AWARD NUMBER: W81XWH-16-1-0085

TITLE: Using High-Precision Signaling Activity Imaging to Personalize Ras Pathway Inhibition Strategies in Neurofibromatosis

PRINCIPAL INVESTIGATOR: John Albeck

CONTRACTING ORGANIZATION: University of California, Davis
Davis, CA 95618

REPORT DATE: June 2017

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
June 2017	Annual	1 Jun 2016 - 31 May 2017

4. TITLE AND SUBTITLE

Using High-Precision Signaling Activity Imaging to Personalize Ras Pathway Inhibition Strategies in Neurofibromatosis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-1-0085

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John Albeck, Taryn Gillies, Heather Blizard, Michael Pargett

E-Mail: jgalbeck@ucdavis.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UNIVERSITY OF CALIFORNIA, DAVIS
1850 RESEARCH PARK DR, STE 300
DAVIS CA 95618

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of this project is to develop reporter cells and to perform real-time ERK and Akt activity measurements in cell types and genetic contexts relevant to NF1. During this award period, we have improved methodologies for introducing genetically encoded signaling reporters into cells and for performing quantitative analysis of signals in individual living cells. We have developed an astrocyte cell line expressing ERK and Akt reporters and have characterized the response of this cell line to growth factor stimulation and to pharmacological inhibitors of Ras pathway effectors, revealing a surprisingly high level of basal (unstimulated) ERK activity in these astrocytic cells relative to epithelial cells and fibroblasts. The methods and initial data collected in this period will allow us to perform a detailed characterization of Ras effector signaling in normal and NF1-mutant cells over the next year of the project.

15. SUBJECT TERMS

none provided

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	12	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

INTRODUCTION: Many of the pathological effects of NF1 mutations result from failure to regulate the activity of the Ras signaling pathway. Inhibition of this pathway is one of the major therapeutic strategies currently being explored for NF1. The two main effectors of Ras, the Raf/MEK/ERK and PI3K/Akt kinase pathways, stimulate proliferation and control the expression of many downstream genes. Recent cell biology studies have shown that the kinetics of ERK and Akt activation, including the intensity, duration, and probability of response, are critical for deciding how the cell responds to Ras activation and which genes are expressed. We have also found that different inhibitors of the Ras pathway have unique effects on these kinetic features and on cellular proliferation and gene expression. Therefore, an understanding of kinetic changes in kinase activity and their effects is important in designing effective inhibition strategies. Our preliminary studies demonstrate our ability to resolve these kinetic differences at high temporal resolution using high-throughput live-cell imaging of ERK and Akt, while similar measurements are not possible using traditional biochemical methods. The goal of this project is to develop reporter cells and perform real-time ERK and Akt activity measurements in cell lines and genetic configurations relevant to NF1. We will assess whether NF1 mutants cells differ in signaling intensity, duration, threshold, or basal activity for both of these effector kinases. We will also measure the ERK and Akt kinetics in wild type and NF1 mutant cells in response to a panel of pathway inhibitors to determine which dose levels and inhibitors represent the best strategy for normalizing mutant signaling activity and cell responses.

KEYWORDS: Ras, ERK, Akt, live-cell imaging, FRET reporter, inhibitor, kinetics.

ACCOMPLISHMENTS

What were the major goals of the project?

The major goals for the reporting period are as follows:

Specific Aim 1 - Identify the kinetic differences in Ras effector activity resulting from NF1 mutations	**Timeline (original)**	**Completion Status**
Major Task 1 ***Establish and validate isogenic reporter cell line models carrying NF1 mutants***	Months	
Obtain CRISPR reagents, generate NF1+/- and -/- astrocyte, Schwann cells, and C2C12s	1-3	30%
Transfect/transduce C2C12 and MPNST cell lines, select, and test by live-cell microscopy.	4-6	50%
Transfect immortalized astrocyte and Schwann cells, test by live-cell microscopy.	6-9	100%
Milestone(s) Achieved		
Stable reporter cell lines for initial test, NF1	3	100%
Optimized imaging protocol and analysis pipeline	6	100%
All stable reporter cell lines ready	9	25%
Major Task 2 ***Measure and compare kinetic modes of ERK and Akt activity in NF1 wild type and mutant cell lines***		

Collect data for different growth factor stimulation conditions	6-12	25%
Extract data and perform quality control on dataset.	6-14	10%
Perform statistical comparisons	12-16	0% (next reporting period)
Milestone(s) Achieved:		
Full dataset collected	15	20%
Grid of statistical comparisons complete	16	0% (next reporting period)

What was accomplished under these goals?

