This document is provided as a sample application. Some pages and text have been redacted.

<table>
<tr><td>PI: White, Katharine Alice</td><td colspan="2">Title: Roles for increased intracellular pH and heterogeneity in ca</td></tr>
<tr><td>Received: 08/26/2019</td><td>Opportunity: RFA-RM-19-006
Clinical Trial:Optional</td><td>Council: 05/2020</td></tr>
<tr><td>Competition ID: FORMS-E</td><td colspan="2">FOA Title: NIH Directors New Innovator Award Program (DP2 Clinical Trial Optional)</td></tr>
<tr><td>1DP2CA260416-01</td><td>Dual: RM,OD</td><td>Accession Number: 4340958</td></tr>
<tr><td>IPF: 6169301</td><td colspan="2">Organization: UNIVERSITY OF NOTRE DAME</td></tr>
<tr><td>Former Number: 1DP2OD029432-01</td><td colspan="2">Department: Chemistry and Biochemistry</td></tr>
<tr><td>IRG/SRG: ZRG1 MOSS-R (70)R</td><td>AIDS: N</td><td>Expedited: N</td></tr>
<tr><td><u>Subtotal Direct Costs</u>
<u>(excludes consortium F&A)</u>
Year 1: 1,500,000</td><td>Animals: N
Humans: N
Clinical Trial: N
Current HS Code: 10
HESC: N</td><td>New Investigator: Y
Early Stage Investigator: Y</td></tr>
<tr><td colspan="3"></td></tr>
<tr><td>Senior/Key Personnel:</td><td>Organization:</td><td>Role Category:</td></tr>
<tr><td>Katharine White</td><td>University of Notre Dame</td><td>PD/PI</td></tr>
</table>

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE | **State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ● Application ❍ Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number
2 CB; 6 MCB

2. DATE SUBMITTED
2019-08-26

Application Identifier

c. Previous Grants.gov Tracking Number

5. APPLICANT INFORMATION **Organizational DUNS*:** [redacted]

Legal Name*: University of Notre Dame
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]

Person to be contacted on matters involving this application
Prefix: First Name*: Mary Middle Name: Last Name*: Greene Suffix:
Position/Title: Preaward Program Manager
Street1*: [redacted]
Street2:
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
ZIP / Postal Code*: [redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* O: Private Institution of Higher Education

Other (Specify):
Small Business Organization Type ❍ Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*
● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
Roles for increased intracellular pH and heterogeneity in ca

12. PROPOSED PROJECT
Start Date* Ending Date*
08/01/2020 06/30/2025

13. CONGRESSIONAL DISTRICTS OF APPLICANT
[redacted]

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: First Name*: Katharine Middle Name: Last Name*: White Suffix:
Position/Title: Assistant Professor
Organization Name*: University of Notre Dame
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested*	$1,500,000.00
b. Total Non-Federal Funds*	$0.00
c. Total Federal & Non-Federal Funds*	$1,500,000.00
d. Estimated Program Income*	$0.00

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ○ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

○ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: First Name*: David Middle Name: Last Name*: Ross Suffix:
Position/Title*: Senior Director, Pre-Award Administration
Organization Name*: University of Notre Dame
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
ZIP / Postal Code*:
Phone Number*: Fax Number: Email*:

Signature of Authorized Representative*	Date Signed*
David Ross	08/26/2019

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ○ Yes ● No

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ○ Yes ○ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6 _ 7 _ 8

If NO, is the IRB review Pending? ○ **Yes** ○ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ○ Yes ● No

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ○ Yes ○ No

IACUC Approval Date:

Animal Welfare Assurance Number

3. Is proprietary/privileged information included in the application?* ○ Yes ● No

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ○ Yes ● No

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ○ Yes ○ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ○ Yes ● No

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ○ Yes ● No

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	ProjectSummary1016729739.pdf
8. Project Narrative*	Narrative1016729732.pdf
9. Bibliography & References Cited	
10.Facilities & Other Resources	Facilities_Final1016729733.pdf
11.Equipment	

Tracking Number: GRANT12924227

Funding Opportunity Number: RFA-RM-19-006. Received Date: 2019-08-26T10:50:53.000-04:00

Abstract

One of the greatest challenges in cancer biology is how to overcome issues of tumor heterogeneity and a dynamic microenvironment to select effective therapeutic treatments, reduce therapy resistance, and produce better patient outcomes. While genetic sequencing as standard of care for solid tumors has expanded the therapeutic toolbox, the molecular mechanisms underlying tumor heterogeneity, metastasis, and drug resistance remain poorly understood. For example, failures of targeted therapies like PI3 kinase inhibitors highlight the need for better understanding of how genetic and phenotypic heterogeneity work in concert with microenvironment pressures to enable cancer cell survival, metastasis, and evolution. Although less well studied in terms of heterogeneity, increased intracellular pH (pHi) is a feature of most cancers and enables a host of cancer phenotypes, including increased cell proliferation, metastasis, evasion from apoptosis, migration, and drug resistance. While the constitutively higher pHi of cancer has been shown to enable these behaviors on a population level in various models, little is known about how single-cell spatiotemporal pHi dynamics or pH heterogeneity might influence or drive single-cell cancer phenotypes. Here, I will use an innovative optogenetic tool to spatiotemporally manipulate pHi in living cells and elucidate the role of pHi dynamics in initiating or supporting single-cell cancer behaviors. I hypothesize that increased pHi is a critical indicator of cancer cell function and directly relevant to promoting single-cell invasion and drug resistance. Furthermore, I predict that pHi heterogeneity correlates with other more cryptic markers of heterogeneity including metabolic changes, stem cell markers, and epithelial and mesenchymal markers. In this work, I propose the rigorous investigation of the roles of pHi in supporting cancer cell behaviors through two complementary approaches. First, I will use an optogenetic tool to increase pHi in single cells to determine whether increased pHi is sufficient to drive single-cell invasion and drug resistance in 2D and 3D cancer models. Second, I will obtain pHi heterogeneity maps of 3D cancer models to determine if monitoring pHi increases can be predictive of individual cells that are likely to migrate, invade, or acquire drug resistance. The ability to dynamically measure pHi in living cells makes pHi an attractive biomarker for aggressive tumor subpopulations. Completion of these studies will transform our understanding of how pH dynamics support and promote cancer progression while revealing new routes for monitoring pHi as a diagnostic or prognostic tool or for identifying appropriate therapeutic interventions.

Narrative

Improvement of cancer patient outcomes requires earlier detection, better targeted therapeutics, and reduction of recurrence. We will study how the increased intracellular pH of cancer promotes cancer cell migration and drug resistance at the single-cell level. We will also determine whether pHi heterogeneity can reveal information about the likelihood of a single cancer cell to metastasize or survive drug treatment.

Facilities and Resources

The White lab has an approximately 1000 ft^2 wet lab located on the first floor of the Harper Cancer Research Institute. The lab also has access to shared warm room, cold room, cell culture room, and a microscope room. The lab has or has access to all biochemical, cell biological, and biophysical equipment needed for this project. This includes a Nikon spinning disk confocal with digital micromirror device, cell culture hoods, shakers, incubators, -20 freezers, -80 freezers, BioRad ChemiDocMP gel imager, LYNX4000 centrifuge, and a MolecularDevices fluorescent plate reader.

Major Equipment:

The White lab shares a Nikon spinning disk confocal microscope system with the Notre Dame Integrated Imaging Facility. The White lab has guaranteed 70% time on the microscope, distributed annually with no restricted days or hours. The microscope includes a spinning disk confocal head (CSU-X1, Yokogawa), high power solid-state lasers (395 nm, 488 nm, 56 nm, and 640 nm), a scientific grade CMOS camera (ORCA-Flash4.0), an inverted microscope stand with integrated optical focus feedback (Ti2-ND-P PFS, Nikon), a high-precision motorized stage, and a stage-top environmental chamber to control temperature and CO_2 (Tokai). This microscope is also equipped with a digital micromirror device patterned illumination system (Polygon 4000, Photometrix) for rapid photoactivation of intracellular regions of interest. This microscope system allows us to perform timelapse imaging of tumor spheroids and perform technically challenging live-cell light activation experiments with robust reproducibility over timescales of seconds to days.

