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# Impedance-Based Real-Time Measurement of Cancer Cell Migration and Invasion

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| **Corresponding Author:** | Giridhar Mudduluru<br>Children's Mercy Hospitals and Clinics<br>Kansas City, MO UNITED STATES |
| **Corresponding Author's Institution:** | Children's Mercy Hospitals and Clinics |
| **Corresponding Author E-Mail:** | mudduluru@gmail.com;gmudduluru@cmh.edu |
| **Order of Authors:** | Giridhar Mudduluru |
| | Neka Large |
| | Taeju Park |
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Cover Letter

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2401 Gillham Road
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(816) 234-3000

To

11/07/2019

The Editor,
JoVE Methods Collections.

**Sub:** Submission of methods manuscript with the title "**Impedance-Based Real-Time Measurement of Cancer Cell Migration and Invasion**"

Dear Editor,

We would like to submit the methods manuscript for **"Methods to study cancer cell phenotypes and drug efficacy *in vitro* and *in vivo*"** edition with the title "Impedance-Based Real-Time Measurement of Cancer Cell Migration and Invasion". We summarized here the advantages of real-time measurement of cell migration and invasion over traditional methods. We hope this method manuscripts suite here.

We sincerely hope that you will take our findings into consideration for publication in the **JoVE methods collection**.

Yours sincerely,

Giridhar Mudduluru

PD Dr. Giridhar Mudduluru, PhD
Research Scientist
Department of Pediatrics
Children's Research Institute
P: (816) 302-8571 | M: (609) 334-1118
E: gmudduluru@cmh.edu | W: childrensmercy.org

**TITLE:**

# Impedance-Based Real-Time Measurement of Cancer Cell Migration and Invasion

**AUTHORS AND AFFILIATIONS:**

Giridhar Mudduluru[1], Neka Large[1], Taeju Park[1,2]

[1]Children's Research Institute, Children's Mercy Hospital, Kansas City, Missouri, USA
[2]Department of Pediatrics, University of Missouri Kansas City School of Medicine, Kansas City, Missouri, USA

**Corresponding Authors:**

Giridhar Mudduluru (gmudduluru@cmh.edu)
Taeju Park (tjpark@cmh.edu)

**Email Addresses of Co-authors:**

Neka Large (nlarge@cmh.edu)

**KEYWORDS:**

cancer, migration, invasion, impedance-based, real-time measurement, glioblastoma, Crk

**SUMMARY:**

Cancer is a lethal disease due to its ability to metastasize to different organs. Determining the ability of cancer cells to migrate and invade under various treatment conditions is crucial to assessing therapeutic strategies. This protocol presents a method to assess the real-time metastatic abilities of a glioblastoma cancer cell line.

**ABSTRACT:**

Cancer arises due to uncontrolled proliferation of cells initiated by genetic instability, mutations, and environmental and other stress factors. These acquired abnormalities in complex, multilayered molecular signaling networks induce aberrant cell proliferation and survival, extracellular matrix degradation, and metastasis to distant organs. Approximately 90% of cancer-related deaths are estimated to be caused by the direct or indirect effects of metastatic dissemination. Therefore, it is important to establish a highly reliable, comprehensive system to characterize cancer cell behaviors upon genetic and environmental manipulations. Such a system can give a clear understanding of the molecular regulation of cancer metastasis and the opportunity for successful development of stratified, precise therapeutic strategies. Hence, accurate determination of cancer cell behaviors such as migration and invasion with gain or loss of function of gene(s) allows assessment of the aggressive nature of cancer cells. The real-time measurement system based on cell impedance enables researchers to continually acquire data during a whole experiment and instantly compare and quantify the results under various experimental conditions. Unlike conventional methods, this method does not require fixation, staining, and sample processing to analyze cells that migrate or invade. This method paper emphasizes detailed procedures for real-time determination of migration and invasion of glioblastoma cancer cells.

## INTRODUCTION:

Cancer is a lethal disease due to its ability to metastasize to different organs. Determining cancer genotypes and phenotypes is critical to understanding and designing effective therapeutic strategies. Decades of cancer research have led to the development and adaptation of different methods to determine cancer genotypes and phenotypes. One of the latest technical developments is real-time measurement of cell migration and invasion based on cell impedance. Cell adhesion to substrates and cell-cell contacts play an important role in cell-to-cell communication and regulation, development, and maintenance of tissues. Abnormalities in cell adhesion lead to the loss of cell-cell contact, degradation of extracellular matrix (ECM), and gain of migratory and invading capabilities by cells, all of which contribute to metastasis of cancer cells to different organs[1,2]. Various methods are available to determine cell migration (wound healing and Boyden chamber assays) and invasion (Matrigel-Boyden chamber assay)[3-5]. These conventional methods are semiquantitative because cells need to be labeled with a fluorescent dye or other dyes either before or after the experiment to measure cell phenotypes. In addition, mechanical disruptions are needed in some cases for creating a wound for measuring the migration of cells to the wound site. Moreover, these existing methods are time-consuming, labor-intensive, and measure the results at only one time point. In addition, these methods are prone to making inaccurate measurements due to inconsistent handling during the experimental procedure[6].

Unlike conventional methods, the real-time cell analysis system measures cell impedance in real-time without requiring pre- or poststaining and mechanical damage of cells. More importantly, the duration of an experiment can be extended so that biological effects can be determined in a time-dependent manner. Executing the experiment is time-efficient and not labor-intensive. Analyzing data is relatively simple and accurate. Compared to other methods, this method is one of the best real-time measurements to measure cell migration and invasion[6-9].

Giaever and Keese were the first to describe the impedance-based measurement of a cell population on the surface of electrodes[10]. The real-time cell analysis system works on the same principle. The area of each microplate well is approximately 80% covered with an array of gold microelectrodes. When the electrode surface area is occupied by cells due to adherence or spreading of the cells, the electrical impedance changes. This impedance is displayed as the cell index, which is directly proportional to the cells covering the electrode surface area after they penetrate the microporous membrane (the median pore size of this membrane is 8 µm)[11].