One major activity of this period centered on developing the reporter lines necessary for making measurements of Ras pathway activity. The specific goal of this activity was to create reporter cell lines of cell types relevant in NF1 that can be used for live-cell analysis of signaling kinetics and drug responses. Some unanticipated difficulties in culturing and in reporter gene delivery were encountered, and were addressed, as discussed below in the Changes/Problems section. After identifying appropriate protocols, we stably integrated ERK and Akt reporter genes into astrocyte cells, isolated these cells by flow sorting, and performed image analysis by live cell microscopy. Similar procedures have been initiated and are currently in progress for MPNST and C2C12 cell lines. The outcome from this activity thus far has been the creation of an astrocyte reporter line that performs well for imaging signaling responses to both growth factors and inhibitors (see below), making a useful platform for signaling experiments to be conducted for the remainder of the project. The methodology developed for this activity contributed significantly to a submitted manuscript (item #1 under Products/Journal Publications).

A second major activity in this period was the characterization of signaling kinetics in astrocyte reporter lines. Using the isolated astrocyte reporter cell lines (C8-D1A-EA), we performed a series of experiments to characterize growth factor and drug responses. During live imaging, cells were exposed to EGF, FGF, HGF, IGF, PDGF, or Heregulin. Inhibitors for EGFR (gefitinib), Raf (TAK-632), MEK (trametinib), ERK (SCH-772984), and mTORC1/2 were applied, either preceding or following growth factor treatment. Images were acquired for approximately 100-200 cells per condition, every 6 minutes for experiments ranging in duration from 6-24 hours. We applied our image processing algorithm to all images, extracting data on fluorescence intensity for each cell over the time course of the experiment. The outcome of this activity was the creation of datasets of hundreds of individual cell responses for each condition. Several findings resulted from this data. First, we noted that ERK activity in C8-D1A-EA cells responds robustly to FGF (Fig. 1), weakly to HGF, IGF-1, EGF and Heregulin, and not to PDGF. Second, we found that, as expected, inhibitors of the Raf, MEK, and ERK kinases downstream of Ras potently blocked ERK activation within 5 minutes following addition. Surprisingly however, we found that application of these inhibitors even in the absence of growth factors and serum sharply reduced ERK levels (Fig. 2).

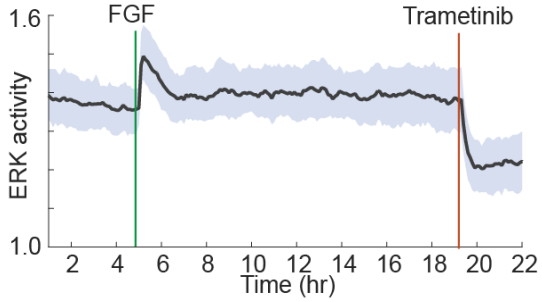

Figure 1. Response of C8-D1A-EA cells to growth factors. The black line represents the mean, and the shaded region the 25th-75th percentile of ERK reporter signal in a population of >100 cells cultured in the absence of growth factors and then treated at the indicated time with FGF. Following FGF treatment, the MEK inhibitor Trametinib was applied. These responses demonstrate the ability of the reporter cell line to indicate both positive and negative changes in ERK activity with high temporal resolution.

This observation contrasts sharply with data we have collected from other cell types, including both epithelial and fibroblast cells, where ERK activity is already at basal levels in the absence of growth factors and cannot be further suppressed even with high doses of inhibitors. This finding indicates that these immortalized astrocytes maintain a high basal level of ERK signaling. Because they were originally isolated from normal mouse cerebellum, these cells are not likely to harbor mutations in the Ras pathway. It may be that the high basal level of ERK activity results from a lower expression level of a negative pathway regulator, such as phosphatases that act on the Raf/MEK/ERK cascade. This possibility suggests a potentially interesting hypothesis: astrocytes may have a gene expression profile that gives them an intrinsically higher basal level of ERK activity relative to other cell types, and thus may be more sensitive to the modest increase in Ras activity resulting from heterozygous NF1 status, explaining why these cells are one of the primary target tissues for this disease. However, it is also possible that mutations in signaling arose during the immortalization process for this cell line. We plan to follow up on this initial finding by testing whether basal ERK pathway activity is high in additional astrocyte cell lines and potentially in primary astrocyte cultures. We will also perform sequencing to confirm the wild type status of Ras/MAPK pathway genes in the astrocyte cell lines and, importantly, will test whether deletion of a single NF1 allele results in a further increase in basal ERK activity.

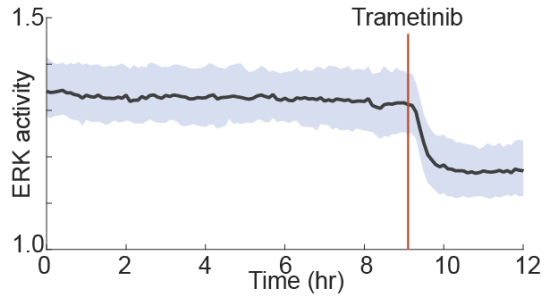

Figure 2. Indications of high basal ERK activity in C8-D1A-EA cells. Mean and 25th-75th percentile range are shown as in Figure 1. Cells were treated only with EGF, a non-stimulating growth factor, prior to receiving the MEK inhibitor Trametinib. The ability of MEK inhibitor to suppress reporter signal even in the absence of a stimulating growth factor indicates that basal ERK activity is high in this cell type.

