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TITLE: Autophagosomal Sequestration of Mitochondria as an Indicator of Antiandrogen Therapy Resistance of Prostate Cancer (PCa)

PRINCIPAL INVESTIGATOR: George Wilding, M.D.

CONTRACTING ORGANIZATION: University of Texas MD Anderson Cancer Center Houston, TX 77030-4009

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Fort Detrick, Maryland 21702-5012

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Purpose: We are investigating if sequestration of metabolically dysfunctional mitochondria by the autophagosomes (mitophagy) imparts anti-androgen resistance and if this phenomenon can be applied in circulating tumor cells in patient blood samples as a biomarker for development of drug resistance.

Method: Effects of the anti-androgen enzalutamide on the autophagy and mitophagy of androgen-dependent LNCaP and –independent C4-2 and CWR22v1 cells are studied first. Autophagy is monitored by fluorescence of cells with anti-LC3B antibody. Cellular fluorescence due to Mitosox dye oxidation is used to identify mitochondria producing high superoxide (O2-). Mitophagy is monitored using fluorescence resonance energy transfer (FRET) by visualization of FRET images and quantitation of FRET image intensities using a Leica Sp8 fluorescence STED confocal microscope and Image J software.

Results and Discussion: Our data show that the degree of mitophagy is more in androgen-dependent LNCaP cells than in –independent C4-2 cells, when grown in androgen-depleted media. Enzalutamide treatment induces mitophagy in both cell lines. However, the increase in mitophagy is more pronounced in the enzalutamide-resistant C4-2 than in the –sensitive LNCaP cells. Mitophagy in circulating tumor cells (CTCs) isolated from patient blood samples are being quantitated to identify drug resistance.

15. SUBJECT TERMS
Mitophagy, prostate cancer, drug-resistance, fluorescence resonance energy transfer (FRET), Circulating Tumor Cells

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1. INTRODUCTION

Subject: Autophagy is activated in cells in response to stresses such as nutrient deficiency and/or oxidative stress for survival. Cancer cells with high metabolic activities require relatively higher levels of nutrients than do normal cells. They often use some of the autophagic signaling pathways such as mTOR or FoxO that are switched on by nutrient deficiency and oxidative stress for survival or self-destruction depending on the magnitude of the stress conditions. Androgens as well as anti-androgens such as bicalutamide, enzalutamide, etc. modulate oxidative stress in prostate cancer cells that affects mitochondrial functions. Our preliminary data suggest that autophagy of dysfunctional mitochondria (mitophagy) in response to anti-androgen therapy may play a critical role in Androgen Deprivation Therapy (ADT)-resistance in prostate cancer cells. We believe, this response can be observed in circulating tumor cells (CTCs) in patients undergoing ADT using a fluorescence resonance energy transfer (FRET) based high-resolution confocal fluorescence microscopy. With the development of methodology for isolating CTCs from patient blood samples, this microscopic method can now be standardized to observe this response, which may identify non-responding patients and may also predict the onset of therapy-resistance in patients at an early-stage. The therapy can then be quickly switched to maximize efficacy as well as reduce unnecessary pain and suffering.

Purpose: We have focused on establishing the FRET confocal microscopy to analyze the degree of mitophagy of the high superoxide producing dysfunctional mitochondria in prostate cancer cells and CTCs isolated from patient blood samples. This information, once firmly established by a positive correlation with patient therapy outcome, could be used as an early-indicator of ADT-resistance. A successful establishment of the methodology and initial data on predictability of patient outcome should provide the basis of two new avenues of future investigations – i) a prospective clinical trial for validation and application of this method to identify therapy-resistant patients (this should help initiate personalized trial for patients, thus identified, with autophagy inhibitors such as metformin or chloroquine) and ii) a basic scientific research on some of the underlying mechanisms of the development of ADT-resistance in patients. CTCs isolated from patients using our standardized method are metabolically active and detailed genomic, proteomic and metabolomic mapping of these cells are possible.