Crk and CrkL are adaptor proteins containing SH2 and SH3 domains and play important roles in various cellular functions, such as cytoskeleton regulation, cell transformation, proliferation, adhesion, epithelial-mesenchymal transition, migration, invasion, and metastasis by mediating protein-protein interactions in many signaling pathways[1,12-18]. Therefore, it is important to determine the Crk/CrkL-dependent migratory and invasive capabilities of cancer cells. Real-time cell analysis was performed to determine the migratory and invasive abilities of glioblastoma cells upon gene knockdown of Crk and CrkL.

This method paper describes detailed measurements of Crk- and CrkL-mediated migration and invasion of human glioblastoma cells.

**PROTOCOL:**

NOTE: All cell culture materials need to be sterile and the entire experiment must be performed in a biosafety cabinet under sterile conditions.

**1. Culture and electroporation of the U-118MG glioblastoma cell line**

1.1. Culture the U-118MG cell line in 5% fetal bovine serum (FBS) containing Dulbecco's Modified Eagle Medium (DMEM) (culture medium) and Maintain at 37 °C in a humid atmosphere containing 5% $CO_2$ incubator (culture conditions).

1.2. Use 70– 80% confluent healthy cells for electroporation.

1.3. For harvesting cells, wash the cells growing in culture dishes with 1x PBS and add 2 mL of 0.05% trypsin-EDTA. Place in the incubator for 30 s and remove the trypsin-EDTA. Collect cells in the culture medium into a 15 mL tube.

1.4. Count the cells using a handheld automated cell counter, centrifuge cells at 100 x *g* for 5 min, and discard the supernatant.

1.5. Suspend the cell pellet in the culture medium and take 600,000 cells for each condition into a microcentrifuge tube. Adjust the cell number depending on experimental designs and growth rates.

1.6. Transfer the cell suspension to a microcentrifuge tube and add 800 µL of Dulbecco's phosphate-buffered saline (DPBS). Spin down using a minicentrifuge for 30 s and discard the supernatant.

1.7. Add 60 µL of resuspension buffer R to the cell pellet and add the siRNAs (i.e., non-targeting siRNA, Crk siRNA, CrkL siRNA, or both Crk and CrkL siRNAs) at a concentration of 6 µM to the respective microcentrifuge tube. Mix them gently by tapping.

1.8. Electroporate 10 µL of cells with an electroporation system at 1,350 V for 10 ms with three pulses and transfer the electroporated cells into 5 mL of the culture medium. Repeat the electroporation for the rest of the cells prepared for each condition.

1.9. Complete all respective electroporation. Transfer electroporated cells into two 35 mm dishes per condition and culture them under culture conditions for 3 days.

1.10. On the third day, treat all the electroporated cells in 0.5% FBS containing DMEM (low serum medium) for 6 h prior to the actual cell impedance measurement.

**2. Preparation of the real-time cell analysis system, cell invasion and migration (CIM) plates, and electroporated U-118MG cells for plating**

2.1. Place the real-time cell analysis system in a $CO_2$ incubator under culture conditions 5–6 h prior to the start of experiment to stabilize the system to the culture conditions.

2.2. For the invasion assay, plate 50 µL of DMEM (plain medium) containing extracellular matrix (ECM) gel at 0.1 µg/µL in each well of the upper chamber of the CIM plate. To avoid having any air bubbles, use the reverse pipetting method. Immediately remove 30 µL of ECM gel, leaving 20 µL in the well.

2.3. After the ECM gel coating, keep the plate in the incubator under culture conditions for 4 h with its lid on. During the ECM gel coating and drying, take preventive measures to avoid direct contact of the electrodes of the upper chamber of the plates with the hands, the surfaces of the biosafety cabinet, or the $CO_2$ incubator.

2.4. To set up the impedance measurement program, double-click the associated software icon (see **Table of Materials**) to open the system software application (control unit). Each cradle has an individual window with different tabs to set the experimental conditions, the impedance measuring time interval and duration, and data analysis.

2.5. Under the **Layout** tab, set quadruplicate wells for each biological condition and set two-step cell impedance measurements under the **Schedule** tab. The first step is for a one-time baseline measurement (one sweep with a 1 min interval), and the second step is to measure the cell impedance in respective individual cradles for an actual experiment. For migration, use 145 sweeps with a 10 min interval, and for invasion, 577 sweeps with a 10 min interval, individually set) in respective individual cradles.

2.6. One h prior to the start of the cell impedance measurement, add 160 µL of DMEM with 10% FBS as a chemoattractant in the wells of the lower chamber of the plate. Assemble either the upper chamber containing the ECM gel-coated wells or uncoated wells with the lower chamber to measure invasion and migration, respectively.

2.7. Fill the wells in the upper chamber with 50 µL of low serum medium and place them in the cradle of the system. Check whether all the wells are recognized by the control unit by clicking the **Message** tab. If the message displays as **OK**, the plate in the cradle is ready for the experiment.

2.8. Preincubate the completely packed plates in the incubator under culture conditions for 1 h prior to measurements in the real-time cell analysis system cradle, which is essential for acclimation of a packed plate to the cell culture conditions.

2.9. For harvesting cells treated with low serum medium, trypsinize the cells as in step 1.3, collect them in low serum medium, and count the cells as in step 1.4.

2.10. After counting, centrifuge cells at 100 x *g* for 5 min and discard the supernatant.

2.11. Resuspend 800,000 cells in 800 µL of low serum medium for the migration and invasion assays. Additionally, resuspend 300,000 cells in 2 mL of culture medium and seed in a 35 mm dish for Western blot analysis to confirm the regulated knockdown of Crk and CrkL.