A third major activity in this period was to perform a comparative analysis of the kinetics of ERK or Akt activity under different conditions. The specific objective of this activity was to create a protocol for identifying the most prominent and relevant kinetic differences between populations of cells. While delays were encountered in producing astrocyte or MPNST reporter cells, we were able to use an existing reporter cell line, MCF10A epithelial cells, as a test case for developing the computational and analytical methods needed to quantitatively compare kinetics between conditions. Existing MCF10A datasets, in which cells were exposed to different growth factor and inhibitor conditions, were used as a test case. MATLAB routines based on peak identification methods were used to extract all potentially relevant kinetic parameters from single-cell ERK activity trajectories, including peak amplitude, average and maximum slope, average pulse width, inter-peak interval, and average peak frequency. While it is straightforward to compare each of these extracted parameters individually between different populations of cells, this approach ignores the possibility that combinations of multiple parameters may drive differences in the output of the pathway. We therefore developed a methodology to use partial least squares regression (PLSR) to develop multivariate models of the contribution of each kinetic parameter to the expression of a target gene (monitored using a knock-in fluorescent protein sequence in the ERK target gene FOSL1). With this approach in place, we will be able to use principal component analysis perform a similar analysis of the signaling kinetics in each experiment going forward. The methods developed for this activity contributed very substantially to a submitted manuscript (item #2 under Products/Journal Publications).

In summary, while initial difficulties in cell culture impeded reaching the objectives for some of the cell lines initially planned for this period, we have been able to successfully carry out the analysis of one of the planned cell types (immortalized astrocytes). In the process we have made methodological innovations that should substantially improve the speed and quality of analysis in the additional cell lines slated for Major Tasks 1&2, as well as for the primary patient cells to be analyzed (Major Task 3 in the original SOW) over the next year of the project. Thus, we do not anticipate a significant change in the overall objectives of the project.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

As discussed above, we will use the live-cell methodologies now in place to continue the analysis of immortalized astrocytes, MPNST tumor cell lines, and NF1 patient fibroblasts as detailed in the original SOW. We plan to complete the publication process for the submitted manuscripts and to prepare and submit a new manuscript detailing the data already collected and to be collected. Dr. Albeck will attend the International RASopathies Symposium in July 2017 to present these data and to speak with collaborators and others in the field.

IMPACT

What was the impact on the development of the principal discipline(s) of the project?

While the results of this work have not yet been published, single-cell analysis has not yet been used to distinguish different kinetics or basal (unstimulated) levels of signaling activity between different types of cells (e.g., astrocytes vs. fibroblasts), or between cells from healthy and diseased individuals. We therefore expect that the methods and data reported here will demonstrate that this new technology can be used to more accurately study how differences in ERK signaling impact the behavior of cells

What was the impact on other disciplines?

The methodologies developed here are applicable to highly parallel time-course measurements from any reporter or similar measurement method. They can therefore be expected to be of use to scientists using imaging-based kinetic measurements across a range of disciplines.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

There have been some unanticipated difficulties in the tissue culture and reporter delivery for the cell lines originally chosen for this proposal. Initially, proliferation of the immortalized astrocyte and MPNST cell lines was very slow, making it difficult to obtain enough cells for the reporter experiments. After experimentation with different culture protocols, we have found that laminin coating of tissue culture vessels substantially accelerates their proliferation and provides an adequate growth rate.

Delivery of reporter genes by transfection was also found to be less efficient than with standard cell lines, making it particularly difficult to generate cells expressing multiple reporters. To address this problem we have developed electroporation protocols that allow a higher percentage of cells to be transfected with reporter plasmids, and we have re-engineered the reporter DNA to create a single piggyBac transposon vector expressing both ERK and Akt reporter genes using an IRES system. This plasmid, when cotransfected with a piggyBac transposase plasmid, allows both reporters to be integrated into genomic DNA in one step.

A third issue that has arisen is difficulty in verifying loss of NF1 protein expression in CRISPR-targeted cell lines. The large size of the protein has created difficulties in western blotting, and we have not been able to identify an antibody that provides a specific signal by immunofluorescence. To address this problem we are working with Frank McCormick's group at UCSF, who have experience in deleting the NF1 gene in cell lines. We will obtain better protocols for NF1 detection, and we have already received from them mouse fibroblasts deleted for NF1 to serve as controls in our experiments.

