in order to stabilize network activity states*. [Table XX] summarizes the project. **In Specific Aim 1**, we will prepare 4 optogenetic constructs to in order to control the activity level of DCNs in 4 physiologically different manners. We will tailor a feedback control architecture around each construct to ensure fast dynamic response and stability in tracking firing rate set-points for long time periods [Astrom]. **In Specific Aim 2**, we will use the population clamp to manipulate the homeostatic state of DCNs. We will perform experiments in which the activation of excitatory and inhibitory neuronal sub-populations are used to clamp network firing rate. The effect of each population clamp will be compared to changes in synaptic strength to determine the most effective means of modulating homeostatic plasticity within the DCN. Finally, using the neuronal subpopulation capable of inducing the most robust homeostatic effect, we will stabilize bursting activity in DCNs by adjusting their homeostatic state. The experimental flow is outlined in [Fig XX].

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**Fig XX.** General experimental flow. Sister DCNs are plated on MEAs then transfected with various optogenetic constructs and given time to grow and express. The networks are subsequently clamped to a specific population firing rate or left uncontrolled. The effect on synaptic excitability, network stability and firing rate statistics is then quantified.

**Aim 1: Implement and tune 4 forms of the optogenetic population clamp**

**1-A. MANIPULATING NETWORK ACTIVITY USING DIFFERENT NEURONAL SUBPOPULATIONS**

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|  |  |  | **Construct** | **Excitation Wavelength** | **Transfected****Cell Activity** | **Network****Activity** | **Bidirectional****Combos** |
| **Cell Type** | **Pyramidal Cells (Glutamatergic)** | **A** | CaMKIIα-ChR2-mCherry | **480 nm** | $$⬆$$ | $$⬆$$ | 1234 |
| **B** | CaMKIIα-ArchT-eGFP | **532 nm** | ⬇ | ⬇ |  |
| **Parvalbumin+****Interneurons (GABAergic)** | **C** | fPV-C1V1-eGFP | **532 nm** | $$⬆$$ | ⬇ |  |
| **D** | fPV-Mac-eGFP | **620 nm** | ⬇ | $$⬆$$ |  |

Rationale:A high performance population clamp should allow for control of network dynamics over a wide range of activity setpoints, both above and below spontaneous network firing rates. However, the physiological means by which network activity is modulated will have consequences on the degree of homeostatic change induced [22]. Certain neuronal subpopulations may be more effective handles for controlling network-wide firing rate over long time periods. We will investigate four optogenetic constructs as handles for network activity that employ distinct neural circuitry.

**Table XX:** Four optogenetic strategies for bi-directional population control using genetically specified neuronal subpopulations. The separated action spectrum of construct pairs allows co-excitation without interference.

Methods:

*Viral constructs.* In collaboration with Robert Gross at Emory University (see attached letter of support) we have employed AAV2-CaMKIIα-ChR2-mCherry to drive ChR2 expression in neocortical PY cells, and verified its expression through imaging, MEA recordings, and whole-cell patch clamp recordings (Preliminary Data). Using constructs obtained from Karl Diesseroth, Ed Boyden, and Edward Callaway, we will create custom plasmids for the remaining three constructs shown in Table XX. In order to generate pAAV2-CamKIIα-ArchT-eGFP, cDNAs encoding the CaMKIIα promoter will be subcloned by PCR into the SmaI and Xbal restriction sites of the vector pAAV2-ArchT-eGFP. For pAAV2-fPV-C1V1-eYFP, cDNAs encoding the fPV promoter will be subcloned by PCR into pAAV-C1V1-eYFP at PacI and AgeI. For pAAV2-fPV-Mac-eGFP, Mac cDNAs will be subcloned by PCR into pAAV-fPV-eGFP at AgeI and NcoI (insert restriction sites). All of these plasmids will be validated in HEK 293 cells. Viral constructs will then be generated and amplified at the UNC Viral Vector Core (<http://genetherapy.unc.edu/services.htm>) to a titer of 1012 c.f.u mL-1.