**3. Baseline reading, seeding of the cells, and cell impedance measurement and visualization**

3.1. Measure the baseline reading by clicking the cradle **Start** button. The baseline should be read after preincubating the plates for 1 h in the cradle of the real-time cell analysis system under culture conditions in the $CO_2$ incubator and before seeding the cells to the respective wells in the upper chamber.

NOTE: Once the cradle **Start** button is clicked, the control unit will ask whether to save the experimental file. After the file is saved, the cradle analyzer measures the baseline cell impedance as set in the program initially and enters a pause mode until the cradle **Start** button is clicked again to measure cell impedance in the second step.

3.2. Take out both plates for migration and invasion from the cradles after the baseline measurement and keep them in the biosafety cabinet.

3.3. Seed 100 µL of cells (100,000 cells) in quadruplicates for each biological condition in the upper chamber of the CIM plate in the respective wells as programmed in the control unit of the cradle by reverse pipetting to avoid air bubbles.

3.4. After seeding, keep the plate under a biosafety cabinet for 30 min at room temperature to allow cells to evenly settle down to the bottom. Transfer the plate back to the respective cradle and click the cradle start button to start measuring cell impedance as programmed in the second step of 2.5.

3.5. After the last sweep, the experiment is finished, and the results are saved automatically.

3.6. Under the **Data Analysis** tab, visualize the changes in cell impedance as cell index in a time-dependent manner during or after completion of the experiment. Each of the respective conditions of quadruplicates can be visualized either individually or as averages and/or standard deviations by clicking the **Option** boxes for average and standard deviation.

3.7. To export cell index data to a spreadsheet file, open an empty spreadsheet file, place the cursor in the middle of the **Data Analysis** window, and right-click. In the dialog box that appears, choose the option **Copy Data into List Format**, and paste the data into the open spreadsheet.

3.8. Adjust the time in the raw data to represent the actual start time of the cell impedance measurement. The second step start time is set as zero.

NOTE: The control unit has the option to obtain the cell index with or without normalization (i.e., normalized cell index) and visualize the results as a graphical presentation in a time-dependent manner. In this example, cell index data are exported without normalization for processing and graphical presentation.

3.9. Release the experiment by clicking the **Release** button in each cradle.

**REPRESENTATIVE RESULTS:**

It has been suggested that Crk and CrkL are important for cell migration and invasion in different cancer cell lines[13,17]. Although Crk and CrkL proteins are structurally and functionally similar to each other and play essential overlapping functions[16,19-21], many gene knockdown studies for Crk and CrkL have not clearly addressed whether the knockdown is specific to either Crk, CrkL, or both. Therefore, it is unclear which of the two proteins contributes to cell migration and invasion. As a proof-of-principle study, we used siRNAs specific to Crk or CrkL and studied their effects on migration and invasion of the U-118MG GBM cell line. The knockdown of Crk decreased CrkII and CrkI protein levels by 85% and 86%, respectively, without reducing the CrkL protein level. The knockdown of CrkL reduced the CrkL protein level by 85% (**Figure 1**). CrkL knockdown slightly reduced the CrkII and CrkI levels, too. Combined use of siRNAs for Crk and CrkL reduced CrkII, CrkI, and CrkL levels by more than 80% (**Figure 1B**). On the other hand, knockdown of Crk and CrkL did not affect the vinculin and $\alpha$-tubulin levels (**Figure 1**).

The U-118MG cells migrated to high serum (10% FBS), reaching the maximal level of migration at 13 h, which served as the experiment internal control (**Figure 2A**). With Crk knockdown, cell migration was delayed, and the cells continued to migrate until 23 h. CrkL knockdown substantially inhibited cell migration. U-118MG cells lost their migratory ability upon knockdown of both Crk and CrkL (**Figure 2A**), suggesting that Crk and CrkL play essential overlapping roles in cancer cell migration. However, this conclusion is not clearly evident if cell migration is examined at a fixed time point. When cell migrations at 6 or 13 h were compared, inhibitions by Crk and CrkL knockdowns were obvious (**Figure 2B**,**C**). In contrast, Crk knockdown did not have an inhibitory effect on cell migration at 18 h (**Figure 2D**), leading to conflicting results depending on the time point selected for comparison. The inhibitory effects of CrkL knockdown and Crk/CrkL double knockdown were clearly visible at all three time points. These results clearly demonstrate that cell migration must be assessed over the entire period of cell migration to accurately analyze effects by genetic manipulations or drugs.

The U-118MG cells invaded high serum, reaching the maximum level of invasion at 52 h, which served as the experiment internal control (**Figure 3A**). With Crk knockdown, cell invasion was delayed, but it reached a similar maximum level at 60 h. With CrkL knockdown, U-118MG cells showed delayed and reduced invasion compared with the control cells. Combined knockdown of Crk and CrkL further inhibited cell invasion (**Figure 3A**). Comparison of cell invasion at 36 h, when

the control cells were actively undergoing invasion, clearly demonstrated inhibition by individual knockdown of Crk and CrkL and a synergistic inhibition by Crk/CrkL double knockdown (**Figure 3B**). However, a comparison of cell invasion at 52 or 60 h exhibited a slight or no inhibitory effect by Crk knockdown (**Figure 3C**,**D**). These results clearly support the suggestion that cell invasion should be analyzed over the entire period of the experiment.

These results demonstrate that both Crk and CrkL mediate cell migration and invasion, and that the real-time cell analysis system has a clear advantage over the traditional methods in understanding the different kinetics of cell migration and invasion and the specific effects on cell phenotypes in a time-dependent manner.