Other Resources:

The White lab is a member of Harper Cancer Research Institute and the University of Notre Dame. This gives the lab access to any core facility on campus including, but not limited to: Flow Cytometry Core, Biophysics Instrumentation Core, Genomics and Bioinformatics Core, Mass Spectrometry and Proteomics Facility, Magnetic Resonance Research Center, and the Center for Social Science Research. As a member of the University of Notre Dame, the White lab has access to these core facilities at a reduced charge or no charge.

These facilities provide state-of-the art equipment and are staffed full-time by knowledgeable scientists who provide expertise and guidance from experiment preparation to data acquisition and analysis. Equipment available through the Flow Cytometry Core (partially housed in Harper Hall) includes a BD FACS AriaIII sorter and a BD LSRFortessa X-20 FACS analyzer that enable simultaneous detection of up to 16 colors and allows us to perform single-cell sorting to isolate stably expressing clonal cell lines. This proposed work will also be taking advantage of standard sequencing analysis offered by the Genomics and Bioinformatics Core Facility (GBCF) at Notre Dame.

OMB Number: 4040-0001
Expiration Date: 10/31/2019

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: First Name*: Katharine Middle Name Last Name*: White Suffix:

Position/Title*: Assistant Professor
Organization Name*: University of Notre Dame
Department:
Division:
Street1*:
Street2:
City*:
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: Fax Number:

E-Mail*:

Credential, e.g., agency login:

Project Role*: PD/PI Other Project Role Category:

Degree Type: PhD Degree Year: 2012

Attach Biographical Sketch*: File Name: Biosketch_White1016729738.pdf

Attach Current & Pending Support: File Name: White_OS_FINAL1016729736.pdf

Tracking Number: GRANT12924227 Funding Opportunity Number: RFA-RM-19-006 . Received Date: 2019-08-26T10:50:53.000-04:00

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Katharine White, Ph.D.

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Clare Boothe Luce Assistant Professor of Chemistry and Biochemistry, University of Notre Dame

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Saint Mary's College Notre Dame, IN	B. S.	05/2007	Chemistry
Massachusetts Institute of Technology Cambridge, MA	Ph. D.	09/2012	Chemistry
University of California San Francisco (Postdoc) San Francisco, CA		10/2018	Cell Biology

A. Personal Statement

One of the greatest challenges in cancer biology is how to overcome issues of tumor heterogeneity and a dynamic microenvironment to select proper therapeutic treatment, reduce therapy resistance, and produce better patient outcomes. Increased intracellular pH (pHi) is a feature of most cancers and enables a host of cancer phenotypes, including increased cell proliferation, metastasis, evasion from apoptosis, migration, and drug resistance. While the constitutively higher pHi of cancer has been shown to enable these global cancer cell behaviors, little is known about how single-cell spatiotemporal pHi dynamics initiate or maintain cancer. My research group is investigating how pH heterogeneity may identify invasive cancer cells, predict acquisition of drug resistance, and lead to retention of specific subclasses of somatic mutations.

During my postdoc, performed with Diane Barber at UCSF and funded by an NIH F32 fellowship, I showed that recurrent arginine-to-histidine cancer mutations in p53 and EGFR confer adaptive pH-sensitive function to the mutant proteins, enhancing cancer phenotypes specifically at increased pHi. I also elucidated novel pH-sensitive molecular mechanisms for wild-type proteins including the sodium proton exchanger NHE1, β-catenin, and the Ras GEF RasGRP1. As a graduate student in Alice Ting's lab at MIT, I used protein design and evolution to create new tools and methods for live-cell protein labeling and applied those tools to label and image proteins and inter-cellular protein-protein interactions. While in training, I successfully mentored 8 students (3 women, 2 other URM). Three of my prior mentees have won research poster and presentation awards at national conferences based on the work performed with me.

In my new position as Clare Boothe Luce Assistant Professor of Chemistry and Biochemistry at the University of Notre Dame, my research platform focuses on mechanisms by which pH dynamics regulate proteins, pathways, and cell behaviors. With projects across experimental scales, we are addressing questions on how dysregulated pHi dynamics regulate cellular behaviors and enable human diseases such as cancer. The ultimate goal of our work is to understand dysregulated protein function at the molecular and cellular levels and apply that understanding to the development of new strategies for limiting disease progression. My training background provides expertise in protein biochemistry, live-cell microscopy, protein engineering, and cancer cell biology. I am uniquely positioned address significant questions on the biological effects of spatiotemporal pH dynamics in cancer initiation, maintenance, and acquisition of drug resistance.

B. Positions and Honors

2007-2012	Graduate Research Associate; Massachusetts Institute of Technology; Cambridge, MA Advisor: Alice Y. Ting
2012-2018	Postdoctoral Fellow; University of California San Francisco; San Francisco, CA Advisor: Diane L. Barber
2019-	Assistant Professor of Chemistry and Biochemistry, University of Notre Dame; Notre Dame, IN

Honors

2007	Outstanding Undergraduate Research Award, American Chemical Society
2007	B.S. awarded, *summa cum laude*
2007	HHMI-MIT Teaching Assistant Fellow
2008	Outstanding Teaching Award, MIT Department of Chemistry
2013	American Cancer Society Postdoctoral Fellowship* *declined due to prior acceptance of NIH F32 Fellowship
2013	NIH F32 NRSA NCI Postdoctoral Fellowship
2019	Clare Boothe Luce Assistant Professorship

Invited presentations

2007	113th Annual Meeting of the American Mathematical Society	New Orleans, LA
2010	Chemical Biology & Novel Therapeutics Program Meeting	Boston, MA
2011	2011 Annual Meeting of the American Society for Cell Biology	Denver, CO
2014	2014 Annual UCSF Biomedical Sciences Retreat	Tahoe City, CA
2016	2016 West Coast Epithelial Biology Society 35th Annual Meeting	Avila Beach, CA
2016	2016 Keystone Symposia Cancer Pathophysiology: Integrating the Host and Tumor Environments	Breckenridge, CO
2017	2017 Telluride Science Research Center Workshop: Protein Electrostatics	Telluride, CO
2019	Western Washington University, Department of Chemistry Seminar	Bellingham, WA
2019	Purdue University, Department of Chemistry Seminar	West Lafayette, IN
2019	Midwest Tumor Microenvironment Conference	South Bend, IN
2019	Telluride Science Research Center Workshop: Protein Electrostatics	Telluride, CO

Professional Societies and Public Advisory Committees

American Chemical Society	2005-2012, 2014-current
Sigma Xi	2007-current
American Society for Cell Biology	2011-current
American Association for Cancer Research	2018-current

C. Contributions to Science

1) ***Protein engineering, tool development, and methodology development.*** My early publications focused on protein engineering and methodology development. Chemical fluorophores have superior photophysical properties to fluorescent proteins and are much smaller. However, to use these probes for live-cell protein imaging, highly specific labeling methods are required. When I began this work, there was no intracellular protein labeling method combining high specificity and sensitivity with a peptide tag. I utilized rational design and directed evolution to re-engineer *E. coli* lipoic acid ligase (LplA) to ligate small-molecule probes onto peptide tags for fluorescent live-cell labeling. This PRIME (Probe Incorporation Mediated by Enzymes) methodology resulted in several high-impact first author publications (1A, 1B). The PRIME method was the first intracellular method capable of labeling cellular proteins with high specificity, sensitivity, and minimal perturbation of the biology of the protein of interest. Compartmentalized labeling of protein subpopulations is

a unique feature of the PRIME methodologies and gives biologists access to experiments that are impossible using any other existing labeling method. This work has been adapted in other co-authored publications (1C-D)

Primary publications:

1A. Uttamapinant C*, **White KA***, Baruah H, Thompson S, Fernandez-Suarez M, Puthenveetil S, Ting AY. A fluorophore ligase for site-specific protein labeling inside living cells. *PNAS.* 107:10914-9. 2010
**Equal contributions*
Featured on the journal cover and highlighted in *Nature Methods* 7,584 (2010) and F1000.

1B. **White KA**[#], Zegelbone, PM. Directed evolution of a probe-ligase with activity in the secretory pathway and application to imaging intercellular protein-protein interactions. *Biochemistry*. 52: 3728-3739. 2013.
[#]*senior and corresponding author*

1C. Liu DS, Loh KH, Lam SS, **White KA**, Ting AY. Imaging Trans-Cellular Neurexin-Neuroligin Interactions by Enzymatic Probe Ligation. *PLoS One*. 8.e52823. 2013.