Scope: A few of these methods such as LC3B protein expression will be used in this proposal to confirm the autophagy and CTC identification, respectively. More detailed mechanistic studies such as initiation of autophagy due to ER-stress, Warbürg effect, mTOR dependency, etc. and the role of glucose metabolism modulators and autophagy inhibitors fall beyond the scope of this proposal.

2. KEYWORDS

1. ADT = Androgen-Deprivation Therapy
2. AR = Androgen Receptor
3. CK = Cytokeratin

4. CRPC = Castrate-resistant Prostate Cancer
5. CTC = Circulating Tumor Cells
6. ER = Endoplasmic Reticulum
7. FRET = Fluorescence Resonance Energy Transfer
8. ICC = Immunocytochemistry
9. LC3 = Light chain 3
10. MDC = Monodansyl Cadavarine
11. Mito-S = Mitosox Red dye
12. PBMC = Peripheral Blood Mononuclear Cells
13. ROS = Reactive Oxygen Species

3. ACCOMPLISHMENTS

What were the major goals of the project?

Specific Aim 1. Standardize confocal microscopy FRET analysis to quantitate mitophagy in androgen-dependent and castrate-resistant prostate cancer cells exposed to antiandrogens in the presence or absence of androgen.

Major Task 1. Standardize confocal microscopy FRET assay and compare degree of mitophagy in anti-androgen –sensitive and –resistant prostate cancer cells.

Subtask 1: Use 96-well plate based FRET assay to determine effect of anti-androgens on androgen-dependent LNCaP and androgen-independent C4-2 cells. (1-6 months)

Subtask 2: Use confocal microscopy FRET assay to confirm results of 96-well plate based assay. (3-9 months)

Subtask 3: Verify 96-well plated based and confocal microscopy FRET assay results using other androgen-dependent (LAPC-4) and androgen-independent (CWR22Rv1) prostate cancer cells. (6-15 months)

Specific Aim 2: Apply the standardized method to live circulating tumor cells (CTCs) for prostate cancer patients undergoing ADT to determine correlation of degree of mitophagy in CTCs with response to ADT/ development of resistance to ADT.

Major Task 2: Optimization of CTC isolation device protocols for fixation, permeabilization and staining of CTCs.

Subtask 1: Submit documents for HRPO approval. (1-2 months)

Subtask 2: Utilize CTCs from estimated 15 patients to test variations in fixation buffer/time, permeabilization buffer/time, and stain concentration/time. (4-9 months)

Major Task 3: Perform confocal microscopy FRET assay of mitophagy on CTCs from patients pre- and post- Enzalutamide therapy.

Subtask 1: Quantitate mitophagy in CTCs from 30 patients' pre-treatment and at the time of radiographic progression of the disease. (9-32 months)

Subtask 2: Analyze data for correlation with disease status. (33-36 months)

What was accomplished under these goals?

Standardization of a new high throughput assay for mitophagy. Effects of anti-androgen treatment on mitophagy of all mitochondria as well as only superoxide producing mitochondria have been assessed. For this, a new HTS assay has been designed during the course of this year. Prostate cancer cells are grown in DMEM with 1% FBS and 4% charcoal-stripped serum (F1C4) for at least 2 days to show strong androgen and/or anti-androgen response. Eighty microliters of cell suspension containing ~4,000 cells are then seeded in F1C4 in each well of a 96 well plate except blanks, which received only media. A day after seeding, cells are treated with 10 µL of graded concentrations of enzalutamide. After 96 h incubation at 37ºC in a 5% CO_2/air incubator, cells are treated with previously standardized concentrations of 10 µL of Mitosox (Mito-S) that only fluoresces after oxidation by superoxide and incubated for 4 hours for dye oxidation as standardized in Year 1. The cells are then stained for 30 minutes with Mitotracker green (Mito-G) dye with non-overlapping fluorescence with that of oxidized Mitosox to stain all functional mitochondria with normal membrane potential following a published procedure. The cellular fluorescence is assayed using a Clariostar 96-well plate reader. During the course of this year, conditions have been standardized that estimates the number of viable cells per well using a resazurin fluorescence (Alamar Blue) assay. All mitochondrial dye fluorescence assay data are normalized to the resazurin fluorescence. The effect of 96 h treatment with anti-androgen enzalutamide on cellular mitochondrial density and on the fraction (superoxide producing mitochondria): (total mitochondria in live cells) are shown in **Figure 1**.