**FIGURE LEGENDS:**

**Figure 1: siRNA-mediated knockdown of CrkI, CrkII, and CrkL in U-118MG cells.** (**A**) Total cell lysates were prepared 4 days after U-118MG cells were electroporated with non-targeting control siRNA (NT), Crk siRNA, CrkL siRNA, or both Crk and CrkL siRNAs, and protein levels were determined by Western blot analyses as described previously[1]. (**B**) The signal intensities of respective bands were quantified using the imaging system and calculated as percentages of NT. Their mean ± SD values are shown in the graph. Vinculin and $\alpha$-tubulin served as internal controls. Statistical analyses of data were performed using unpaired two-tailed Student's t-test for comparison between the two experimental groups. *$p < 0.05$ and **$p < 0.01$, compared to NT.

**Figure 2: Effects of Crk/CrkL knockdown on U-118MG cell migration:** (**A**) Three days after U-118MG cells were electroporated with non-targeting control siRNA (NT), Crk siRNA, CrkL siRNA, or both Crk and CrkL siRNAs, cells were harvested and cell migration was examined using the real-time analysis system. Migration of U-118MG cells was inhibited with a single knockdown of Crk or CrkL in a time-dependent manner. The knockdown of both Crk and CrkL completely blocked cell migration. Cell index values at 6 (**B**), 13 (**C**), and 18 h (**D**) are presented to compare cell migration at different time points (arrows). At 13 h the control cells (NT) reached the maximal migration. At 18 h both control and Crk knockdown cells showed similar levels of cell migration. Statistical analyses of data were performed using unpaired two-tailed Student's t-test for comparison between the two experimental groups. **$p < 0.01$, compared to NT.

**Figure 3: Effects of Crk/CrkL knockdown on U-118MG cell invasion:** (**A**) Three days after U-118MG cells were electroporated with non-targeting control siRNA (NT), Crk siRNA, CrkL siRNA, or both Crk and CrkL siRNAs, cells were harvested and cell invasion was examined for 4 days using the real-time analysis system. The invasion of U-118MG cells was inhibited with a single knockdown of Crk or CrkL in a time-dependent manner. The knockdown of both Crk and CrkL in the U-118MG cell line reduced its invasive capacity up to 48 h compared to NT. Cell index values at 36 (**B**), 52 (**C**), and 60 h (**D**) are presented to compare cell invasion at different time points (arrows). At 52 h, the control cells (NT) reached the initial peak of invasion. At 60 h, Crk knockdown cells reached the initial peak of invasion. Statistical analyses of data were performed using unpaired two-tailed Student's t-test for comparison between the two experimental groups. *$p < 0.05$ and **$p < 0.01$, compared to NT.

## DISCUSSION:

The real-time measurement of cell migration and invasion using the real-time cell analysis system is a simple, quick, and continuous monitoring process with multiple, significant advantages over the traditional methods that provide data at a single time point. As with the traditional methods, experimental conditions must be optimized for each cell line for the real-time cell analysis system, because each cell line may be different in terms of its adhesion to the substrate, growth, cell-to-cell contacts, and migratory and invasive abilities. Due to these differences, each cell line may show different cellular kinetics and cell impedances. Impedance is greatly influenced by the number of cells seeded in a well, the time for cell adhesion, the lag time before cells start to migrate or invade, and the concentration of ECM gel on CIM plates. First, real-time cell analysis makes the optimization easier because it provides results in real time over a specific time period, enabling researchers to identify the time point when the control cells show active cell migration and invasion and when the control cells reach the maximal levels of migration and invasion. Second, ectopic gene overexpression or gene knockdown studies may need additional optimizations, because the cells need to adopt the phenotypes from the modified genotypic changes. In addition, effective drug concentrations and the efficacy of drugs can be determined in combination with normal or modified genetic conditions using the real-time cell analysis system.

Traditional methods such as wound healing, soft agar, Boyden chamber migration, or invasion assays have been used to determine that knockdown of either Crk or CrkL leads to reduced migration and invasion in different cancer cell lines[13,17]. In this study, we induced single or double knockdown of Crk and CrkL in the U-118MG cell line and investigated cell migration and invasion. Real-time measurement of cell impedances over the entire experiment provided in-depth information about the kinetics of cell migration and invasion, allowing us to identify two different modes of inhibition. Whereas Crk knockdown delayed migration and invasion, CrkL knockdown inhibited migration and invasion over the entire time period. Furthermore, the double knockdown of both Crk and CrkL completely blocked cell migration and substantially inhibited cell invasion.

This study provides a proof-of-concept that combining the systematic knockdown approach to induce single and double knockdown of Crk and CrkL with real-time analyses of cell migration and invasion over the entire period of the experiments is necessary for comprehensive analyses of Crk- and CrkL-mediated functions in cancer cells. The data presented in this study suggest that this method can also be used to test candidate drugs for their inhibitory effects on Crk and CrkL. Overall, the real-time cell analysis system is useful in setting up experiments for cell migration or cell invasion and makes real-time, in-depth, and comprehensive analyses possible.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