*Cell culture and transfection*. Primary neuronal cultures are derived from neocortex of embryonic day 18 rats and plated on glass-bottom petri dishes (MatTek, P50G-1.5) or 60 channel MEAs (Multi Channel Systems, Reutlingen, Germany) as described in our publications [xxx]. Briefly, cortical tissue will be enzymatically dissociated for 30 minutes in 2.5 U/mL papain solution. Dissociated cells will be plated onto culturing surfaces precoated with polyethyleneimine and laminin [potter and demarse]. Viral transfections will be performed at 1 DIV by adding 2 uL of viral solution to 1 mL of cell culture medium for each DCN. Virus will be allowed to incubate with the culture for 3 days before removal. No antibotics or glial growth inhibitors will be added to culturing media since these practices may have confounding effects on neurotransmission [x], cell health [Potter Demarse], and homeostatic mechanisms [for glia: Stellwagen & Malenka, 2006, nature]. All DCNs will be stored in light-tight dishes. DCNs will be imaged on a Zeiss LSM 510-META confocal microscope every three days to monitor the expression of fluorescent reporter proteins.

*Intracellular photocurrents.* At 14-17 DIV whole-cell voltage-clamp recordings will be used to measure light-induced photocurrents. Recordings will be conducted on an upright Nikon Eclipse E600FN microscope using an Axopatch voltage-clamp amplifier, Digidata A/D converter and pClamp recording software (Molecular Devices, Sunnyvale, CA). Photocurrents will be induced by driving the appropriate LED on our 4-channel optical stimulator (preliminary results) coupled to the trans-illumination port of the microscope using digital signals produced by pClamp. Light intensities will be adjusted to appropriate levels before experimentation by manually adjusting power on our LED driver and measuring optical power using a Newport 1830c meter (Newport Corp., Irvine, CA). All recordings will be performed in the presence of 0.5 uM tetrodotoxin (TTX) to prevent post-synaptic currents from interfering with the measurement of light-induced currents. Photocurrents will be verified against published levels for each optogenetic protein type (insert citations for [Chr2 - 2a][C1V1 - 13][ArchT/Mac - 7]).

*MEA Recordings and Optical Stimulation.* After verification of construct efficacy, DCNs on 4 MEAs will be transfected as described previously, each with one of the four constructs listed in Table XX. Starting at 4 days post transfection (DPT), MEA recordings will be performed daily using a commercial 60-channel amplifier (Multi Channel Systems, Reutlingen, Germany) and our NR acquisition platform. Interleaved 30 second epochs of pulsatile stimulation trains will be applied; each train is generated from a random combination of three parameters: stimulation frequency, pulse-width, and light power.

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|  |  | **Construct** |
|  |  | ChR2, V1C1 | ArchT, Mac |
| **Stimulation****Parameter** | $F=Stim Frequency (s^{-1})$  | {1,5, 10, 20, 40} | {0.5, 1, 5, 10} |
| $$P=Pulse Width (msec)$$ | {0.1, 0.5, 1.0, 5} | {10, 100, 500,1000} |
| $$I=Irradiance (mW\* mm^{-2})$$ | {0.5, 1, 1.5, 2, 4} @ 480 nm | {1, 2, 5, 10} @ 590 nm |

**Table XX:** Stimulation parameters to explore for each construct type. Values are chosen to cover a wide range of parameters relevant to the on/off kinetics and light-power requirements of each construct type [2a] [13] [7].

*Analysis.* Spike detection and sorting will be conducted online with NR. Sorting will be performed first using principal component analysis for dimensional reduction of spike waveforms and then unsupervised Gaussian mixture modeling for classification (real-time C# port of Charles Bowman's 'Cluster', written in C [Bowman]). Data generated will consist of three binary file types: spike files containing spike objects (time, channel, 3-ms voltage waveform, unit #); digital files containing digital objects denoting the 32 bit port state and encoding stimulation timing, frequency, pulse-width and amplitude; and 'analog' files containing raw traces of LED current measurements. The normalized steady state firing rate of unit $i$, $\overline{f}\_{i}=(|n\_{j})/max⁡(f\_{i}|n\_{j})$, will be calculated for each construct combination at each DIV. Here, $f\_{i}|n\_{j}$ is the steady-state firing rate for a particular combination of control values $n\_{1} =F| \left\{P,I\right\}, n\_{2} =FP|I$, or $n\_{3} =IPF$. $n\_{1}$ is stimulation frequency at a given pulse-width and irradiance,$ n\_{2}$ is duty-cycle at a given irradiance and $ n\_{3} $ is average light power over time. The ensemble average of the set of normalized per unit firing rates, $\left〈\overline{f}\_{i}|n\_{j}\right〉$, will be compared with each prospective control parameter, $n\_{j}$. This method examines the effect of a control signal on an 'average unit' within each DNC, and therefore accounts for variability in the number of neurons that happen to be picked up by a given MEA. The monotonicity, signal to noise ratio, and dynamic range of the relationship between $n\_{j}$ and $\left〈\overline{f}\_{i}|n\_{j}\right〉 $will be considered for a given construct and the highest performing control signal $n\_{j}^{\*}$ will be designated as the control signal for a that construct.