1D. Uttamapinant C, Sanchez MI, Liu DS, Yao JZ, **White KA**, Grecian S, Clark S, Gee K, Ting AY. Site-specific protein labeling using PRIME and chelation-assisted click chemistry. *Nat Protoc*. 8:1620-34. 2013.

2) ***Understanding how dysregulated pH dynamics support cancer.*** A significant unresolved question in cancer is how somatic mutations are retained and what selective pressures direct this process. In my postdoctoral work, I made a significant contribution to this unresolved question through the investigation of how pH-sensing Arg>His provide an adaptive advantage to the increased intracellular pH (pHi) common to nearly all cancers regardless of genetic background or tissue origin (2A). I showed that recurrent Arg>His mutations EGFR-R776H and p53-R273H can enhance cancer phenotypes specifically at increased pHi by conferring a gain in pH sensing to the mutant protein. I also summarized the effect of dysregulated pHi on various cell behaviors in an invited review at the Journal of Cell Science (2B). Work in collaboration with Ryan Hernandez lab at UCSF, analysis of 29 cancers identified 6 amino acid mutational signatures, including 4 that were defined by two classes of charge-changing mutation Arg>His and Glu>Lys. These signatures were independent of driver mutation, CpG site frequency, or nucleotide mutational signatures (2C). This work presents and validates the new idea that the established higher intracellular pH (pHi) of cancers can enable tumorigenic functions of mutant proteins with histidine substitutions. Collectively, this multidisciplinary study supports a new perspective on the functional significance of somatic mutations that will be of critical importance to cancer biologists, cancer geneticists, and those studying protein structure-function relationships in complex environments.

2A. **White KA**, Szpiech ZA, Garrido Ruiz D, Strauli NB, Jacobson MP, Hernandez RD, Barber DL. Arginine to histidine mutations confer dynamic pH sensing and are enriched in a subset of cancers. *Sci Signaling.* 10:eaam9931. 2017*.*

2B. **White KA**, Grillo-Hill BK, Barber DL. Cancer cell behaviors mediated by dysregulated pH dynamics at a glance. *J. Cell Sci.* 130:663-669. 2017.

2C. Szpiech ZA, Strauli NB, **White KA**, Garrido Ruiz D, Jacobson MP, Barber DL, Hernandez RD. Prominent features of the amino acid mutation landscape in cancer. *PLoS One.* 12(8):e018273. 2017.

3) ***Understanding how pH dynamics regulate normal cellular processes.*** Intracellular pH dynamics regulate normal cell processes like cell cycle progression, directed cell migration, epithelial plasticity, and stem cell differentiation. I identified the adherens junctions protein β-catenin as a previously unrecognized pH-sensitive protein with decreased stability at increased pHi mediated by pH-dependent association with the E3 ligase β-TrCP (3A). I elucidated the molecular mechanism for pH-sensitive PIP2 binding in regulating activity of the Na^{+}-H^{+} exchanger (NHE1) (3B), which regulates ion transporter activity in response to growth factor stimulation. I have also contributed to the identification of the RasGEF RasGRP1 as a pH sensor with pH-dependent activation mediated by a conserved histidine residue (3C). This work focused on the molecular

mechanisms of pH sensing utilizing individual histidine residues. Future work will investigate how more complicated networks of ionizable residues might contribute to pH-sensitive protein function.

3A. **White KA*,** Grillo-Hill BK*, Esquivel M, Barber DL. β-catenin stability is pH-sensitive with decreased stability at higher intracellular pH. *JCB.* 217(11):3965-3976*.* 2018. *Equal contributions

3B. Webb BA, **White KA**, Grillo-Hill BK, Schonichen A, Choi CC, Barber DL. A histidine cluster in the cytoplasmic domain of the Na-H exchanger NHE1 confers pH-sensitive PI(4,5)P2 binding and regulates transporter activity. *JBC* 291:24096-104. 2016.

3C. Vercoulen Y*, Kondo Y*, Iwig JS*, Janssen A, **White KA**, Barber DL, Kuriyan J, Roose JP. A histidine pH sensor regulates the activation of the Ras-specific guanidine nucleotide exchange factor RasGRP1. *eLife*. *in press.* 2017. *Equal contributions.

Complete List of Published Work in MyBibliography:
http://www.ncbi.nlm.nih.gov/sites/myncbi/1Vcx2CJCTn_AF/bibliography/45429283/public/?sort=date&direction=ascending

D. Research Support

Current Research Support

Warren Center for Drug Discovery Pilot Grant 01/01/19-01/01/20
Notre Dame Internal
Miniaturization of pH plate reader assay for HTS of cancer therapeutics to lower pHi
Aims: Develop a high throughput screening assay for pHi lowering drugs. These aims do not overlap with those proposed in this application.

University of Notre Dame Startup Funds

Clare Boothe Luce Assistant Professorship (Henry Luce Foundation)

Past Research Support

F32 NRSA Individual Postdoctoral Fellowship 08/01/2013-07/31/2016
NIH-NCI (CA177085)
The pH-dependent adaptive advantage for recurrent histidine mutations in cancer
Aims: Investigate the adaptive gain in pH sensing conferred by recurrent arginine to histidine mutations in cancer.

PHS 398 Cover Page Supplement

OMB Number: 0925-0001

Expiration Date: 03/31/2020

1. Vertebrate Animals Section

Are vertebrate animals euthanized? ○ Yes ○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

○ Yes ○ No

If "No" to AVMA guidelines, describe method and provide scientific justification

2. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

PHS 398 Cover Page Supplement

3. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ❍ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, check the box indicating that one from the registry will be used:

Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

4. Inventions and Patents Section (Renewal applications)

*Inventions and Patents: ❍ Yes ❍ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ❍ Yes ❍ No

5. Change of Investigator/Change of Institution Section

❑ Change of Project Director/Principal Investigator

Name of former Project Director/Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

❑ Change of Grantee Institution

*Name of former institution:

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 03/31/2020

Introduction	
1. Introduction to Application (for Resubmission and Revision applications)	
Research Plan Section	
2. Specific Aims	
3. Research Strategy*	DP2_ResearchEssay_WHITE_FINAL1016729737.pdf
4. Progress Report Publication List	
Other Research Plan Section	
5. Vertebrate Animals	
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support	
10. Resource Sharing Plan(s)	
11. Authentication of Key Biological and/or Chemical Resources	
Appendix	
12. Appendix	

Project Science Areas: 2 CB; 6 MCB

Roles for increased intracellular pH and heterogeneity in cancer

Project Description:

CHALLENGE, INNOVATION, AND IMPACT STATEMENT

One of the greatest challenges in cancer cell biology is how to overcome issues of tumor heterogeneity, drug resistance, mutation, and clonal selection in order to successfully combat the disease[1,2]. Currently, standard of care when treating patients is to perform genetic sequencing of their tumors. Unfortunately, unless the patient has one of a select few mutations for which targeted therapies exist, much of this data is useless in identifying effective treatment options for patients[1]. Furthermore, there exist few feasible assays to meaningfully assess tumor heterogeneity or predict likelihood of acquiring drug resistance. To improve patient outcomes, the field needs a better mechanistic understanding of how single-cell behaviors lead to metastasis and drug resistance.

Constitutively increased intracellular pH (pHi) is seen across cancers (pHi ~7.4-7.6 in tumor cells vs. ~7.1-7.3 in normal cells), and is thought to be an early event in cancer development[3]. At a population level, increased pH has been shown to enable cancer cell proliferation, metastasis, evasion of apoptosis, metabolic adaptation, and drug resistance[4]. However, a current limitation for resolving the effects of altered pHi dynamics in cancer biology is the lack of tools to experimentally change pHi with spatiotemporal control.