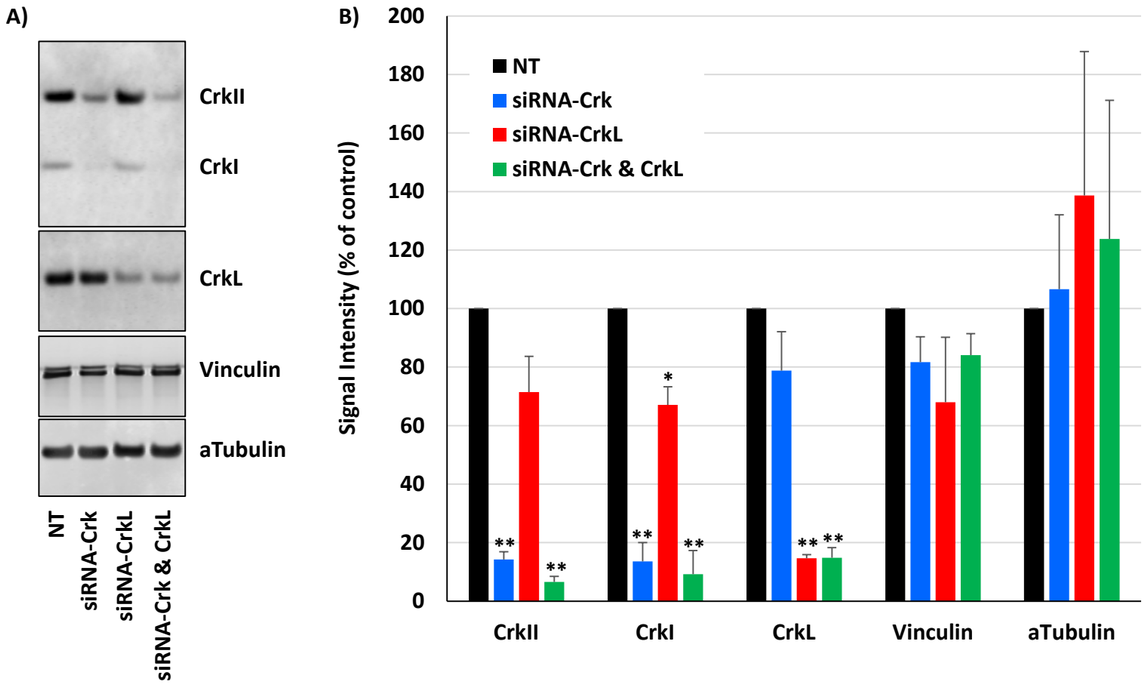


Figure 2



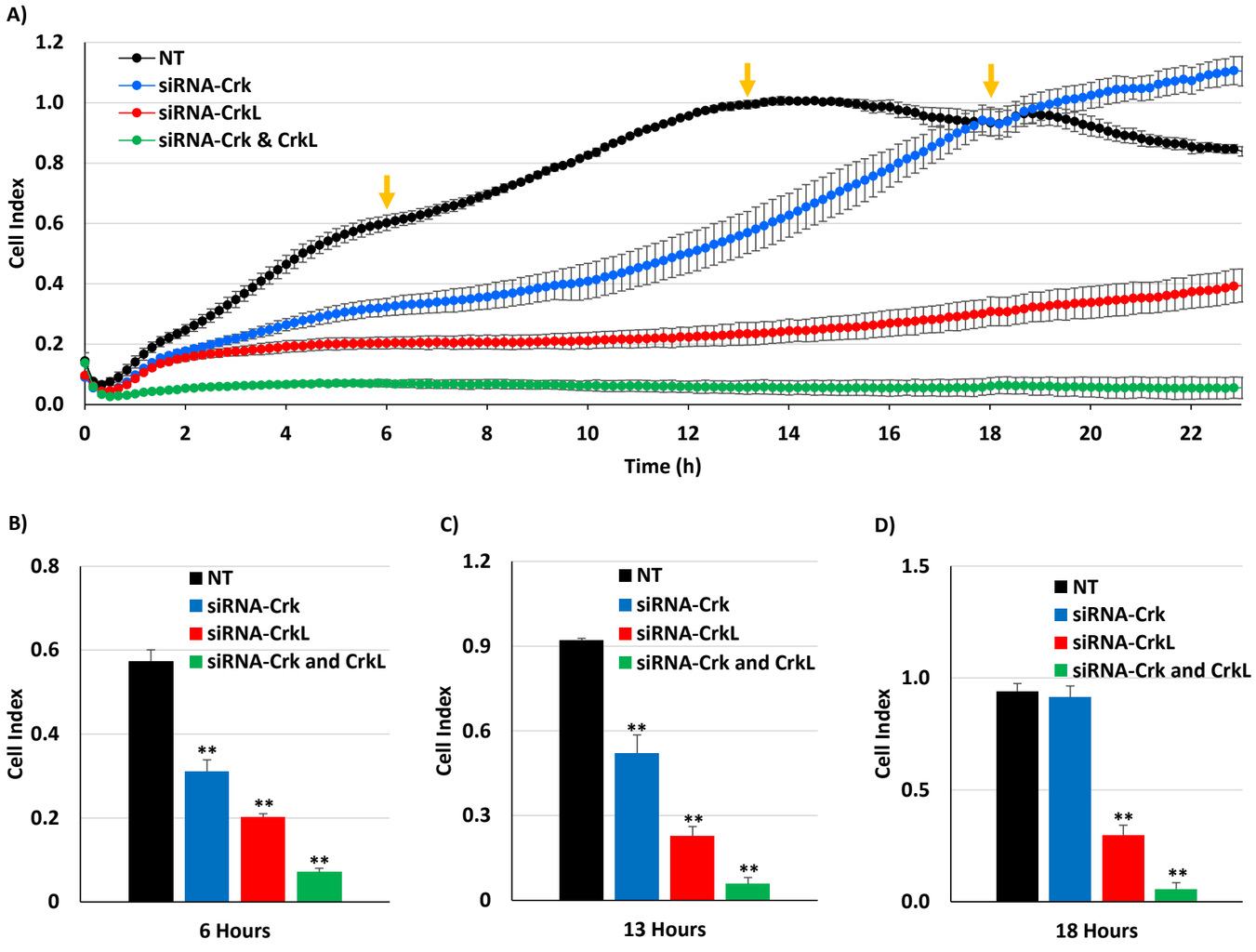
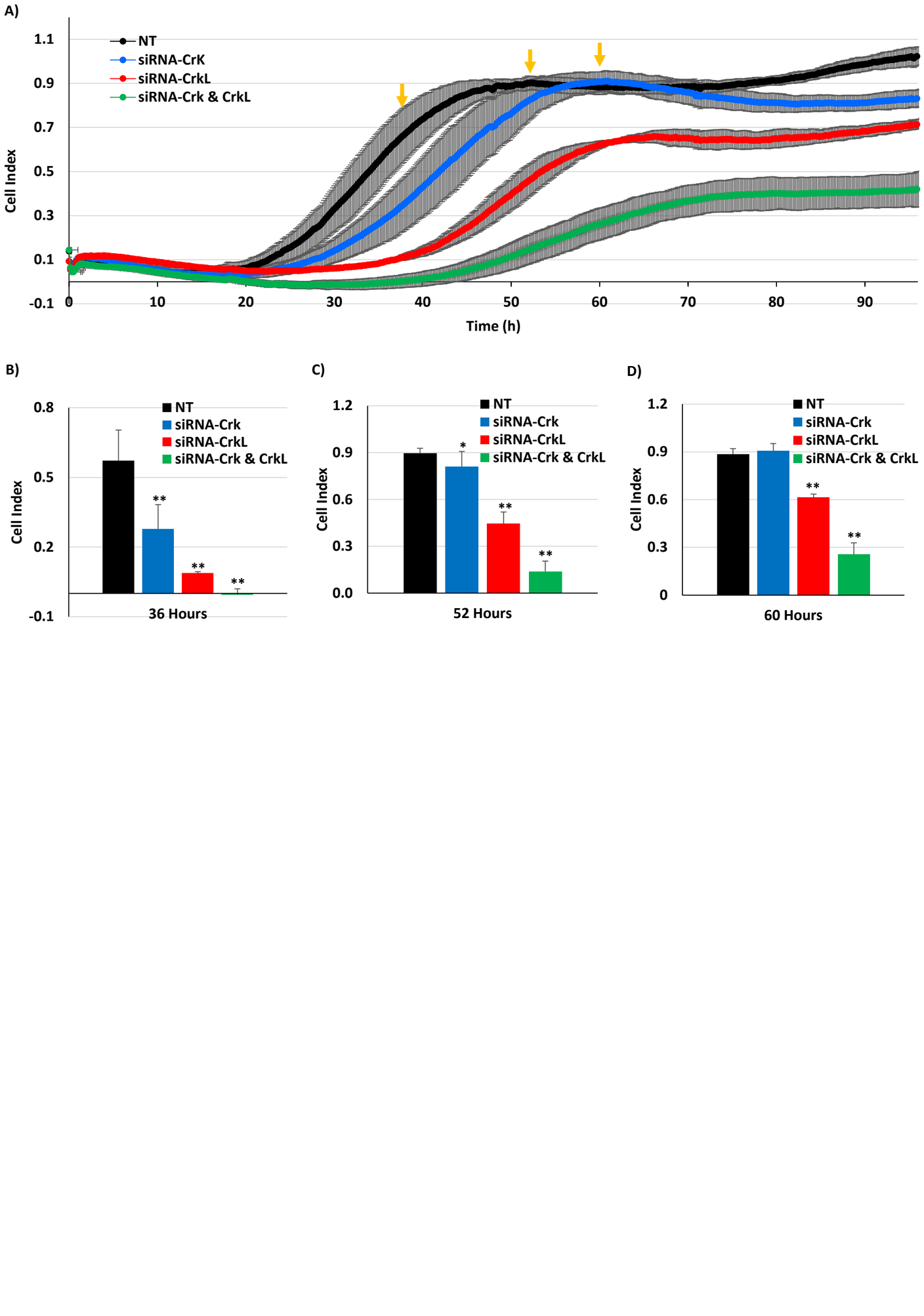