**1-B. OPTIMIZE PERFORMANCE OF POPULATION CLAMP**

Rationale: Control performance for tracking static setpoints is evaluated using step response time and stability metrics (no oscillations around the target value). Since the changes in cellular physiology associated with homeostatic plasticity take place over relatively long time scales (hours to days) our population clamp must be capable of holding firing rates at a desired set-point over these time periods.

Methods: Cell culturing, transfections, and MEA recordings will be performed as described in Aim 1-A.

*Controller optimization.* Using the highest performing control signal $n\_{j}^{\*}$ from Aim 1-A, we will control the average unit firing rate, $f\_{i}$, about the target rate $f^{\*}$, for each construct using our PI controller that successfully controlled firing rate using amplitude-modulated ChR2 excitation (preliminary data). We will aim for a step-response time-constant of 10 seconds and a steady-state tracking error of 0.5 RMS Hz in $f\_{i}$ about $f^{\*}$. We will use our previously written population clamp DLL (preliminary data) to calculate $\left〈f\_{i}[t]\right〉$ online, every 100 ms. A feedback control signal will be sent to the LED of appropriate wavelength according to eq. 2, with the per-unit error term $e\left(t\right)= \left〈f\_{i}[t]\right〉 -f^{\*}$. In [figure xx] the spontaneous firing rate statistics of spontaneous activity from 2331 detected units across 17 DCNs at 14 DIV is presented (data from [p3]). The ensemble average firing rate per unit, $\left〈f\_{i}\right〉\_{spont} $, is calculated across half-hour recordings of spontaneous activity for each DCN. Because there is large variability in the value of $\left〈f\_{i}\right〉\_{spont} $ for different cultures, the value of $f^{\*}$ will be dependent on an estimate of the spontaneous average unit firing rate for a given culture,$ f^{\*}=R\*\left〈f\_{i}\right〉\_{spont}$. For each rate, we will tune the PID algorithm using the Zeiger-Nichols method (preliminary data, fig. XX).

**Fig X:** The meaning of high and low population firing rate is dependent on the DCN being controlled. (A) Histogram of spontaneous firing rates for 2331 units across 17 DCNs (B) The average firing rate of units varies across DCNs.

*Validation for long-term experiments.* To test the efficacy of each construct and corresponding feedback control parameter set, we will first perform 30 minute sessions at set-points increasingly above or below $\left〈f\_{i}\right〉\_{spont}$ until we the effectiveness of the controller diminishes. Next, we will hold DCNs for 24 hour periods at setpoints covering the viable range found in the 30 minute experiments.

*Monitoring apoptosis.* We will perform a live dead stain (DiOC18(3) and propidium iodide for eGFP and eYFP marked constructs or SYTOX® Green dye and C12-resazurin for mCherry marked constructs; Life Technologies, Carlsbad, CA ) to quantify the effect of extended periods of elevated activity on culture health. Numerous studies have shown that decreased levels of activity do not adversely affect DCN health [18].

**EXPECTED OUTCOMES FOR SPECIFIC AIM 1** We expect that each construct will demonstrate strengths and weaknesses in controlling population activity, and this variability will be accounted for in control design. Additionally, we expect that there will be a biological relevant range of population activity levels that we can hold DCNs at for extended (>24 hour) periods without excitotoxicity or control failure.

**ANTICIPATED DIFFICULTIES AND ALTERNATIVE APPROACHES FOR SPECIFIC AIM 1**

*Insufficient activation of inhibitory constructs.* Our preliminary data demonstrate that we can achieve sufficient light power for ChR2-type constructs. However, we have not yet tested the silencing constructs. While ArchT and Mac are more sensitive than previous optogenetic silencers (need citation), it is still possible that we will need a brighter light source than our LED system for effective activation of these constructs. To address this issue, we have included funds for a 200 mW, 532 nm, fiber-coupled laser (Shanghai Laser, Shanghai, China).