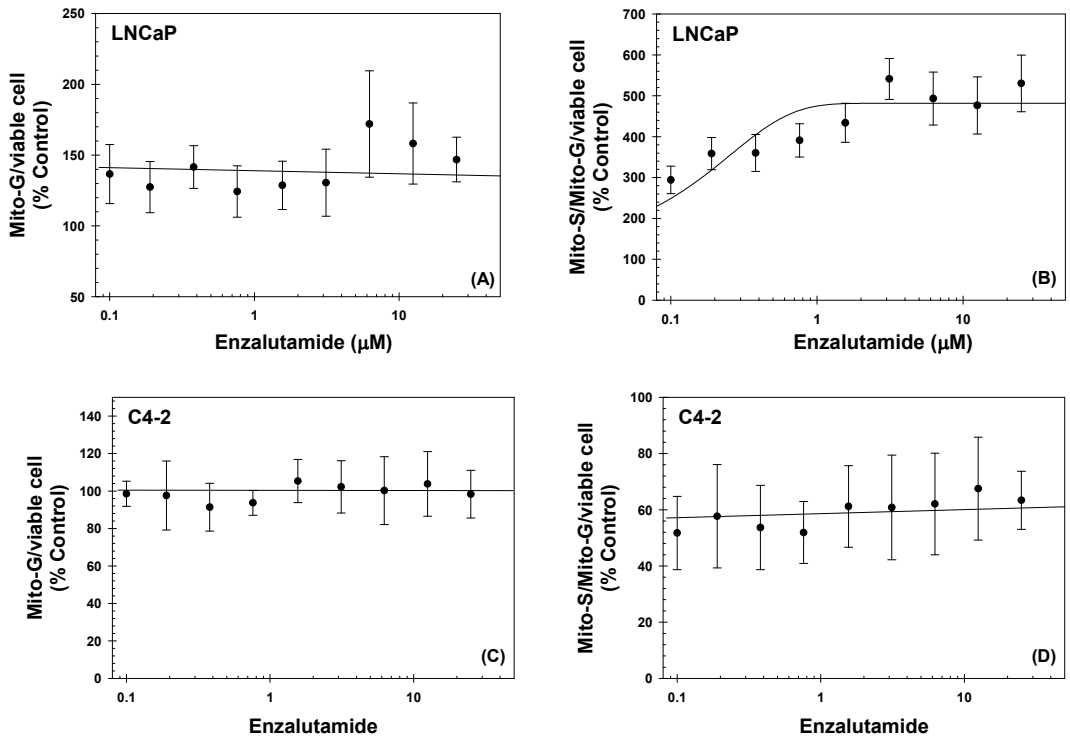

Figure 1. Effect of enzalutamide on total mitochondria (A:LNCaP; C:C4-2) and the ratio of Superoxide producing mitochondria: total mitochondria (B:LNCaP; D:C4-2). The data and the error bars are the means and standard deviations of readings from 12 wells per enzalutamide concentration (in µM) run in duplicates.

It is evident from the data that Enzalutamide treatment does not change the mitochondrial density in any of the cell lines. While the amount of superoxide producing mitochondria remains unchanged in C4-2 cells after an initial drop, that in LNCaP cells increases by nearly 5-fold with increasing concentrations of Enzalutamide. These data suggest that excess superoxide producing mitochondria are removed in Enzalutamide treatment probably by autophagy, it persists in LNCaP cells and may be cause mitophagy.

Standardization of fluorescence imaging for co-localization of the cells in a confocal microscope. During the course of this year, a high-resolution Leica STED-confocal microscope has been installed in our Department and a whole new image analysis software at a very high resolution (50 nm) has been standardized. At this resolution, individual mitochondria and autophagosomes can be visualized and quantified (see **Figures 2 and 3)**.