**Figure 3**





| Name of Material/ Equipment |
| --- |
| Biosafety cabinet |
| CIM plates |
| Crk siRNA |
| CrkL siRNA |
| Dulbecco’s modified eagle’s medium (DMEM) |
| Dulbecco's phosphate-buffered saline (DPBS) |
| Fetal bovine serum (FBS) |
| Heracell VIOS 160i CO2 incubator |
| Matrigel |
| Neon electroporation system |
| Neon transfection system 10 µL kit |
| Non-targeting siRNA |
| Odyssey CLx (Imaging system) |
| RTCA software |
| Scepter |
| Trypsin-EDTA |
| U-118MG |
| xCELLigence RTCA DP |

| Company | Catalog Number |
|---|---|
| ThermoFisher Scientific | 1300 Series Class II, Type A2 |
| Cell Analysis Division of Agilent Technologies, Inc | 5665825001 |
| Dharmacon | J-010503-10 |
| Ambion | ID: 3522 and ID: 3524 |
| ATCC | 302002 |
| Gibco | 21-031-CV |
| Hyclone | SH30910.03 |
| ThermoFisher Scientific | 51030285 |
| BD Bioscience | 354234 |
| ThermoFisher Scientific | MPK5000 |
| ThermoFisher Scientific | MPK1025 |
| Dharmacon | D-001810-01 |
| LI-COR Biosciences | |
| Cell Analysis Division of Agilent Technologies, Inc | |
| Millipore | C85360 |
| Gibco | 25300-054 |
| ATCC | ATCC HTB15 |
| Cell Analysis Division of Agilent Technologies, Inc | 380601050 |

| Comments/Description |
| --- |
| |
| Cell invasion and migration plates |
| |
| |
| Culture medium used for cell culture |
| DPBS used to wash the cells |
| |
| Co2 incubator |
| Extracellular matrix gel |
| Electroporation system |
| Electroporation kit |
| siRNA for non targated control |
| Western blot imaging system |
| Instrument used for experiment |
| Handheld automated cell counter |
| |
| Cell lines used for experiments |
| Instrument used for experiment |

To
12/23/2019

The Editor,
JoVE Methods Collections.

**Sub:** Submission of revised methods manuscript with the title "**Impedance-Based Real-Time Measurement of Cancer Cell Migration and Invasion**" **# JoVE60997**

Dear Editor,

We thank the reviewers and editorial office for reviewing the manuscript and appreciate their positive responses.