*Undesired induction of bursting.* In our experience, suppression of network inhibition via the addition of GABAzine (analogous to expressing Mac in PV interneurons) leads to an extreme propensity for population bursting. This could override our attempts to hold static increases in population firing rate. To address this potential problem, we can co-transfect cultures with AAV2-CaMKIIα::ArchT cultures so that bursts can be suppressed, via concurrent suppressive control via CaMKIIa::ArchT [013b].

*Inappropriate control algorithm.* In Aim 1-B, we will attempt to achieve target step response time-constant and steady-state tracking error using our existing PI design (preliminary data, equations) since there is a large body of literature dedicated to PI/PID tuning [Astrom]. However, if this fails to meet design requirements we turn to more sophisticated methods for handling non-linear plant dynamics, namely, model-predictive control and/or fuzzy control.

excitotoxicity: For the constructs that are used to hold $\left〈f\_{i}\right〉$ at elelvated levels, directly following these control

### Aim 2: Optogenetic population clamp to manipulate the homeostatic state of DCNs.

#### A. TRIGGERING HOMEOSTATIC INCREASES IN SYNAPTIC STRENGTH

Rationale: An appropriate balance of excitation and inhibition is necessary for the proper functioning of neural circuits. Reduced levels of network spiking, e.g., due to pharmacological deafferentation, lead to compensatory increases in excitatory synaptic strength and cellular excitability in neocortical pyramidal neurons (Turrigiano et al., 1998; Desai et al., 1999).  In addition, homeostatic plasticity mechanisms decrease inhibitory input to pyramidal cells via compensatory alterations in mIPSC frequency, probability of release, and number of GABAergic neurons (Bartley, 2008; Hartman, 2006; Kilman, 2002; Rutherford,1997). In these studies, activity was suppressed using pharmacological blockade of spiking activity using TTX.  This approach does not tell us whether the homeostatic plasticity mechanisms that lead to an increase in activity are triggered by too little excitation or too much inhibition. Answering this question is crucial in the development of therapeutics aimed at inducing long-term changes in pathologically-behaving networks. Changes that might disrupt the balance of excitation and inhibition could be reduced glutamatergic transmission or increased GABAergic transmission. The former has been tested via pharmacological blockade of non-NMDA type glutamate receptors and shown to effectively decrease synaptic strength (Turrigiano et al., 1998).  Increasing GABA drive in order to reduce network activity has not been explored. We propose to test the cases of reduced glutamatergic transmission via optogenetic suppression of activity in CaMKIIα -expressing pyramidal cells, and elevated GABAergic transmission via optogenetic elevation of FPV+ inhibitory interneurons.

Methods: Sister DCNs will be plated on MEAs as previously described. All DCNs will be transfected with either AAV2-fPV-C1V1-eGFP or AAV2-CaMKIIα-ArchT-eGFP. At 14-17 DIV, network firing rates will be clamped to $f^{\*}=0.01 \*\left〈f\_{i}\right〉\_{spont}$ for 24 hours in half of the DCNs and left unregulated in remaining cultures. MEA data will be collected concurrently from clamped and control cultures stored in a single incubator using two NR rigs. Up to three culture pairs will be controlled sequentially in the 14-17 DIV time window. Following 48 hours of population clamp, whole-cell voltage-clamp recordings will be obtained from pyramidal cells from all DCNs. Recordings will be conducted oxygenated aCSF with 0.5 uM TTX.  mPSC amplitude and frequency distributions will be calculated post-hoc using MiniAnalysis (Synaptosoft, Decatur, GA) and MATLAB (The Mathworks; Natick, MA). Distributions from clamped and non-clamped DCNs will be compared. Comparisons of synaptic strength will always be made between sister DCN pairs.

Potential Outcomes: Because the two clamp types (B and C in Table XX) reduces population activity levels using a different neuronal subpopulation, the corresponding increases in synaptic strength can be compared for each clamp type. There are three possible results. **(1)** The best handle for upward SS is firing rate.This is the case if we see no difference in SS between reducing activity using either form of population clamp**. (2)** The best handle for upward SS is reduction of glutamatergic neurotransmission. This is the case if increases in synaptic strength are most dramatic in the CaMKIIα::ArchT controlled cultures. **(3)** The best handle for upward SS is elevation of GABAergic neurotransmission. This is the case if increases in SS are more dramatic in the FPV::C1V1 controlled cultures.

