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Imaging and Analysis of a High Density Cell Migration Assay

Using Agilent BioTek Cytation 5 and Gen5 Image+ Software for the Quantification of Cell Migration into the Cell-free Zone of Oris Pro Microplates

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Introduction

Complex biological or environmental signaling events can lead to cell migration, the movement of cells from one location to another. The migration of cells commonly occurs during both normal biological processes such as the development of an organism or marking pathological events such as cancer metastasis. Automated processes in higher density formats improve throughput greatly increasing the usefulness of performing migration assays at a larger scale. This study evaluated the use of a high density cell exclusion assay in 384-well format for investigation of cell migration using a cell line stably expressing green fluorescent protein (GFP). The use of a biocompatible gel (BCG) deposited in a portion of the well was used to exclude cells during seeding (Figure 1). A cell-free zone was revealed after dissolution of the BCG following media/cell addition to the wells. After cell attachment, compound addition can be made directly to the wells for investigation of cell migration inhibition. The Oris Pro system provides complete visual access to the cells and is suitable for high-throughput analysis through imaging. In this study, images captured from the Oris Pro plates by the Agilent BioTek Cytation 5 were analyzed using automated digital wide field fluorescence microscopy. Quantification of cell migration was performed by onboard Agilent BioTek Gen5 Image+ software which used algorithms to compute % confluence of the cell-free zone in each of the 384 wells. This image analysis was shown to be equivalent to the commonly used % closure of the cell-free zone using a known migratory inhibitor.
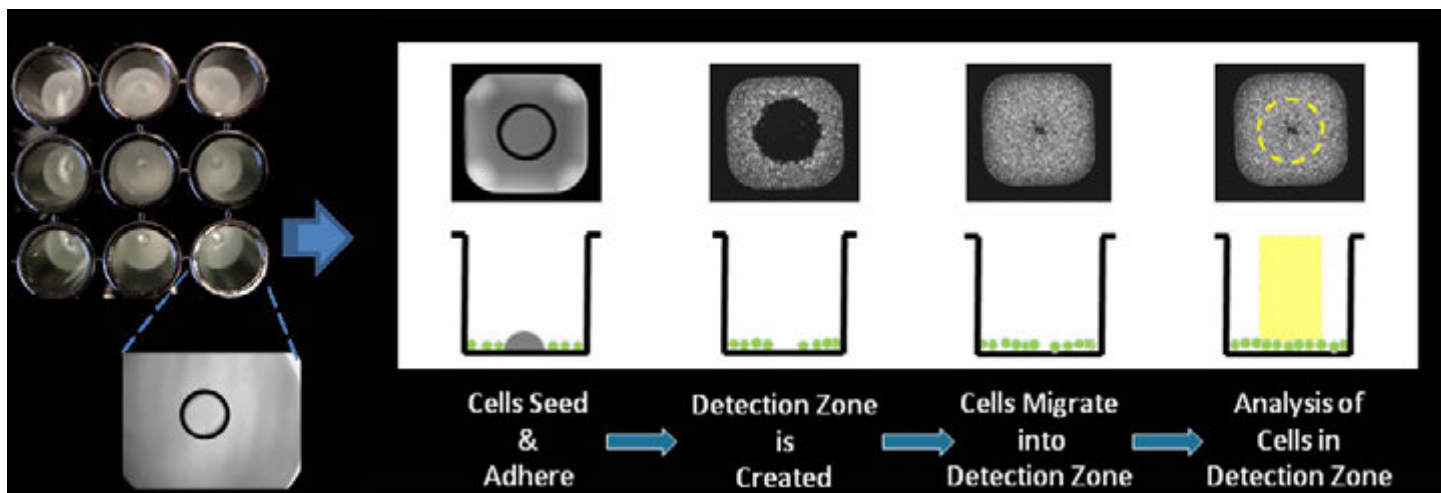
Figure 1. Oris Pro cell migration assay. The Oris Pro assay uses a non-toxic biocompatible gel, BCG to form a cell-free detection zone on culture surfaces. After cells are seeded into the 96- or 384-well migration assay plates, the BCG dissolves permitting cells to migrate into the well centers. Images of cells in the central detection zone can be captured and quantified in real-time using microscopy.

Materials and methods

Cell culture

MDA-MB-231/GFP cells were grown to 80 to 90% confluency in T-75 tissue culture flasks in Advanced DMEM (Gibco part number 12491-015) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Pen-Strep. Cells were harvested using standard methods. MDA-MB-231/GFP cells were resuspended in DMEM/F12, no phenol red (Gibco part number 11039-012) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% Pen-Strep (phenol free complete media or PFCM) and counted.