We have carefully addressed the comments and revised the manuscript. We have included additional, detailed steps in the protocol and have deleted figure 1 as per the reviewer's suggestion. As per the editorial comment, video content of the introduction and the protocol is heighted the manuscript.

We sincerely hope that the current version of the manuscript is clearer than the previous one and is suitable for publication in **JoVE**.

For your convenience, we have detailed our responses to the editorial and reviewers' comments below.

Yours sincerely,

Giridhar Mudduluru and Taeju Park

**Comments and responses:**

**Editorial comments:**
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please provide an email address for each author.
3. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
4. Please provide at least 6 keywords or phrases.

5. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please remove the term xCELLigence and use the generic terms instead. The term can be introduced once in the introduction.
Response: We have updated the manuscript as per the author guidelines and all 5 of the editorial comments above.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example xCELLigence, the Cell Analysis Division of Agilent Technologies, Inc., Matrigel, Corning, CIM plates, etc.
Response: We concur and have minimized the repetitive use of commercial terminology, replacing “xCELLigence DP system” with “real-time cell analysis system,” “matrigel” with “extracellular matrix gel” and so on. We have kept the reference to CIM plate, which stands for “cell invasion and migration plate,” as CIM plates are used often in the experiment.

7. Please do not refer to the manuscript as a video. The text manuscript component is an individual part of the submission.
Response: We made this change as requested and detailed protocol is written under protocol section 3 without referring the video.

8. Please expand all abbreviations during the first-time use.
Response: All abbreviations were expanded when first used in the manuscript.

9. We cannot have non-numbered steps, headings, subheadings, and substeps in the protocol section. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.
Response: We adjusted the numbering of sections and sub-sections as requested.

10. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
Response: We modified the protocol as per the request.

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Please include discrete experimental steps, the knob turns, button clicks, etc to show how the step is performed.
Response: We updated the protocol as requested by the editors.

12. Please make a separate section for the glioblastoma cell culture and electroporation step. Please include details of how it is performed in the imperative tense, including reaction set up, etc. How do you check for the knockdown? These steps may not be shown in the video.
Response: We included a separate procedure section in the protocol for U-118MG cell line culture and electroporation.

13. Please ensure that you describe each action in a stepwise manner.
Response: We updated the protocol to describe each action in a stepwise manner.

14. 1.3: Please make substeps.
Response: We updated the protocol by adding substeps.

15. 1.4: How do you program the cradle? Please provide all the details. How do you ensure that the system is recognizing all the wells?
Response: We have provided the details for the cradle programming procedure in the protocol and described how to check the messages generated by the control unit to ensure the wells are recognized by the control unit.

**Changes or additional text added in the manuscript as response to the above comment:**

Under protocol subsection 3.7 the procedure is updated with the following comment **"**Check whether all the wells are recognized by the control unit by clicking the message tab. If the message displays as OK, the CIM plate in the cradle is ready for the experiment.**"**

16. 1.5: Please detail all steps in the text as well to show the experiment is performed?
Response: We provided details for all the steps that are necessary to start and finish the experiment.

**Changes or additional text added in the manuscript as response to the above comment:**

3.4. To set up the impedance measurement program, double-click the RTCA Software Pro icon to open the system software application (control unit). Each cradle has an individual window with different tabs to set the experimental conditions, the impedance measuring time interval and duration, and data analysis.

4.10. Release the experiment by clicking the release button in each cradle.

17. 2.1: How do you perform the baseline readings- button clicks etc? e.g. move the plate to the detection chamber and click "baseline".
Response: We provided the details to set and read the baseline and actual cell impedances.

**Changes or additional text added in the manuscript as response to the above comment:**

4.1. Measure the baseline reading by clicking the cradle start button. The baseline should be read after pre-incubating CIM plates for 1 hr in the cradle of the real-time cell analysis system under culture conditions in the $CO_2$ incubator and before seeding the cells to the respective wells in the upper chamber. (Once the cradle start button is clicked, the control unit will ask to save the experimental file. After the file is saved, the cradle analyzer measures the baseline cell impedance as set in the program initially and enters a pause mode until the cradle start button is clicked again to measure cell impedance in the second step).

18. 2.2: How do you trypsinize, how is the counting performed etc. Do you keep the cells at 37 degrees for 3 days after electroporation?
Response: We provided elaborated new section 2 under protocol by addressing this comment.

19. 2.3: How do you program for the migration and invasion?
Response: We provided the details on the program settings for migration and invasion.

**Changes or additional text added in the manuscript as response to the above comment:**

3.5. Under the layout tab, set quadruplicate wells for each biological condition and set two-step cell impedance measurements under the schedule tab. The first step is for one-time baseline measurement (1 sweep with a 1-min interval), and the second step is to measure the cell impedance for an actual experiment (for migration, 145 sweeps with a 10-min interval, and for invasion, 577 sweeps with a 10-min interval) in respective cradles.

20. 2.4: How do you monitor the cell impedance?
Response: Details are provided on how to monitor the cell impedances in the protocol.

**Changes or additional text added in the manuscript as response to the above comment:**

4.6. Under the data analysis tab, visualize the changes in cell impedance as cell index in a time-dependent manner during or after completion of the experiment. Each of the respective conditions of quadruplicates can be visualized either individually or as averages and/or standard deviations by clicking the option boxes for average and standard deviation.

21. 2.5: How is this done? Please include all the button clicks, the knob turns, etc.
Response: We provided the details on how to monitor the impedances with different options in the protocol.

22. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We have highlighted the important text in the manuscript and protocol so that it tells a cohesive story of the Protocol.

23. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in Figure Legend, i.e. "This figure has been modified from [citation]."
Response: We are not including any published data.

24. As we are a methods journal, please revise the Discussion to explicitly cover the following in

detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: We rewrote the discussion section to address all the points mentioned in the comment.

25. Please sort the materials table in alphabetical order. Please ensure that the table includes the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
Response: We have sorted the materials in alphabetical order.