Overall, these problems have delayed the timeline by 4-5 months. However, having developed adequate solutions for two of them, and a plan for addressing the third, we expect to be able to catch up and proceed as planned over the next year.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals.

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

PRODUCTS:

Publications, conference papers, and presentations
Nothing to report.

Journal publications.

"Live-cell imaging and analysis with multiple genetically encoded reporters"
Michael Pargett and John G. Albeck. *Current Protocols in Cell Biology*, submitted and under review. Federal support acknowledged.

"Linear integration of ERK/MAPK activity and lack of persistence detection by Fra-1"
Taryn E. Gillies, Michael Pargett, Marta Minguet, Alex E. Davies, John G. Albeck. *Cell Systems*, submitted and under revision. Federal support acknowledged.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers, and presentations.

Nothing to report.

Website(s) or other Internet site(s)
Nothing to report.

Technologies or techniques

- Methodologies for creating reporter cell lines and for performing quantitative live-cell analysis of kinetic trajectories from individual cells. These methods have been described in detail in items #1 and #2 under "Journal Publications" above.

Inventions, patent applications, and/or licenses
Nothing to report.

Other Products

- Reporter cell line C8-D1A-EA, an immortalized astrocytic cell line expressing reporters for both ERK and Akt activity.
- MATLAB scripts (software) for performing image and signal analysis on live-cell reporter data.
- Data sets of individual cells treated with growth factors and Ras pathway inhibitors.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*John Albeck*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	0000-0003-2688-8653
Nearest person month worked:	4
Contribution to Project:	*Supervised all areas of research on the project, and has participated in data generation and analysis.*
Funding Support:	*N/A*

Name:	*Taryn Gillies*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*5*
Contribution to Project:	*Developed signal analysis methods for application to ERK and Akt signals; constructed cell lines and performed microscopy experiments*
Funding Support:	*This award*

Name:	*Heather Blizard*
Project Role:	*Technician*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Maintained cell cultures, generated DNA constructs, developed reporter cells and cell lines; prepared frozen cell stocks*
Funding Support:	*This award*

Name:	*Michael Pargett*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*4*
Contribution to Project:	*Developed, maintained, and debugged image analysis software; developed signal analysis techniques; performed data analysis*
Funding Support:	*This award and National Institutes of Health*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Two pending grants have become active since the beginning of this award. These awards do not impact the amount of effort for this award, and do not directly overlap with the experiments in the SOW for this award. However, there are methodological similarities in these awards, as similar imaging and analysis methods will be used in these other projects to examine related conditions such as Noonan or Costello syndrome. These projects will therefore be synergistic, allowing the signaling kinetics observed in NF1 mutant cells to be compared to the changes in other diseases, increasing our ability to identify common features that underlie the disruption of cell and physiological behavior.

R01 04/01/16-03/31/21
NIH/NIGMS $1,050,000
The role of dynamics in defining the limits of normal developmental signaling
This project will examine signaling alterations caused by mutations in BRAF, RAF1, KRAS, and MAP2K1 which occur in Cardio-facio-cutaneous, Noonan, and Costello Syndromes.
Overlap: none
Role: PI, 20% effort

Stand Up to Cancer Innovative Research Grant 07/01/16-06/30/19
American Association for Cancer Research $680,875
Targeting drug resistance plasticity in basal breast cancer using dynamic single-cell informatics.
This project proposes to examine signaling and gene expression patterns underlying drug response heterogeneity in basal breast cancer cells
Role: PI, 25% effort

The following grants ended during the past reporting period:

Faculty Senate Research Grant 07/01/15-06/30/16
UC Davis Committee on Research $24,306
Guiding treatment for the RASopathies using single-cell imaging of signaling dynamics
This project will examine signaling alterations caused by mutations in BRAF, RAF1, KRAS, and MAP2K1 which occur in Cardio-facio-cutaneous, Noonan, and Costello Syndromes.
Overlap: none
Role: PI, 10% effort

Institutional Research Grant 01/01/16-12/31/17
American Cancer Society $36,000
A pilot study of live-cell imaging technology to identify drivers of resistant lung cancer cell subpopulations
The goal of this project is to use live-cell reporters to identify molecular factors underlying heterogeneous responses to cytotoxic chemotherapy in non-small cell lung carcinoma.
Overlap: none
Role: PI, 10% effort

What other organizations were involved as partners?

- **Organization Name:** University of California, San Francisco
- **Location of Organization:** San Francisco, CA
- **Partner's contribution to the projec**t:

  Collaboration NF patient and control fibroblasts were obtained from Dr. Lauren Weiss; NF1-deficient mouse fibroblasts were obtained from Dr. Frank McCormick. Both Drs. Weiss and McCormick contributed to discussions of experimental planning and preliminary data.

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

APPENDICES

Nothing to report.