

September 18, 2018

*Journal of Visualized Experiments*

*1 Alewife Center, Suite 200*

*Cambridge, MA 02140*

To Whom It May Concern:

Enclosed please find our point-by-point response (in red ink) to the Reviewers’ comments to our manuscript entitled “**Use of an *in vitro* scratch assay to demonstrate the effects of arsenic on skin cell migration**”. We have responded to the reviewers’ requests and we believe these changes have improved the manuscript. Please find the changes highlighted as “track changes” in the newest uploaded document.

This enclosed manuscript is truthful and original work and represents a useful in-vitro assay that will be of interest to your readership. The submitting author has the written consent from all authors to submit the manuscript and all authors accept complete responsibility for the contents of the manuscript.

The manuscript has not been previously published, and is not currently under consideration elsewhere. The work reported will not be submitted for publication elsewhere until a final decision has been made as to its acceptability by ***JoVE***.

Please address all future correspondence regarding this manuscript to myself. Thank you for your help and consideration.

Yours truly,



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**Editorial Comments:**  
  
• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.   
  
• **Protocol Language:**

1) Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Examples NOT in the imperative voice: 7.1, 7.1.2, etc.

-We have made the required changes.

2) Lines 315- 331 should be made into a table for clarity and conciseness of the protocol.

-We have included Table 1 that details these calculations.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.**There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

-We have made numerous changes to the protocol.  
  
• **Protocol Numbering:** Please adjust the numbering of your protocol section to follow JoVE’s instructions for authors, 1. should be followed by 1.1. and then 1.1.1. Some numbering errors are present. –Corrections have been made.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.  
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.  
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.  
4) Notes cannot be filmed and should be excluded from highlighting.  
5) Please bear in mind that software steps without a graphical user interface/calculations (e.g 3.3.5-3.3.9) cannot be filmed.

-We have updated the highlighted sections.

• **Results:** Please mention the statistical tests performed and report sample sizes.

-We have made the requested changes. See “representative results” section.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

-We have edited the discussion to include the relevant information mentioned above.   
  
• **Figures:**  
1) Fig 2: Please use a different panel labeling scheme. We suggest A-L.

-We have made the suggested change.  
  
• **Figure/Table Legends:**  
1) Fig 3: Define error bars and A,B.

-We have made the required changes.  
  
• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are cryovial, TrypLE Express, MatLab  
1) Please use MS Word’s find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names.

-We have made the required changes.  
  
• **Table of Materials:** Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as cell lines, software, microscope, etc.

-We have updated the Table of Materials.  
  
• Please define all abbreviations at first use.  
  
• Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

-We have made the required changes.  
  
• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

**Reviewer #1:**  
  
Manuscript Summary:  
Here the authors present a modification to a very well established protocol to investigate the impact of arsenic contamination on skin cell migration.  
  
Major Concerns:  
1. The steps in the procedure are clearly written and would result in the desired outcome. The critical steps are highlighted and no important information is missing. Anticipated results are reasonable and useful to the reader if more context is provided. However, due to the simple nature of this protocol, the incremental modification and the amount of papers that have been published previously describing this protocol, it is recommended that this publication not be accepted for publication in the current form. Some modifications that could improve the manuscript include:  
  
1. Discussing the broad application of this method/protocol for the study of many types of environmental contaminates, not just arsenic.

-We have included highlights and references from our previous work with the scratch assay and the influence uranium (environmental contaminant) and platelet-rich plasma (therapeutic) independently have on *in vitro* cellular migration. This ref demonstrates that the scratch assay can be used to study compounds that both speed up- and slow down, cellular migration. This proves its value for broad applications.

2. Providing more background on how people are exposed to these contaminates and in what concentrations to make the assay relevant in the context of healthcare.

-Agreed. We have included an additional paragraph in the “introduction’ section that specifically addresses arsenic exposure routes and justification for our selected arsenic concentrations, which come from the literature.

3. Including the procedure for creating and using the automated algorithm to calculate the decrease in open wound area would also advance this protocol beyond what is currently available in the literature.

-We have chosen to remove the use of the automated algorithm because we plan to submit the algorithm work as a separate and unique publication. Additionally, we have updated the “protocol” section, which now describes manual image analysis that utilizes NIH image-j software.

4. Summed area under the curve is used as an output but is not described in detail. Please add more information on how this value is calculated.

-We have addressed this comment and describe, in detail, the mathematical equation for calculating area under the curve using percent wound closure values.  
  
Minor Concerns:  
There are a few spelling errors, please review for grammar.

-All errors have been corrected to the best of our knowledge.

**Reviewer #2:**  
  
Manuscript Summary:

The manuscript by Pinto et al. describes a protocol to evaluate cell migration of human dermal fibroblasts in the presence of arsenic using the in vitro scratch assay. The in vitro scratch assay has been extensively used in particular for screening of compounds which potentially modulate the process of cell migration. Accordingly, several protocols have been published during the last years. Although the paper is well written, the scope of the manuscript is somewhat limited focussing on human dermal fibroblasts and arsenic only. Importantly, there are major issues with the methodology that need to be addressed before this work is publishable.  
  