**Reviewers' comments:**
Reviewer #1:

The authors describe the method for the use of xCELLigence cell-substrate impedance real time analysis methodology on cancer cell migration and invasion, supported by the JoVe video strategy. This complements similar text only online methods articles already available (see Scrace et al 2013, for example).

As well as the protocol for the use of the instrument per se, the authors use a biological question to demonstrate this, focusing on evaluating the role of Crk and CrkL in cancer cell migration and invasion. I think that this angle is not necessary to the degree the authors have used it in the protocol, as it reads more as an evaluation of the later, rather than a detailed protocol for using the xCELLigence system.

Response: We agree with the reviewer's comment. In the resubmitted version, the protocol is updated with more detailed descriptions. However, our method paper emphasizes the advantages of continuous monitoring of cell kinetics after knockdown of two functionally overlapping adaptor molecules. For this reason, we give more emphasis on Crk and CrkL information in the results and discussion sections, which gives fine details and strengths of this method.

Methodology:

The protocols are described, but lack details at points (see specific comments below). It would be desirable to improve on this part of the manuscript, especially due to the nature of the publication as a methodological resource.

The authors also describe (and include figures relating to) the process of knocking down Crk and CrkL in these cells in both the 1st section of the protocol (point 1) as well in the Representative Results section. I propose this level of detail is not required about the specific experimental

design and interpretation of the data as the paper should be methodological in nature, and more attention to detail should be given to the actual setup, use, and data analysis procedures for the xCELLigence system.

Response: As per the reviewer's suggestion, in the presently submitted version the protocol is updated in detail, and some information related to Crk and CrkL is deleted from the figures and discussion sections.

Protocol specific notes:

- No specs are included for cell incubation pre-setup of the CIM plate, including humidity levels.
Response: We provided the details.

**Changes or additional text added in the manuscript as response to the above comment:**

2.1. Culture the U-118MG cell line in 5% fetal bovine serum (FBS) containing Dulbecco's Modified Eagle Medium (DMEM) (culture medium) and maintain at 37°C in a humid atmosphere containing 5% $CO_2$ incubator (culture conditions).

3.1. Place the real-time cell analysis system in a $CO_2$ incubator under culture conditions 5 to 6 hr prior to the start of experiment to stabilize the system to the culture conditions.

3.3. After the ECM gel coating, keep the CIM plate in the incubator under culture conditions for 4 hr with its lid on. (During the ECM gel coating and drying, take preventive measures to avoid direct contact of electrodes of the upper chamber of CIM plates with either the hands or the surfaces of the biosafety cabinet or the $CO_2$ incubator.)

- There is no note re humidity levels for xCELLigence RTCA DP system stabilization
Response: We updated the protocol with this information.

- The assembly of the CIM plates lacks some crucial details regarding avoiding air bubbles and plate manipulation, and these need to be included. See Scrace et al 2013 for more details.
Response: The details are provided on how to avoid having air bubbles while coting the plates with matrigel and seeding the cells for impedance. As the reviewer stated, Scrace et al. provided the details in their book chapter since they were using both E-plates and CIM plates. The present method paper is describing only the cell migration and invasion using CIM plates. Due to this reason, we have not included more details on plates and plate manipulate issues.

- The well background measurement procedures before adding the cells need to be more detailed.
Response: We have provided the details.

**Changes or additional text added in the manuscript as response to the above comment:**

4.1. Measure the baseline reading by clicking the cradle start button. The baseline should be read after pre-incubating CIM plates for 1 hr in the cradle of the real-time cell analysis system under culture conditions in the $CO_2$ incubator and before seeding the cells to the respective wells in the upper chamber. (Once the cradle start button is clicked, the control unit will ask to save the experimental file. After the file is saved, the cradle analyzer measures the baseline cell impedance as set in the program initially and enters a pause mode until the cradle start button is clicked again to measure cell impedance in the second step).

- The experimental setup on the computer is too brief. I am aware this is noted as per manufacturer's instructions and will be demonstrated in the video, but more detail needs to be included, including perhaps screen grabs of the process.
Response: In the revised version, we have provided more details.

- Baseline readings setup and measurements need to include more detail.
Response: We have provided more details on baseline reading in the protocol.

- The calculation of the normalized cell index needs to be explained in more detail.
Response: In this method paper, we have not included the normalized cell index. We simply plotted the graphs using the cell index, because we think normalization is not required if you analyze the trend of biological effects over the entire time period. Therefore, we did get into the details of the normalized cell index.

Data specific notes:

- I suggest that Figure 1 is removed from the paper, and only include Figures 2 and 3 (in particular 2A and 3A) are included from the current version, Figure 1 is not truly relevant to the methodological nature of the paper.
- Similarly, references to the actual biological relevance of the role of Crk and CrkL should be removed from the paper (Data and Discussion).

Response: We thank the reviewer for this suggestion and agree. Accordingly, we have removed Figure 1 and some information about Crk and CrkL from the discussion section. However, Figures 2b, c, and d and 3b, c, and d, which represent cell impedance values at specific time points, provide valuable information and help readers understand clearly the importance and benefit. For this reason, we kept these subfigures in the present revised version.

Reviewer #2:

Manuscript Summary:

Mudduluru et al. submitted an interesting method paper for the field with the title "Impedance-based real-time measurement of cancer cell migration and invasion". Measuring real-time cell kinetics under loss or gain of gene function are essentials to estimate the cancer cell behavior. This method procedure facilitates the researcher to obtain real-time data in a time-dependent manner under different experimental conditions. The authors clearly mention the advantages of real-time measurement cell impedance over traditional single-point determination.

The complete procedure is explained in detail, which can be performed elsewhere by flowing step by step.

Response: We thank this reviewer for the positive response to our method paper. With all the additional details in the protocol, we hope that this reviewer will appreciate the revised version.