Our innovation is to apply an optogenetic tool (a light-activatable proton pump) to spatiotemporally manipulate pHi in individual cells and monitor cell biological effects. State-of-the-art for spatiotemporal pHi manipulation are microinjection experiments where scientists load cells with a pH indicator dye and then use a micropipette filled with a high or low pH solution to poke holes in the cells and inject the buffer to manipulate pHi and monitor by light microscopy. These experiments are slow, technically challenging, irreversible, and damaging to the cell. Conversely, this optogenetic tool allows us to reversibly increase intracellular pHi in living cells using spatially-restricted light activation (Figure 1). This tool allows us to answer previously intractable questions about how spatiotemporal pHi dynamics and pHi heterogeneity influence cancer cell behaviors.

In this proposal, we will test the hypothesis that single-cell spatiotemporal pHi dynamics contribute to key global cancer cell behaviors by investigating three critical questions (Figure 1):

1) Is increased pHi sufficient for or predictive of single-cell cancer behaviors like invasion and drug resistance?
2) Does pHi heterogeneity in 3D spheroid models correlate with phenotypic heterogeneity?
3) Is pHi heterogeneity in 3D spheroids predictive of invasive or drug resistant populations?

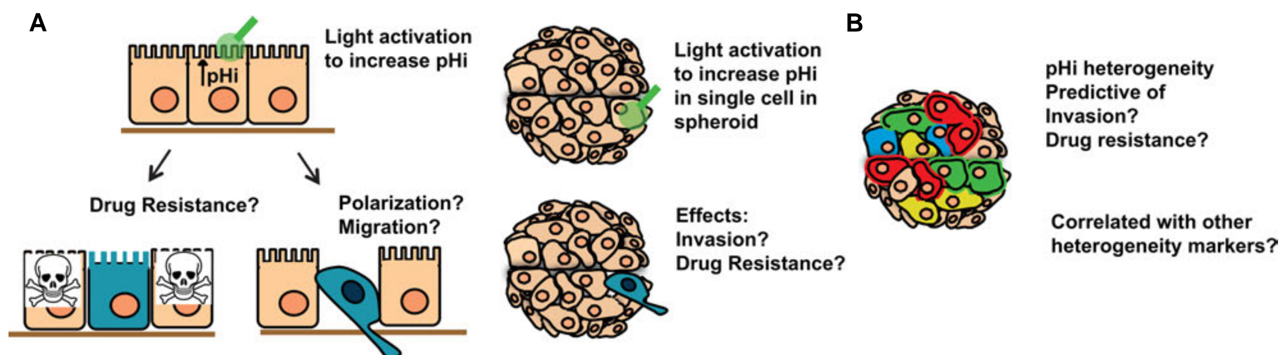

Fig 1. Outline of proposed work A) Investigate roles for increased pHi in single-cell behaviors like drug resistance and cell migration and invasion will be investigated through spatiotemporal manipulation of pHi in 2D and 3D. B) Correlate pHi heterogeneity maps with other markers of tumor heterogeneity. Investigate whether pH heterogeneity is predictive of single-cell behaviors like invasion and drug resistance.

The potential impact for basic cancer biology is to validate pHi as a straightforward marker for assessing phenotypic heterogeneity and drug resistance in cancer model systems. It will also determine whether increased pHi is a sufficient regulator of cell migration and drug resistance. If successful, this work would support measuring pHi in patient tumors as a possible predictive tool and would improve understanding of how changes in cell physiology and tumor environment contribute to cancer progression.

RATIONALE

The key to our approach is a genetically-encoded optogenetic tool that allows us to spatiotemporally manipulate pHi in living cells. Such an approach has never been previously explored for understanding how increases in pHi affect single-cell cancer behaviors.

Various light-activatable ion transporters have been utilized as tools to manipulate neuronal function by depolarizing plasma membranes and inducing neurotransmitter release[5,6]. For example, rhodopsins are light activatible proton pumps that pump an intracellular proton to the extracellular space in response to activation with red light (Figure 2). Unlike most pH homeostatic mechanisms, rhodopsins are electrogenic. However, these proteins are natively light-activatable, expressed on the plasma membrane, and are feasible starting points for a proposed intracellular pH manipulation tool.

For the proposed technology, the following requirements must be met: 1) Tool must be genetically encoded for ease of use; 2) tool must increase intracellular pHi; 3) light-activation and proton pumping must be fast and reversible; 4) dark-state leaky proton pumping should be minimal. We started with Archaerhodopsin (ArchT) as a potential pHi manipulation tool because it meets criteria 1, 2, and 4. Additionally, one paper reported localized pHi changes observed in the pre-synaptic bouton, but these transient pHi changes were not persistent under those authors' imaging conditions[7]. However, these results suggested that ArchT would be a good platform to develop a tool to manipulate pHi in living cells.

Proof of principle: characterization of Archaerhodopsin as a spatiotemporal pHi manipulation tool

We used Archaerhodopsin (ArchT) as a starting point for our spatiotemporal pH manipulation tool (Fig 2A). We pair the tool with a pH biosensor (mCherry-pHluorin) to spatiotemporally manipulate and monitor pHi in living cells in real time. In proof-of-principle experiments, we used the ArchT optogenetic tool to spatiotemporally raise pHi in human embryonic kidney cells (HEK293) cells. Using confocal microscopy and a digital micromirror device, we can illuminate and activate the proton pump in a small spatially-restricted region of a single HEK293 cell (Fig. 2B). Local response happens within 1 second, but sequential illumination (brief pulses of 561 nm light every 5 seconds) allows us to increase pHi over longer experimental timeframes (Fig. 2C).

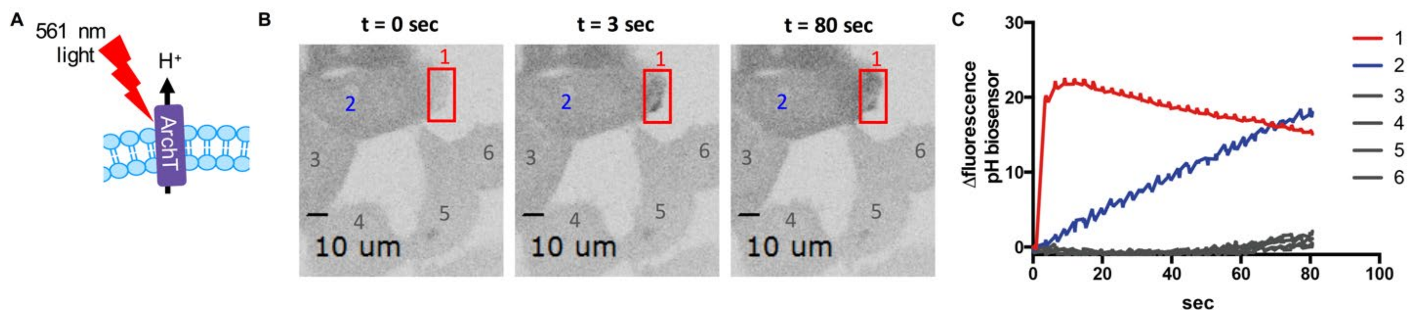

Fig 2. ArchT tool to spatiotemporally manipulate pHi. A) The ArchT tool pumps protons out of the cell when stimulated with 561 nm light. B) Confocal images of HEK293 cells stably expressing a pH biosensor (mCherry-pHluorin) and light-activatable proton pump (ArchT). The proton pump is activated at t=3 by illuminating only in the red box (1) on an individual cell (2). The plot shows change in fluorescence of the pH biosensor (higher fluorescence = higher pHi). C) Quantification of images in B) over time. Only the illuminated cell (2) has increasing pHi over this 2-minute experiment.

Importantly, cells expressing the pH biosensor alone do not exhibit increased pHi upon stimulation with 561 nm light (Fig. 3). The initial stimulation effect produces a very local increase in pHi, which is then followed by slower whole-cell response. Sequential stimulation allows us to raise pHi in stimulated HEK293 cells from 7.25 +/- 0.08 to 7.55 +/- 0.11, on average. A unique feature of this approach is that we can perform analysis at the single-cell level to measure heterogeneity or bin individual cells to determine if there is a pH “tipping point” at which physiological changes are initiated.