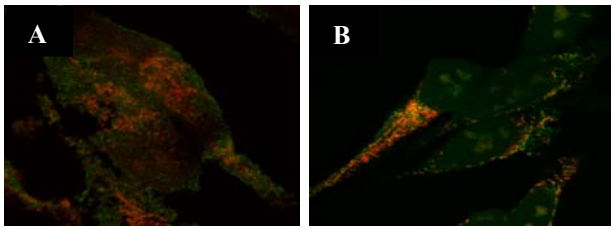

Figure 2. An optical section of STED confocal microscopic image at 50 nm resolution. LC-3B ICC autophagosomes (green) and Mitosox-red stained mitochondria (red) and co-localized (yellow). (**A**) C4-2 control; (**B**) C4-2 + 10 μM Enzalutamide.

For the high-resolution microscopy, LNCaP and C4-2 cells are seeded separately in 300 μL F1C4 in each chamber of 8-chamber slides. Anti-androgen-sensitive LNCaP cells have been treated with 1 μM (IC_{50}) and –resistance C4-2 cells have been treated with 10 μM enzalutamide for 96 h. Cells in different chambers are then treated with Mito-O alone or Mito-S alone and the slides are incubated for 4 h at 37°C in a CO_2/air incubator to allow for Mito-S oxidation. Cells are then fixed and stained with anti-LC3B antibody and a secondary Alexa488 conjugated antibody. The co-localization of the mitochondria with autophagosomes is observed at 1,600x magnification in 0.5 μm optical sections using a Leica SP8 STED confocal microscope. These images are shown in **Figure 2**. The data show considerable separation between red and green stained organelles in control C4-2 cells. Appearance of yellow zones shows considerable co-localization of the two organelles in enzalutamide resistant C4-2 cells growing in the presence of 10 μM enzalutamide. These data are distinct from what we presented in the Year 1 report, where only qualitative separation at a relatively lower resolution (~200 nm) of MDC and Mitosox dye was demonstrated. Switching from MDC to LC-3B protein staining defines a more specific characterization of the autophagosome.

Standardization of FRET imaging of the cells for co-localization in high-resolution microscopy. The FRET images using 488 nm excitation/595 nm emission for Alexa488 labeled LC-3B antibody and Mitosox red dye in the Leica Sp8 STED confocal microscope at 1,600x magnification and 0.5 μm optical sections are shown in **Figure 3**. The fluorescence intensities of the FRET signals from all optical sections have been quantified and integrated using Image J software with appropriate threshold to contour map each cell and are analyzed as shown in **Figure 4** (next page).

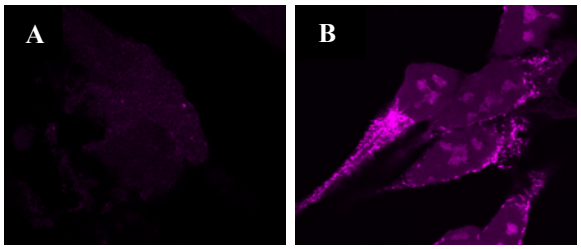

Figure 3. An optical section of confocal microscopic FRET signal between LC-3B and Mito-S stained C4-2 cells (pseudo-colored). (**A**) Control, (**B**) 10 μM Enzalutamide.