#### B. TRIGGERING HOMEOSTATIC DECREASES IN SYNAPTIC STRENGTH

Rationale: Elevated levels of network spiking lead to compensatory decreases in excitatory synaptic strength in neocortical pyramidal neurons (Turrigiano, 1998) and increassed inhibitory input to pyramidal cells via compensatory increases in mEPSC amplitude in inhibitory interneurons (Chang, 2010; Doyle, 2010).  In these studies, a chronic activity elevation was achieved via elevation of glutamatergic transmission (using kainate injection) or blockade of inhibitory transmission (using bicuculline or gabazine). Two recent studies used genetic targeting of cation channels to elevate activity in individual cells and saw cell-autonomous changes (i.e. changes not requiring changes in activity at the network level) in synaptic strength.  In the first study, activation of overexpressed TRFPV1 channels via capsaicin resulted in Increased in mIPSC amplitude in pyramidal cells (Peng et al., 2010).   In the second, activation of ChR2 channels using pulsatile blue light led to reductions in miniature and evoked EPSC amplitude (Goull & Nicoll, 2010).  However, these manipulations have not been performed at the network level, nor have activity states been clamped to set levels. In this aim we take inspiration from studies that alter glutamatergic or GABAergic transmission at the network level, but use the cell-type specific targeting approach to better tease apart circuits that are involved in homeostatic plasticity mechanisms. To increase network activity we test two approaches: elevation of glutamatergic transmission via optogenetic excitation of pyramidal cells, or suppression of GABAergic transmission via optogenetic inhibition of FPV+ inhibitory interneurons.

Methods: Sister DCNs will be plated on MEAs as previously described. Both DCNs will be transfected with either AAV2-FPV-C1V1-eGFP or AAV2-CaMKIIα-ArchT-eGFP. network firing rates will be clamped to $f^{\*}=10 \*\left〈f\_{i}\right〉\_{spont}$ for 24 hours in half of the DCNs and left unregulated in remaining cultures for 24 hours. Intracellular recordings will be conducted as in aim 2A.

Potential Outcomes: Because the two clamp types (A and D in Table XX) increases population activity levels using a different neuronal subpopulation, the corresponding increases in synaptic strength can be compared for each clamp type. There are three possible results. **(1)** The best handle for downward SS is firing rate.This is the case if we see no difference in SS between reducing activity using either form of population clamp. **(2)** The best handle for downward SS is the elevation of glutamatergic neurotransmission. This is the case if increases in synaptic strength are most dramatic in the CaMKIIα::ChrR2 controlled cultures. **(3)** The best handle for downward SS is the reduction of GABAergic neurotransmission. This is the case if increases in SS are more dramatic in the FPV::C1V1 controlled cultures.

**C. BIDIRECTION CONTROL OF SYNAPTIC STRENGTH BY CLAMPING POPULATION ACTIVITY OVER A RANGE OF SETPOINT FIRING RATES.**

Rationale: Many diseases and disorders of the cortex may be due to maladaptive plasticity mechanisms (homeostasis gone wrong).  In order curb these problems, a system capable of controlling the homeostatic state of the heterogeneous cortical networks is required.  This includes being able to drive synaptic strength up or down across a spectrum of values. Aim 2A and 2B will reveal what strategies are best for increases or decreases in synaptic strength, so the purpose of aim 2C is to combine these strategies for smooth bidirectional control of synaptic strength over a wide range.

Methods: We will co-transfect DCNs on MEAs with the two viruses that show the greatest effect on synaptic strength from Aims 2A and 2B. At 14-17 DIV, network firing rates will be clamped one of the set of rates $f^{\*}=R\*\left〈f\_{i}\right〉\_{spont}, R= \{0.01, 0.1, 0.5,2,5,10\} $ or left unregulated. Directly following the clamping period, intracellular recordings will be conducted as in aim 2A.

Potential Outcomes: We envision two potential outcomes for this aim: **(1)** There is a smooth monotonic and inverse relationship between clamped firing rate and changes in excitatory synaptic strength. If this is the case, using the bidirectional population clamp, we should be capable of driving the homeostatic state of a DCN to a specific set point over this range. **(2)** The relationship between clamped firing rate and changes in excitatory synaptic strength is monotonic and inverse, but very steep. It this is the case, we will see scaling occur for dramatic increases or decreases in firing rate, but not for smaller deviations from $\left〈f\_{i}\right〉\_{spont}$ activity. This would indicate that achieving a functional effect in terms of curbing aberrant bursting activity in a DCN will require fairly drastic changes in the network's firing rate.