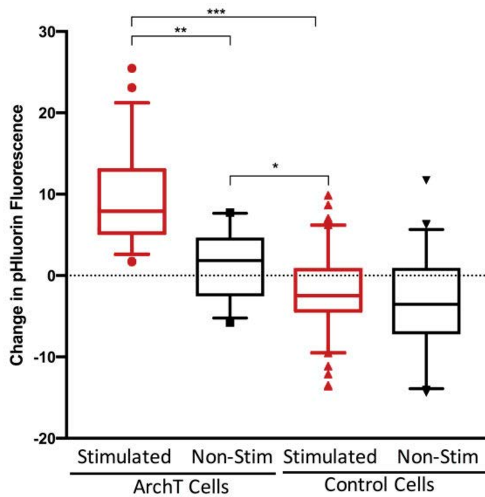

Figure 3. Quantification of pHi manipulation in living HEK293 cells. Change in fluorescence after 2 minutes of sequential stimulation with 561 nm light. Cells expressing the ArchT tool have increased fluorescence when stimulated, whereas control cells do not. N = 100 cells per condition. Quantification of change in fluorescence after 2 minutes of sequential stimulation with 561 nm light. Cells expressing the ArchT tool have increased fluorescence (pHi) when stimulated, whereas unstimulated cells and all control cells not expressing the ArchT tool do not.

Proof of principle: Determine how spatiotemporal pH dynamics promote single-cell membrane protrusion.

As proof of principle experiment to determine whether our ArchT tool can probe pH-dependent cell behaviors, we first investigated membrane protrusion. An important debated question in the field is whether local pH gradients can exist across the cell and how this might alter or reinforce pH-dependent cellular processes. Some groups argue that local pH changes (particularly near the negatively-charged inner leaflet of the plasma membrane) will be immediately communicated across the cell at the rate near that of free proton diffusion[8]. Others argue that buffering components in spatially-restricted regions of the cell (like the lamella) will enable localized increases in pHi that can produce localized changes in actin remodeling[910]. With our optogenetic pH manipulation tool, we are uniquely positioned to address this long-standing debate in the field.

Preliminary data generated by current graduate student [redacted] and summarized in Figs. 2-3 above show that our spatiotemporal pH manipulator is applicable for experiments that explore pH-dependent cell behaviors on a short timescale (up to ~2 minutes). Actin cytoskeleton remodeling occurs on the timescale of seconds to minutes and has been shown to be a pH-dependent process on a population level[11]. Actin remodeling is also a key first step in cell invasion. Therefore, we first sought to determine whether localized changes in pHi can induce immediate effects on membrane protrusion. We plan to explore this right away, in parallel with efforts (described below) to investigate the role of pHi in cancer cell migration and acquisition of drug resistance in 2D and 3D cultures.

In preliminary results, we used the ArchT tool to raise pHi in a spatially restricted region such as the lamella. We observed immediate and localized membrane protrusion and ruffling effects that are not rapidly communicated outside the stimulation window over the 2-minute experiment (Fig. 4A). While we observed localized membrane protrusion effects in 10/12 stimulated cells expressing ArchT and the mCherry-pHluorin biosensor, localized membrane ruffling was not observed in any control cells expressing the pH biosensor mCherry-pHluorin alone (0/14 cells, Fig. 4B). This is the first experimental result to demonstrate that increased pHi is sufficient for membrane protrusion and show that the membrane protrusion is rapid and local. We will further characterize these results, but we are very excited to apply the ArchT tool in more complex experiments investigating how increased pHi may drive cancer cell behaviors like single-cell migration, invasion, and drug-resistance.

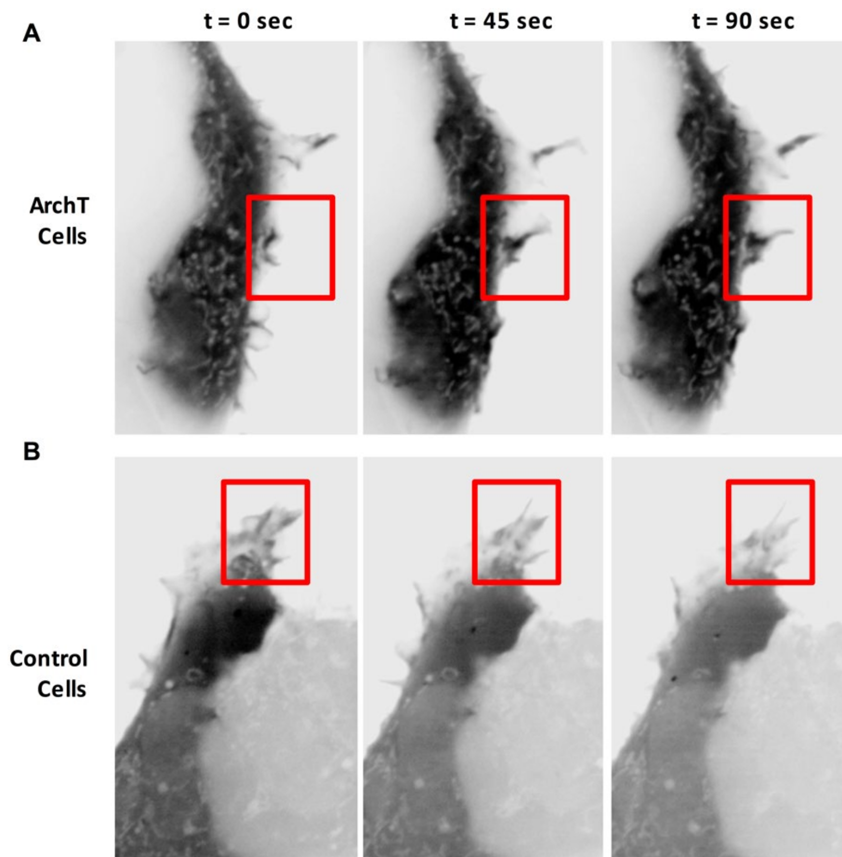

Fig 4. ArchT tool to activate localized membrane ruffling events A) HEK293 cells expressing the pH biosensor (mCherry-pHluorin) and the ArchT tool are stimulated with 561 nm light only in the red box at t=1 sec. Membrane protrusion happens quickly over the course of this experiment, with significant pHi-driven protrusions observed at t=45 and t=90. B) Experiment was performed identically to that described in A on HEK293 cells expressing only the pH biosensor (Control cells). pHi does not change and no membrane protrusions were observed.

Proof of principle results: classification of pHi heterogeneity in 3D cancer spheroids

In proof-of-principle experiments, generated by current graduate student Julia Spear, we have shown that cancer cells grown in 2D have high but relatively homogeneous pHi, while cells grown in 3D spheroids have heterogeneous pHi (Fig 5A). We also have three observations that support investigating a role for pHi in defining invasive population subtypes. First, we observe that stimulation of tumor spheroids with EGF increased average pHi significantly while decreasing pHi heterogeneity (Figure 5B). Second, we observe that knockdown of the pH homeostatic ion transporter the sodium proton exchanger (NHE1) inhibits the spheroid protrusions we observe in response to growth factors (Figure 5C). Third, we observed increased pHi in single-cells migrating out of a spheroid (Figure 5D). These data suggest that pHi heterogeneity may reflect physiological or microenvironment pressures and be a meaningful reporter or predictor of migratory cell phenotypes. These proof of principle data support our proposed work investigating whether pHi heterogeneity is predictive of cell phenotype or cell behavior in 3D tumor spheroid models.

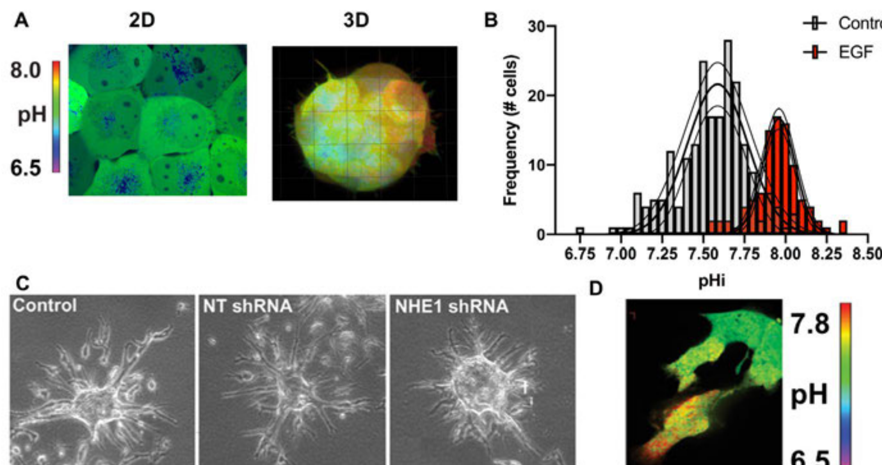

Fig 5. pH heterogeneity in 3D cancer spheroids A) H1299 cells expressing the ratiometric pH biosensor (mCherry-pHluorin) are grown in 2D and in 3D. pHi is back-calculated after standardizing the biosensor with buffers containing protonophores. B) Histogram quantification of 3D data shown in A. When cells are stimulated with 20 ng/mL EGF, average pHi increases and heterogeneity (spread) decreases. C) NHE1 shRNA lowers pHi and attenuates protrusions from spheroids stimulated with growth factor. Control and NT spheroids have similar protrusions. D) pHi quantified in a single protruding cell from a spheroid suggests that invasive cells may have higher pHi than neighboring cells.