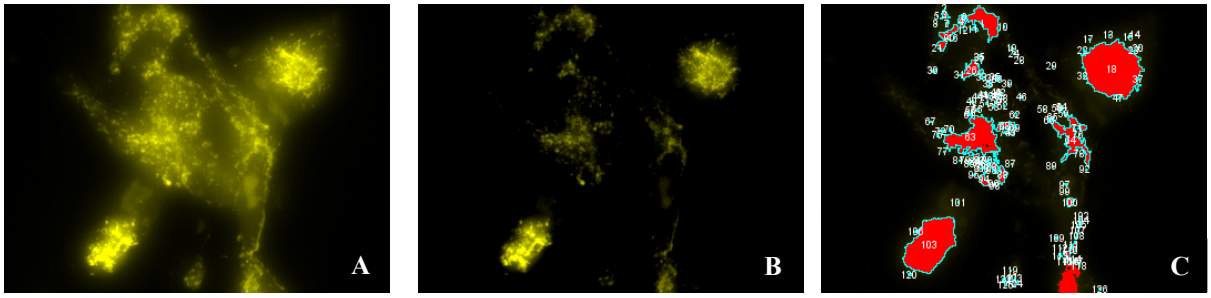

Figure 4. A representative FRET image of C4-2 cells treated with 10 mM Enzalutamide. (**A**) Raw FRET image, (**B**) Background corrected FRET image, and (**C**) Image J Segmentation and pixel intensity quantitation.

Similar FRET signal intensities from LC-3B and Mito-O dye combination are also obtained for normalization (data not shown). The mean FRET intensities calculated from enzalutamide-treated and untreated LNCaP, C4-2 and CWR-22v1 cells are shown in **Figure 5**. Where there is no appreciable change in FRET intensities for Mito-S and LC-3B and in LNCaP cells, in both enzalutamide-resistant cell lines, C4-2 and CWR22v1, an appreciable increase in FRET intensities has been observed in Enzalutamide treated cells as compared to that in the control vehicle treated cells.

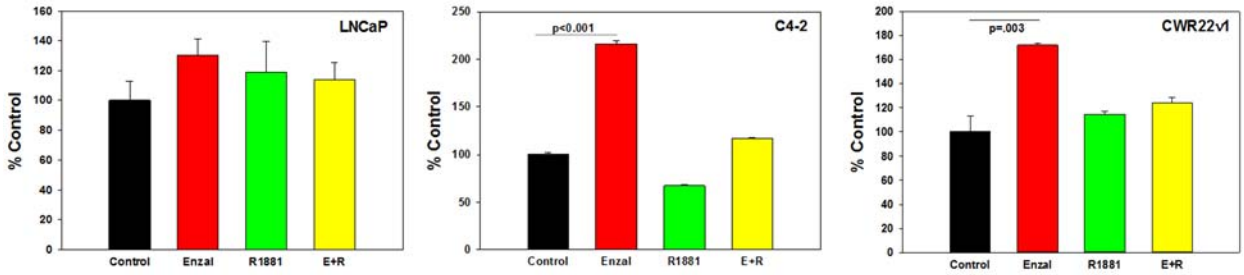

Figure 5. Mean of 2 repeat experiments determining the relative changes in mean pixel intensities of FRET image segments of 0.5 mm optical sections (10-12) in 10-15 cells/field. All data are normalized to FRET intensities from images of LC-3B and Mito-O with error bars representing standard deviations.

Standardizing a method for Circulating Tumor Cell (CTC) isolation from patient blood samples. During this year, methods have been standardized to isolate CTCs from patient blood using a size exclusion microfluidic device that has been standardized and employed routinely for CTC and epithelial cell isolation in our collaborator Dr. Reuben's laboratory under an MD Anderson IRB protocol (PA015-0956). These cells are viable for at least 24 hours after sorting.

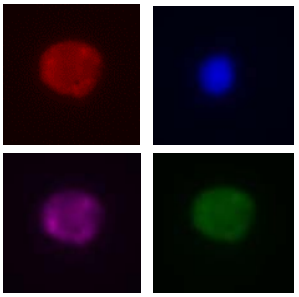

Figure 6. Prostate cancer patient CTC as identified by ICC staining with 3 antibodies and a nuclear stain–anti-EpCam (purple), anti-CK (red), DAPI (blue) and anti-AR (green) (1,000x).