Optimization of cell seeding density

MDA-MB-231/GFP cells were diluted to the appropriate cell plating density in PFCM. For the Oris Pro 384 Cell Migration Assay, Collagen I Coated, 20 µL of MDA-MB-231/GFP were seeded in replicates of 12 at densities of 2.5, 5, 7.5, 10, 12.5, 15, and 17.5K cells/well. The assay plate was centrifuged briefly at 1,000 rpm in an Eppendorf 5810R centrifuge immediately following the addition of cells to facilitate seeding (Figure 2). Cells were allowed to attach for a minimum of 1 hour in a humidified incubator at 37 °C, 5% CO_2 prior to compound addition.[2]

For cell seeding density optimization studies, half of the seeded wells at each density were treated with cytochalasin D (CD) to a final concentration of 1 µM while wells in the other half were treated with vehicle alone (0.1% DMSO/PFCM). Cells were imaged kinetically for up to 48 hours in an Agilent BioTek Cytation 5 cell imaging microplate reader with incubation at 37 °C and a gas control module set to 5% CO_2 using the settings outlined in Table 1 and described in further detail below. Images were captured and analyzed using Agilent BioTek Gen5 Image+, as described in Appendix A (Winooski, VT), Microsoft Excel (Redmond, CA), and plotted with GraphPad Prism (La Jolla, CA) software. Briefly, Gen5 Image+ determines cell percent confluence migrating into the defined cell-free detection zone. Control wells consisting of cells treated with a known migration inhibitor are used for comparison to untreated cells for determination of migration expressed as percent closure.
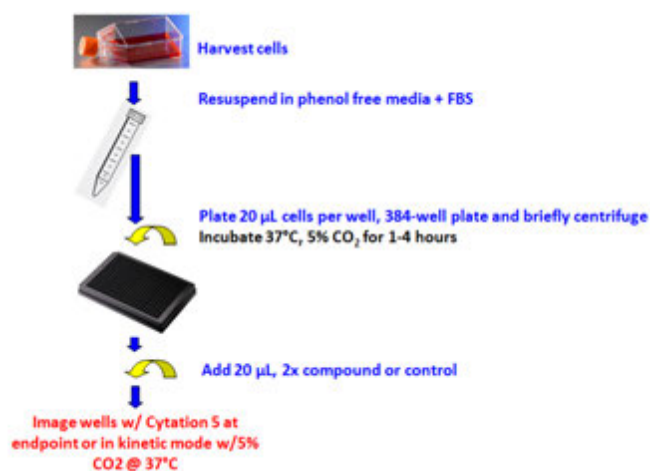

Figure 2. Assay workflow. MDA-MB-231/GFP cells were harvested and prepared in phenol free media for seeding.

Table 1. Agilent BioTek Cytation 5 read parameters. The 384-well plate format required a vertical offset to insure the cell-free zone was centered in the image field. The 2.5x Zeiss objective was sufficient to capture the entire cell-free zone in a single image.

Agilent BioTek Cytation 5 Read Parameters	
Plate Type	384-well
Mode	Image
Objective	2.5x
Color	GFP
Exposure	Auto
Horizontal Offset	0 µm
Vertical Offset	−406 µm

Cytochalasin D dose response

Cells were harvested and seeded at optimal density as described above. Following the 1-hour incubation period, cytochalasin D was serial diluted 1:3 and transferred to the assay plate in triplicate. Plates were imaged and analyzed as described above.

Results and discussion

Optimization of cell seeding density

The optimum cell seeding density was determined by treatment of six replicate wells at each cell seeding density with either the known migration inhibitor, 1 µM cytochalasin D, or vehicle alone. Kinetic data allowed the optimal incubation time and seeding density to be determined for further experiments. Percent confluency of the cell monolayer in the cell-free zone was automatically calculated in the Gen5 Image+ software to determine the seeding density giving the largest change in percent confluence in a timely fashion for use in future experiments (Figure 3). It was determined that MDA-MB-231/GFP cells plated at a density of 12,500 cells/well gave reproducible results after a 36-hour incubation period (Figure 4). Cells seeded at a lower cell density failed to migrate efficiently, likely due to lack of an adequate confluent monolayer at the start of the experiment. Conversely, cell seeded at a higher density showed a tendency to migrate less efficiently, likely due to insufficient adherence to the plate. Additionally, high seeding densities can lead to nonadherent cells settling in the detection zone at the beginning of the assay. Therefore, cells were seeded at the optimal density for dose response experiments using the migration inhibitor cytochalasin D.
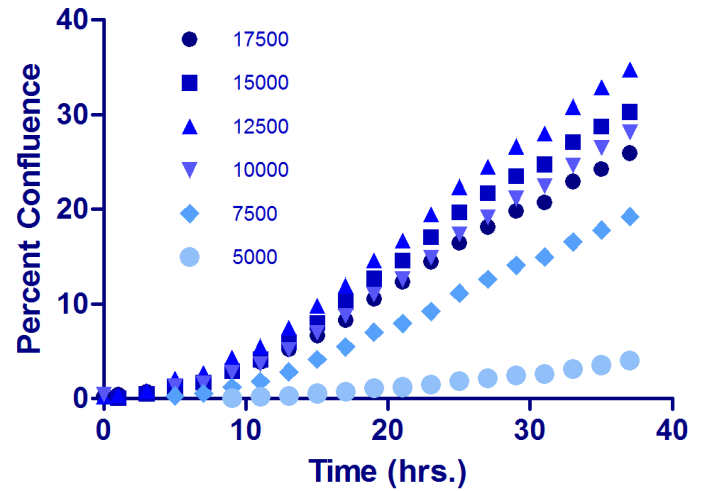