APPROACH

Extend Archaerhodopsin capabilities to enable longer-term imaging experiments

We consider the pH-dependent actin remodeling experiments described above to be "proof of principle" and though we plan to ask critical biological questions and further characterize these data, we are most excited about applying this technology to study longer-term pH dependent cancer behaviors like pH-dependent single-cell migration, invasion, and drug resistance.

To study these behaviors, we will need to optimize our imaging experiments with the current ArchT tool to enable longer-term maintenance of increased pHi in normal epithelial cells. While we can achieve increased pHi from ~7.25 to ~7.55 for 2 minutes, longer experiments require further optimization. Considerations must be made to minimize the total amount of light the cells are exposed to over an experiment as excess light has shown to be toxic to cells. We will therefore need to optimize our photoactivation protocols to enable long-term imaging and maintenance of increased pHi.

Our preliminary optimization shows that 5 second photoactivation of ArchT every 2 minutes is sufficient to induce increased pHi over multi-hour imaging times. However, targeted cells begin to sicken from light exposure (or heat) after this point, and begin to undergo apoptosis at 12 hours. Optimization focused on reduction of LED illumination power or longer wait times between photostimulations should enable longer-term experiments. However, we will be able to monitor initial phases of cell migration and invasion, as previous published work suggests loss of cell-cell contacts and actin stress-fiber formation both occur within 4-6 hours[12][13].

Determine whether increased pHi is sufficient for single-cell migration and acquisition of drug resistance in 2D and invasive cell behaviors in 3D

Increased pHi has been correlated with increased cell invasion[14,15] and the acquisition of chemotherapeutic drug resistance at the population level[16–18]. However, it is unknown whether increased pHi is sufficient to induce these cancer cell behaviors at a the single-cell level. We will use the above reagents to determine effects of pHi dynamics on cancer cell behaviors at the single-cell level. In our first experiments, we will express our ArchT tool and pH biosensor in normal mammary epithelial cells (MCF10A cells) grown in 2D and in a 3D matrigel sandwich. We will light-activate ArchT in a single cell, monitor pHi increase by microscopy, and quantify cell migration and invasion phenotypes (Figure 6A). Our prediction is that increased pHi is a sufficient driver of single-cell migration in 2D and sufficient for single-cell migration out of an organized 3D epithelium. We are using non-transformed cells to mimic effects of the increases in pHi that occur at the earliest stages of cellular transformation in cancer, but we will also compare these results to effects of spatiotemporal pHi changes in invasive breast cancer cells (MDA-MB-231) which have a constitutively increased pHi of 7.55. We predict that increased pHi will still produce a single-cell migratory effect, even in the cancer cell lines.

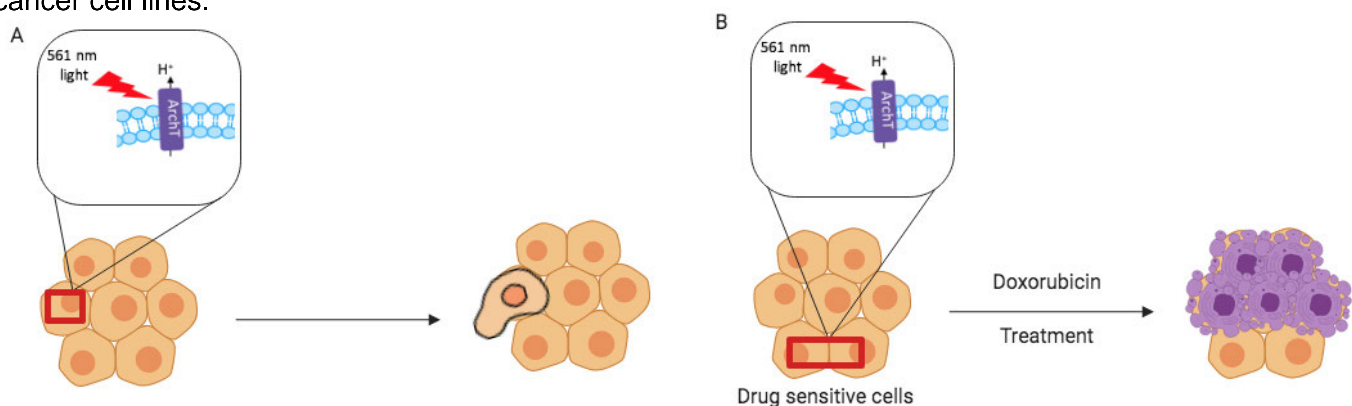

Fig 6. Experimental approach for investigating single-cell pH-dependent behaviors. A) Cells expressing the ratiometric pH biosensor (mCherry-pHluorin) and ArchT tool are grown in 2D (or in 3D). A subset of cells are stimulated (red squares) with 561 nm light to increase pHi. We will monitor single-cell protrusion/migration phenotypes (A). As well as resistance to doxorubicin (B).

For the drug resistance experiments, we will treat cells with doxorubicin while using the ArchT tool to raise pHi over 12 hours in MCF10A cells and MDA-MB-231 cells (both sensitive to doxorubicin) (Figure 6B). We predict that increased pHi will be sufficient to confer doxorubicin resistance to the cells with stimulated ArchT. The beauty of these experimental approaches lies in our ability to isolate single cells for stimulation. This means that we always have matched internal controls (cells with no change in pHi) within each field of view. Thus, we can ensure that any effects we observe are due to increased intracellular pHi produced by activated ArchT.

Outcomes of these experiments include demonstration that increased pHi is sufficient for single-cell migration, invasion, and acquisition of drug resistance. This result would be transformative in linking an early cancer hallmark (increased pHi) to these single-cell fitness advantages. Moreover, this work would motivate targeting pHi as a method to limit single-cell clonal adaptation and expansion and constrain cancer progression.

Determine whether pHi heterogeneity correlates with other phenotypic heterogeneity in 3D

Better understanding of the molecular mechanisms driving phenotypic heterogeneity is critically important for advancing cancer therapeutics[19]. Our proof-of-principle results suggest that pHi heterogeneity is a feature of 3D cancer cell line growth. We predict that pHi heterogeneity may correlate with other markers of phenotypic heterogeneity such as epithelial or mesenchymal markers, hypoxic, metabolic, and stem cell markers. Whereas these traditional markers are very difficult to monitor in living cells, pHi can be measured using cell-permeable pH-sensitive dyes in tumor spheroids[20] or explants or *in vivo* using hyperpolarized MRI imaging techniques[21]. This makes pHi an attractive candidate biomarker for invasive, drug resistant, or tumor initiating populations.

Prior work has suggested that pHi correlates with hypoxia in tumor spheroids[20] but other more recent analyses suggest that this may not be the case[22]. Using our live-cell pHi imaging platform and spheroid expertise, we will create live-cell pHi maps and then fix, stain, and reimage spheroids to prepare correlative maps of other heterogeneity markers on the *exact same spheroids*. We are uniquely positioned to be able to acquire live-cell images of pHi in spheroids of various sizes, while having the expertise to perform post-fixation imaging of the same spheroid for exact cell-matched single-cell data. Because of the conflicting data in the literature on the causal link between hypoxia, pHi, and metabolic changes, any clarifying data we obtain on this objective could be transformative. For example, if we determine that high pHi correlates with a mesenchymal phenotype while low pHi correlates with a “stem-like” tumor initiating phenotype that would support future work developing therapeutics to specifically target subpopulations and monitor pHi and treatment response. In addition to this proposed broad correlative analysis, we have two focused hypotheses that we will be testing, as described below.