Standardization of FRET imaging of the isolated CTCs in a high-resolution microscope. The cells are plated immediately after isolation in 3 chambers of a lysine coated 8 chamber slide in RPMI1640 with 10% FBS. The cells are allowed to attach overnight. The next day, the cells in 2 chambers are treated with Mito-S for 3 hours. All cells are then fixed with paraformaldehyde and the cells in the third chamber are subject to immunocytochemical staining (ICC) with anti-CK, anti-AR, anti-EpCam and DAPI to identify nucleated epithelial cells of prostatic origin, which

we have designated as prostate CTCs as proposed in our application. A representative CTC is shown in **Figure 6** (previous page) as identified by the ICC stains. The cells in the other 2 chambers are stained for anti-AR (Alexa 647) and anti-LC3 (Alexa 488) antibodies (images not shown). A representative FRET image (Ex 488/Em 595) between LC-3 (Ex 488/Em 535) and Mito-S (Ex 540/Em 595) is shown in **Figure 7** (previous page). The FRET signals from the CTCs isolated from six patients thus far are shown in **Figure 8**.

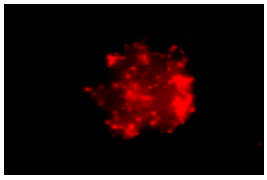

Figure 7. Representative FRET image (pseudo colored red) of a prostate cancer patient CTC stained with Mito-S and LC-3B antibody with Alexafluor-488 stain in a Leica SP8 STED confocal microscope (1,600x).

There is a clear difference in FRET intensities between the two groups of patients. These data are also collected and standardized in the new high-resolution microscope for more accurate analysis of the CTCs. These data for a total of 45 patients will be collected and statistically correlated with patient outcome to determine the threshold of intensity values that may predict the therapy outcome. At this point, the accrual rate is below the expected level due to a major change in prostate cancer therapy in the last 3 years. The IRB protocol has been amended to adjust for the patients undergoing the new therapy. This has slowed down the patient accrual. We expect to increase the rate of accrual during this year to meet the accrual goal.

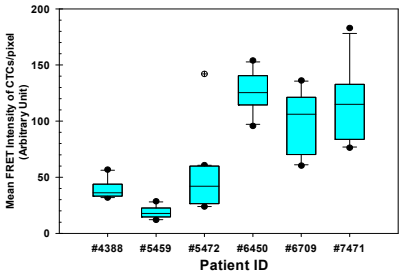

Figure 8. Mean FRET intensities of a minimum of 10 CTCs isolated from each of six prostate cancer patients stained with Mito-S and then with LC3B antibody *ex vivo* following the method described above.

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

Nothing to report

How were the results disseminated to communities of interest?

A manuscript describing the method and data collected using prostate cancer cell lines is currently being written up for publication.

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

The FRET data will be collected longitudinally from advanced prostate cancer patients undergoing enzalutamide treatment (a total of 45 patients in the course of next two years) and will be correlated to their disease and therapy outcome during the course of this grant proposal. We anticipate to determine a threshold FRET signal intensity, above which the patient do not respond to enzalutamide therapy, which is the ultimate goal of this proposal.

4. IMPACT

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

The FRET method for detecting enzalutamide resistance in prostate cancer CTCs will be translated as one of the clinical biomarkers for development of enzalutamide therapy resistance as proposed.

During the course of this year, the FRET technology has also been extended to live primary prostate cancer cells isolated from patient prostatectomy tissues under a separate NIH grant proposal (R01 CA185251). One of the PIs of that proposal Hirak Basu, who was a key personnel in this application will be integrating this method with the prostatectomy method to elucidate a role of mitochondria in cell invasion and metastasis and thus, establishing a role of mitophagy in prostate cancer progression and drug resistance.

What was the impact on other disciplines?

We believe these studies will open up a new avenue of research in the field of mitochondrial function (or dysfunction) in cancer progression, in general and may lead to application of this technology in the prognosis and prediction of drug resistance in other types of cancers in addition to prostate cancer. In addition, accurate measurement of mitochondrial function and mitophagy in CTCs could be broadly adopted as a pharmacodynamic (PD) marker for the new mitochondria-targeted therapies that are introduced in cancer treatment.

What was the impact on technology transfer?

Mitochondrial function in CTCs is currently being standardized as a PD marker for patients currently undergoing Phase I clinical trial for an oxidative phosphorylation inhibitor IACS-010759.

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

Changes in approach and reasons for change

No significant change of technology and/or goal has thus far been necessary.