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#### D. LONG-TERM PLASTICITY IN THE HOMEOSTATIC STATE OF A DCN THAT REDUCES OR ELIMINATE THE NETWORK’S TENDENCY TOWARD PATHOLOGICAL BURSTING.

Rationale: DCNs are de-afferented cortical networks that develop synchronized population-wide bursting activity after two weeks in vitro (Wagenaar, BMC Neuro, cortical slab).  These bursts are reminiscent of pathological bursting that develops in vivo after cortical  de-afferentation.  It is likely that in both in-vivo and in-vitro networks, loss of afferent induces homeostatic mechanisms to overcompensate for the lack of excitatory drive [16] [16a] [16d].  A potential therapeutic approach is to control the homeostatic state of the cortical network to prevent pathological overcompensation.  In Aim 2C we will establish precise control over homeostatic mechanisms using our optogenetic population clamp. In Aim 2D the goal is to use this new tool to induce plastic change in DCNs that eliminates pathological bursting for extended time periods after the population clamp has been removed.

Methods: DCNs will be prepared as in Aim 2C. At 13 DIV, the spontaneous spiking activity of each DCN will be continuously recorded for 12 hours. Starting at 14 DIV, population firing rates will be clamped to one of the following target rates: $f^{\*}=R\*\left〈f\_{i}\right〉\_{spont}, R= \{0.01, 0.1, 0.5,2,5,10\}$ for two days.  Every 12 hours, the population clamp will be paused to allow a 30 min recording of spontaneous activity.  At 16 DIV, the population clamp will be stopped and a 2nd 12 hour recording of spontaneous network activity will be performed. Following these recordings, an offline analysis of the changed induced in network firing patters will be performed for the recording of spontaneous data. Specifically, the firing rate of the network, the width of the average cross-correlation between detected units, inter-spike interval distribution [14], and the fraction of spikes that occur within bursts [] will be compared though the 48 hour protocol.

Directly following the final 12 hour MEA recoding, concurrent whole-cell voltage-clamp and MEA recordings will be performed on pyramidal cells in each DCN (Fig. XX). Electrical stimuli [] will be delivered to random MEA electrodes at 0.5 Hz. A peri-stimulus histogram of network activity will then be used to quantify the average rise time and amplitude of the network response, giving an indication of the of excitatory recruitment following each clamping protocol. Analogously, a peri-stimulus histogram of the synaptic currents received by voltage-clamped cells will indicate the magnitude of an average synaptic response to external excitation of the DCN. All of these measures will be compared across time and values of $f^{\*}$.

Potential Outcomes:

* + **12 hours of stimulation is sufficient to prevent bursting**, e.g. we see tons of bursting during the pre-recording, but no bursting after the first 12 hours.  same idea applies to 24 and 36 hour markers.
	+ **Clamping at a high firing rate is the most effective way to maintain burst-free activity**, e.g. during the 12 hour post-recording, bursting comes back that latest for the culture clamped at the highest FR.  same idea applies to other clamped firing rates.

**ANTICIPATED DIFFICULTIES AND ALTERNATIVE APPROACHES FOR SPECIFIC AIM 2**

* Our use of $\left〈f\_{i}\right〉\_{spont}$ as a basis of a DCNs nominal firing rate is based on a large set of spontaneous data at 14 DIV. however, it is possible that the large variability in $\left〈f\_{i}\right〉\_{spont}$ across cultures is simply due to random variations in the number of fast spiking interneurons that are picked up by the MEA. If this is the case, we would expect to see poor correlations between the relationship between a given value of R and a predictable change in synaptic strength. If this occurs, we will switch to absolute values for F\* = {0.01 0.25, 0.5, 2, 5, 10} Hz.
* If AAV2-CamKIIα-ArchT-GFP and AAV2-fPV-Mac-GFP are co-expressed in the same cultures, it will notbe possible to visually distinguish between the fluorescent tags. If co-expression of these two constructsbecomes necessary, we will subclone eYFP of mCherry into the \_?\_ and \_?\_ restriction sites of the AAV2-fPV-Mac vector.
* 48 hours not enough? Do it longer.