Determine whether increased pHi is a predictive marker of invasive cells in 3D

We will prepare spheroids of lung cancer cells (H1299) and breast cancer cells (MDA-MB-231). We selected these lines because they are very heterogeneous in terms of morphology and pHi when grown in spheroids. We will perform time-lapse imaging of spheroids over 12-24 hours to determine whether increased pHi is predictive of cells that are migrating out of the spheroid (invasive cancer cells). We can quantify these large 3D datasets with ease using powerful Imaris software. A membrane dye allows us to segment and quantify individual cells in large tumor spheroids. Using the Imaris software, we can easily obtain single-cell volumetric quantification of size, pHi, position, circularity, and other morphological features. Our hypothesis is that high pHi is a predictor of migratory or invasive cancer cells. To test this, we will perform our analysis and bin cells based on pHi and determining % of high pHi cells that migrate vs. % low pHi cells. We also predict that high pHi is a necessary signal for cell migration from a tumor spheroid. To test this, we will perform our analysis by identifying all invasive cells and then go back through time to determine what the maximum pHi that cell experienced prior to beginning migration.

Determine whether increased pHi is a predictive marker of drug resistant cells in 3D

We will prepare spheroids of lung cancer cells (H1299) and breast cancer cells (MDA-MB-231), both of which are sensitive to the chemotherapeutic drug doxorubicin. We will perform time-lapse imaging of spheroids over 12-24 hours of doxorubicin treatment while monitoring pHi and cell death (apoptosis). Our hypothesis is that high pHi is predictive marker of drug resistant cells. We expect that the cells with the highest measured pHi will also have the highest survival in response to doxorubicin treatment. Because we’ll have time-lapse quantification of pHi and cell death over time, we can determine whether resistance to doxorubicin-induced cell

death correlates linearly with pHi or whether it's a digital response where a certain pHi is reached that confers drug resistance.

Our ability to perform high-resolution single-cell pHi measurements in large tumor spheroids uniquely positions us to investigate the hypothesis that increased pHi might be a predictive marker of cell phenotype in heterogeneous cancer models. Outcomes of these experiments include identifying high pHi as a predictive marker of cells that are likely to invade, metastasize, or acquire drug resistance. This result would motivate expanding these experiments to primary cell tumor organoids to further characterize this predictive link in a more complex system. Furthermore, if the link between pHi and other heterogeneity markers is highly correlative, these results would motivate using an easily screenable feature (pHi) as a proxy for more cryptic cell markers like stem cell or tumor initiating cell populations.

CAVEATS, ALTERNATIVES

The studies to generate new protocols for longer pHi control are challenging but tractable. If image acquisition and microscope optimization are not sufficient, an alternative is to perform random mutagenesis of Archaerhodopsin using low-throughput screening for slower dark-state recovery (to increase protons pumped per photon). Another alternative is to create a light-activatable version of the sodium proton exchanger NHE1. I have extensive experience in characterizing the mechanism of this ion transporter, and insertion of a light-activatable LOV2 domain at the c-terminal tail with a dark-state binding peptide (ZDK) to sequester the tail away from the plasma membrane should produce an "inactive" protein, that could then be activated by blue light release of LOV2 and ZDK. As we have already shown membrane ruffling in response to increased pHi, extending these experiments to investigate and quantify cell migration should be straightforward. The pH heterogeneity aims do not require the ArchT tool, which makes those goals independent of any engineering or optimization problems. Photobleaching of our pH biosensor could be an issue during long-term time-lapse experiments along with cell autofluorescence when imaging the green biosensor. In the event these issues arise, transitioning to a red-shifted pH biosensor pHred should minimize both of these concerns, although that protein has some redox-dependence which makes it less desirable as a first-choice probe. Finally, if spheroid fixation, staining, and quantification becomes too cumbersome on larger spheroids, we will explore live-cell-compatible biosensors of other tumor heterogeneity markers.

INNOVATIVENESS

Many cancer biologists wish for a better way to predict or identify whether a cancer will undergo metastasis or acquire drug resistance. For example, can we determine from a biopsy or functional MRI assay whether a patient's tumor is likely to metastasize or acquire resistance? Genetic sequencing has been revolutionary in the classification and origins of cancer, and organoid drug screening is a potentially transformative approach for screening therapeutics prior to administering to a living patient. However, these analyses are limited by the fact that spatial and dynamic information is frequently lost. Moreover, organoid screening helps a single patient without necessarily revealing the molecular mechanisms driving that tumor's pronounced response to drug while genetic screening is frequently not translatable even when patient's share a mutation. The varied clinical response of PI3K inhibitors is just one telling example of this.

It is clear the phenotypic and genotypic heterogeneity function in concert with microenvironment pressures to create a perfect storm of cancer survival, metastasis, resistance, and evolution. Key to improving patient outcomes are a better understanding of molecular mechanisms driving these cancer hallmarks. We propose that the increased intracellular pHi of cancer is an understudied driver of heterogeneity, resistance, and metastasis. The work proposed herein will transform our understanding of the molecular mechanisms driving heterogeneity in cancer and identify pHi heterogeneity as a proxy marker for other tumor heterogeneity markers. The benefit of using pHi as a diagnostic, prognostic, or therapeutic stratification tool is that pHi is comparatively easy to measure in tumor organoids and can be done in living cells. Moreover, completion of this project would motivate future development and optimization of better tools for in vivo pHi imaging. Completion of this work will also solidify the role of intracellular pHi dynamics in supporting cancer and for the first time demonstrate that increased pHi is a sufficient regulator of single-cell cell migration, invasion, and drug resistance at the single-cell level. This result could revolutionize the way clinicians think about therapeutic interventions targeting these cancer fitness advantages that result from single-cell acquisition, clonal expansion, and competition.

INVESTIGATOR QUALIFICATIONS

The goal of my research platform is to understand how spatiotemporal pHi dynamics drive single-cell cancer behaviors and tumor heterogeneity. I am uniquely positioned to investigate the role of increased pHi as a driver and predictor of single-cell behaviors. This project relies on my strengths in developing imaging tools and methodologies as well as my expertise in pH-dependent cancer cell behaviors. I have recruited a multidisciplinary team that complements my areas of expertise in terms of the heterogeneity work. This includes a postdoctoral scholar, [redacted], with expertise in tumor biology and metabolism, and a graduate student, [redacted], with spheroid culture and imaging experience. The multidisciplinary environment of Harper Cancer Research Institute is an ideal setting for completing the multidisciplinary work proposed. I am equipped to guide the work proposed herein, but have access to experts in relevant cancer biology fields for consultation. [redacted] is an expert in ovarian cancer tumor-stromal microenvironment interactions and has extensive expertise in spheroid growth. [redacted] has expertise in in vivo tumor heterogeneity analysis and will be a fantastic resource for experimental design and first reader for our resulting manuscripts.

My lab is one of the few in the world that is studying the effects of pHi dynamics at the single-cell level. My past expertise in characterizing pH dependent protein function[23,2425] and revealing pH dependent cancer cell behaviors[26] position me to be able to bridge molecular understanding with cell biological observations. The proposed work is innovative and ambitious, and we have significant proof-of-principle experiments as support. My lab opened in January 2019 and we have made significant progress (all proof-of-principle data shown in this proposal was collected in my lab at Notre Dame and all except Figure 2 were acquired by my graduate students). This demonstrates my ability to train and mentor students on these ambitious projects as well as the abilities of the talented students I've recruited to the team.

SUITABILITY FOR THE NEW INNOVATOR PROGRAM

The proposed work is appropriate for the following reasons:

- This interdisciplinary proposal represents a major departure from traditional basic cancer cell biology research. State of the art in the field consists of applying better and better methods to screen drugs in organoid models, interpret or predict population-level effects, and approach questions of heterogeneity with combinatorial or sequential therapeutics[27]. Here, the proposed strategy is determining the role of increased pHi in mediating key cancer behaviors like migration/metastasis and drug resistance. The proposed work involves enzyme engineering, cell biology, and high-resolution imaging and is unlikely to be pursued in a traditional cancer genomics or translational cancer laboratory.