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

This award experienced an initial delay when the PI Dr. Wilding and a Key Personnel Dr. Basu transferred from the University of Wisconsin to The University of Texas MD Anderson Cancer Center on 09/01/2015 and 03/16/2016, respectively. The award was transferred to MD Anderson and a revised agreement was provided to extend the effective period to 10/31/2019.

We have also encountered some delay in patient accrual due to change in prostate cancer therapy in the last three years. Methodology has been properly adjusted to account for adopting to the change. These factors have reduced the number of patients anticipated to be accrued for this study. We believe we will increase the speed of accrual during 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

None

Significant changes in use or care of vertebrate animals.

Not applicable. No vertebrate animal work in this project.

Significant changes in use of biohazards and/or select agents

Not applicable. No use of biohazard or select agent in this study.

6. PRODUCTS

Journal publications

Nothing to report

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

Nothing to report

Other publications, conference papers, and presentations

Poster summarizing the data reported above has been presented in AACR Prostate Cancer symposium in Orlando, Fl.

Website(s) or other Internet site(s)

Nothing to report

Technologies or techniques

FRET assay using high-resolution confocal fluorescence microscopy for prostate cancer cell and patient CTC has been standardized. The technology has been described in detail in the AACR poster and will be published in a peer-reviewed journal when all the data are collected.

Inventions, patent applications, and/or licenses

Nothing to report

Other Products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

The following individuals worked on this project during the current reporting period.

The University of Texas MD Anderson Cancer Center

Name:	**George Wilding**
Project Role:	Principal Investigator
Person months worked:	1.20 calendar months
Contribution to Project:	Dr. Wilding directs the research and supervise personnel in his laboratory. He is primarily responsible for data analysis and interpretation, troubleshooting, writing and editing all reports and publications, and overall completion of the project.
Funding Support	Dr. Wilding was supported by this DOD award

Name:	**Hirak Basu**
Project Role:	Collaborator
Person months worked:	0.60 calendar months
Contribution to Project:	Dr. Basu's expertise in autophagy and metabolism is considered invaluable for the success of this project. He assisted the PI in troubleshooting and report writing.
Funding Support:	Dr. Basu was supported by this DOD award

Name:	**James Reuben**
Project Role:	Collaborator
Research Identifier:	0000-0001-8972-2103
Person months worked:	0.12 calendar months
Contribution to Project:	Dr. Reuben is collaborating with Dr. Zurita for the last several years in isolating and detecting CTCs in prostate cancer patients under an IRB approved protocol. His laboratory has a standardized protocol for prostate cancer CTC identification and isolation that has been adopted for the studies proposed in this project. He is assisting Dr. Wilding with CTC isolation and identification and with troubleshooting and writing reports and publications.
Funding Support:	Dr. Reuben was supported by this DOD award

Name:	**Amado Zurita**
Project Role:	Collaborator

Person months worked:	0.12 calendar months
Contribution to Project:	Our collaboration with Dr. Zurita began on 09/01/2017. Dr. Zurita and his clinical team are involved in patient identification, consenting and blood sample collection for CTC isolation. His clinical team will de-identify the samples and store the link for protected patient information to be used for outcome correlation at the end of the project in compliance with the IRB approved protocol.
Funding Support	Dr. Zurita was supported by this DOD award

Name:	**Grace T. Wu**
Project Role:	Lab Manager
Person months worked:	6 calendar months. Ms. Wu will dedicate 0.60 calendar months effective 11/1/2018.
Contribution to Project:	Ms. Wu is directly responsible for maintenance of cell lines and assists in cell culture studies in Aim 1 and microscopy in Aim 2. Additionally, she is primarily responsible for maintenance of laboratory supplies and solutions for this project and assisting the PI in ensuring compliance, coordinating data collection, data analysis, and data and financial management for the entire project.
Funding Support:	Ms. Wu was supported by this DOD award

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

No

What other organizations were involved as partners?

Not applicable

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS

Not applicable

QUAD CHARTS

Not applicable

9. APPENDICES

Not applicable