Major Concerns:  
1. The authors state that confluent cells will typically become growth arrested. However, after scratching the monolayer cells may proliferate again. Thus, it is mandatory to use proper controls (such as mitomycin C) to discard proliferation instead of migration. In addition, migration assays may be performed under serum-free conditions.

-Thank you, that is a good point and something to consider when conducting cellular migration assays. However, upon scratching, we allow cellular migration to take place for 24 hours, at which point we terminate the experiment. Experimentation in our lab has determined that human dermal fibroblasts have an approximate doubling time of 24-28 hours at 7,000 feet elevation where our lab in Flagstaff, AZ is located. This would not give the majority of cells enough time to fully double (proliferate) and significantly change the closure rates that we observe within the 24 hour migration period. With this knowledge, we believe that the majority of the “wound closure” observed is from cellular migration alone, with little effect from proliferation.

2. Since dermal fibroblasts are extensively embedded in ECM, the cells may behave quite differently in an artificial monolayer setting. To mimic the situation in vivo more closely, a protocol (incl. results) should be presented where cells are also seeded on plates coated with proper ECM substrates.

-Yes, we agree that the 3-dimensional *in vivo* environment where dermal fibroblasts reside is much different than that of an *in vitro* monolayer culture. We understand that there are many changes/additions that can be made to this well described assay, which may include coating the growth surface with proteins such as collagen, fibronectin, elastin and hyaluronic acid. However, coating may not be applicable to all scratch assays intended to assess migration. This methods paper is intended to provide a detailed technique to investigators such that it can be readily manipulated and tailored to fit a broad range of projects (i.e. coating). Another piece to consider is that much of any coating added to the wells would likely be removed during the time of scratching, leaving little to no substrate at the scratch site for the cells to adhere to and migrate on; thus not having a significant effect on migration rates. Lastly, we believe that the addition of an ECM coating would be outside the scope of this specific work, and would be a great question to address in future work.  
  
Minor Concerns:  
1. Appropriate positive controls (besides arsenic) which inhibit cell migration should be added in the protocol and results.

-The purpose of this paper was to describe the scratch assay as a model, therefore describing this method we’ve chosen to share representative data sets. We agree with the reviewer that during experimental design, positive and negative controls should be selected based on the study design the investigator is pursuing. We have added a statement to this effect in the body of the manuscript “It is recommended that appropriate positive and/or negative controls be enrolled within the experiment to completely assess the effect of treatment on the cell lines.”

2. Although the manuscript focusses on arsenic, the authors should add details for the protocol when evaluating other environmental contaminants for the modulation of cell migration.

-We have included highlights and refs from our previous work with the scratch assay and the influence Uranium (environmental contaminant) and platelet-rich plasma (therapeutic) independently have on *in vitro* cellular migration. This ref includes a detailed protocol for both compounds, and gives insight on how this simple assay can be readily manipulated for various projects.

3. The authors may want to include discussion on the required modifications of the protocol when using other cell types (e.g. keratinocytes, endothelial cells).

-We have included references in the introduction that describe, in detail, migration assays using the cell lines mentioned in comment #3. Additionally, we describe important details to consider when designing this assay in paragraph 3 of the introduction.

4. What is known about the mechanism of action of arsenic regarding the inhibition of fibroblast cell migration. This should be added in the manuscript.

-Arsenic was used as “representative data” to primarily provide information on how this assay can help investigators understand cellular migration as it pertains to specific questions/hypotheses, such as environmental contaminants in this manuscript. In this journal, we were encouraged to limit discussion regarding arsenic and its toxic effects and focus on the methods. We have added references in the discussion that discuss mechanism of action regarding the inhibition of fibroblast cell migration. Additionally, please see below the instructions for authors regarding the “Discussion” section. Lastly, we have NIH funding in place to explore the mechanism behind arsenic disruption of wound healing (Southwest Health Equity Research Collaborative - NIH RCMI, 1 U54 MD012388-01). Our specific aims involve; 1) elucidating how arsenic impacts dermal fibroblasts *in vitro*; 2) elucidating how estrogen may reverse the negative effects of arsenic *in vitro*; and 3) translating the findings from aims 1 and 2 into a full thickness *in vivo* wound healing model (murine).

**“…DISCUSSION:** *(3-6 paragraphs)*

JoVE is a methods-based journal. Thus, the Discussion of the article should be focused on the protocol and not the representative results.

This section should discuss the following with citations:

* Critical steps in the protocol.
* Modifications and troubleshooting of the method.
* Limitations of the method.
* The significance of the method with respect to existing/alternative methods.
* Future applications or directions of the method.”