- Genomics is advancing at an incredible rate, with innovations in single cell DNA sequencing[28], 3D genome mapping[29], and ribosome profiling. However, with the exception of a few superstars, targeted molecular therapeutics have not been able to overcome issues of tumor heterogeneity and clonal adaptation and selection. Therefore, the field of cancer therapeutics is very much in need of advances that link universal and genome-independent phenotypic outcomes to an easily measurable heterogeneity biomarker. The heterogeneity work will link a quantifiable physiological cue (in this case high pHi) with more cryptic heterogeneity markers like drug resistance, stem or tumor initiating markers, and invasive mesenchymal markers. Therefore, this work will be transformative for how we view tumor heterogeneity and provide a route to characterize and classify phenotypic heterogeneity using pHi.

- State-of-the-art for spatiotemporal pHi manipulation is microinjection experiments. These experiments are slow, technically challenging, irreversible, and damaging to the cell. If successful, the impact of this work will be felt across many fields. The spatiotemporal pH manipulation methodology is likely to be adopted by many basic cell biology laboratories, especially due to the ease of use. The knowledge generated may be transformative for many areas of cell biology, uncovering new pH sensitive proteins and pathways that in turn lead to novel hypotheses for normal and pathological cell function and molecular mechanisms. For example, our current understanding of pH-sensitive proteins has been built from decades of slow and careful one-by-one studies. If a single experiment can double or triple the number of known pH sensing proteins, this would be transformative for both basic and cancer biology.

- The proposed work is high risk, despite the proof of principle data we have already obtained. We still have yet to thoroughly characterize our current technique of increasing pHi spatiotemporally inside cells. Moreover, there remain questions of whether pHi heterogeneity is a cause or a consequence of other microenvironment-sensitive physiological cues like hypoxia, nutrient availability, or cell-substrate cell-matrix adhesion. Our results could resolve this important question, but it is a slow and labor-intensive process to acquire and quantify pHi heterogeneity maps, and additional strategies still need to be explored to improve our ability to correlate these maps to fixed-sample staining.

Bibliography & References Cited

1. Gillies, R. J., Verduzco, D. & Gatenby, R. A. Evolutionary dynamics of carcinogenesis and why targeted therapy does not work. *Nat Rev Cancer* **12**, 487–93 (2012).
2. Orlando, P. A., Gatenby, R. A. & Brown, J. S. Tumor evolution in space: the effects of competition colonization tradeoffs on tumor invasion dynamics. *Front Oncol* **3**, 45 (2013).
3. Reshkin, S. J. *et al.* Na+/H+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes. *FASEB J* **14**, 2185–97 (2000).
4. White, K. A., Grillo-Hill, B. K. & Barber, D. L. Cancer cell behaviors mediated by dysregulated pH dynamics at a glance. *J Cell Sci* **130**, 663–669 (2017).
5. Boyden, E. S., Zhang, F., Bamberg, E., Nagel, G. & Deisseroth, K. Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* **8**, 1263–8 (2005).
6. Chow, B. Y. *et al.* High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* **463**, 98–102 (2010).
7. El-Gaby, M. *et al.* Archaerhodopsin Selectively and Reversibly Silences Synaptic Transmission through Altered pH. *Cell Reports* **16**, 2259–2268 (2016).
8. Deamer, D. Membranes and the Origin of Life: A Century of Conjecture. *Journal of Molecular Evolution* **83**, 159–168 (2016).
9. Tikhonov, A. N. & Vershubskii, A. V. Computer modeling of electron and proton transport in chloroplasts. *Biosystems* **121**, 1–21 (2014).
10. Michard, E., Alves, F. & Feijo, J. A. The role of ion fluxes in polarized cell growth and morphogenesis: the pollen tube as an experimental paradigm. *The International Journal of Developmental Biology* **53**, 1609–1622 (2009).
11. Gov, N. S. & Gopinathan, A. Dynamics of membranes driven by actin polymerization. *Biophys. J.* **90**, 454–469 (2006).
12. Shi, Y. & Massagué, J. Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. *Cell* **113**, 685–700 (2003).
13. Duan, D. & Derynck, R. Transforming growth factor–β (TGF-β)–induced up-regulation of TGF-β receptors at the cell surface amplifies the TGF-β response. *Journal of Biological Chemistry* **294**, 8490–8504 (2019).
14. Lin, Y. *et al.* NHE1 mediates migration and invasion of HeLa cells via regulating the expression and localization of MT1-MMP. *Cell Biochem Funct* **30**, 41–6 (2012).
15. Lauritzen, G. *et al.* The Na+/H+ exchanger NHE1, but not the Na+, HCO3(-) cotransporter NBCn1, regulates motility of MCF7 breast cancer cells expressing constitutively active ErbB2. *Cancer Lett* **317**, 172–83 (2012).
16. Daniel, C. *et al.* The role of proton dynamics in the development and maintenance of multidrug resistance in cancer. *Biochim Biophys Acta* **1832**, 606–17 (2013).
17. Kopecka, J. *et al.* Carbonic anhydrase XII is a new therapeutic target to overcome chemoresistance in cancer cells. *Oncotarget* **6**, 6776–93 (2015).
18. Lauritzen, G. *et al.* NBCn1 and NHE1 expression and activity in DeltaNErbB2 receptor-expressing MCF-7 breast cancer cells: contributions to pHi regulation and chemotherapy resistance. *Exp Cell Res* **316**, 2538–53 (2010).
19. Dagogo-Jack, I. & Shaw, A. T. Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews Clinical Oncology* **15**, 81–94 (2018).
20. Dovmark, T. H., Saccomano, M., Hulikova, A., Alves, F. & Swietach, P. Connexin-43 channels are a pathway for discharging lactate from glycolytic pancreatic ductal adenocarcinoma cells. *Oncogene* **36**, 4538–4550 (2017).
21. Korenchan, D. E. *et al.* Dicarboxylic acids as pH sensors for hyperpolarized ^{13}C magnetic resonance spectroscopic imaging. *The Analyst* **142**, 1429–1433 (2017).

22. Korenchan, D. E. & Flavell, R. R. Spatiotemporal pH Heterogeneity as a Promoter of Cancer Progression and Therapeutic Resistance. *Cancers* **11**, 1026 (2019).
23. Webb, B. A. *et al.* A Histidine Cluster in the Cytoplasmic Domain of the Na-H Exchanger NHE1 Confers pH-sensitive Phospholipid Binding and Regulates Transporter Activity. *J Biol Chem* **291**, 24096–24104 (2016).
24. White, K. A. *et al.* β-Catenin is a pH sensor with decreased stability at higher intracellular pH. *The Journal of Cell Biology* **217**, 3965–3976 (2018).
25. Vercoulen, Y. *et al.* A histidine pH sensor regulates activation of the Ras-specific guanine nucleotide exchange factor RasGRP1. *Elife* **6**, e29002 (2017).
26. White, K. A. *et al.* Cancer-associated arginine-to-histidine mutations confer a gain in pH sensing to mutant proteins. *Science Signaling* **10**, eaam9931 (2017).
27. Muthuswamy, S. K. Organoid Models of Cancer Explode with Possibilities. *Cell Stem Cell* **22**, 290–291 (2018).
28. Khoo, B. L. *et al.* Single-cell profiling approaches to probing tumor heterogeneity. *Int J Cancer* **139**, 243–55 (2016).
29. Tan, H., Bao, J. & Zhou, X. Genome-wide mutational spectra analysis reveals significant cancer-specific heterogeneity. *Sci Rep* **5**, 12566 (2015).

PHS Human Subjects and Clinical Trials Information

OMB Number: 0925-0001 and 0925-0002

Expiration Date: 03/31/2020

Are Human Subjects Involved	❍ Yes	● No
Is the Project Exempt from Federal regulations?	❍ Yes	❍ No
Exemption Number	❑ 1 ❑ 2 ❑ 3 ❑ 4 ❑ 5 ❑ 6 ❑ 7 ❑ 8	
Does the proposed research involve human specimens and/or data	❍ Yes	● No

Other Requested information