Figure 3. Seeding density and incubation time optimization. Cells were seeded as 12 replicates at seeding densities of 2.5, 5, 7.5, 10, 12.5, 15, and 17.5K cells/well. Six replicates of each cell density were treated with 1 µM CD while the remainder were treated with vehicle alone. Each well was imaged every hour for 38 hours. Percent confluence was calculated at 2-hour intervals for each cell density and plotted to determine optimal cell seeding density and adequate incubation period.
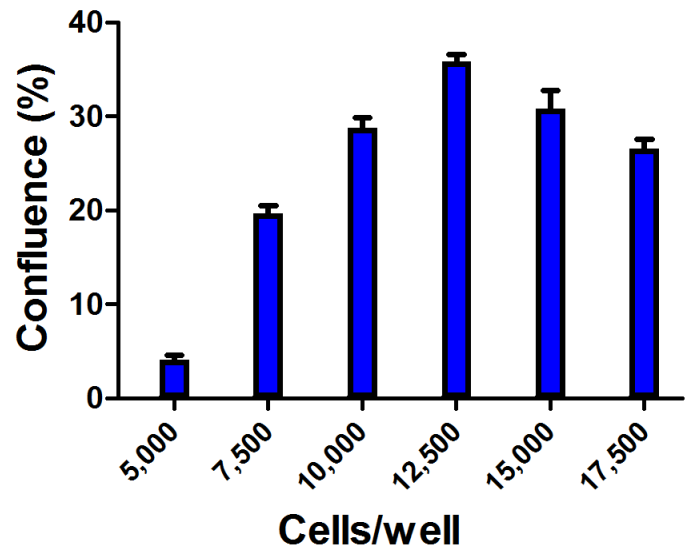

Figure 4. Seeding density and incubation time optimization. Percent confluence was calculated at each cell density to determine optimal cell seeding density and adequate incubation time (representative data shown at 36 hours post-seeding.)

Cytochalasin D dose response

Cells were treated with CD in a dose response format in triplicate and allowed to migrate for the appropriate length of time (36 hours). Data analysis was performed using the method described above for determination of the IC_{50} value (Figure 5). The IC_{50} value for MDA-MB-231/GFP treated cells was 57.0 nM when determined by Agilent BioTek Gen5 using percent confluence. This determinant showed excellent correlation with previously published data[1] and equivalence with the commonly used readout of percent closure.
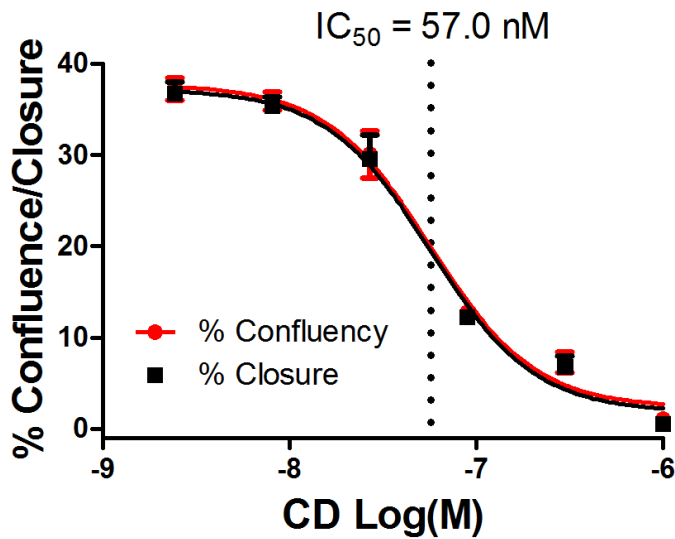

Figure 5. Dose response curves. MDA-MB-231/GFP cells were imaged in the 384-well microplate format after treatment with CD in dose response format following a 36-hour incubation. Plot depicts percent confluency or percent closure versus inhibitor concentration. The resultant IC_{50} determinants were identical.

Conclusion

The Agilent BioTek Cytation 5 cell imaging multimode reader provides an automated method for the quantification of migration assays using systems such as Oris Pro. The Oris Pro cell migration assay is a robust, easily automatable assay that provides complete visual access to the cells and is suitable for high-throughput screening and high content analysis. Image acquisition and analysis is facilitated by Agilent BioTek Gen5 Image+ software, and provides robust assessment of cell migration into the cell-free zone of the Oris Pro plates using either percent confluence or closure as readouts. The above results demonstrate excellent assay performance suitable for a high-throughput assay format.

References

1. Fronczak, J.A., Hulkower, K.I. and Vogt, A. (2011, May) A robust 384-well cell migration assay for high content analysis of cells treated with anti-cancer therapeutics. Poster session presented at American Association of Pharmaceutical Scientists, Washington, DC.

2. Hulkower, K.I. and Gehler S.R. How to measure area closure using the Oris Pro cell migration assay Technical Memo. Platypus Technologies, LLC. Madison, WI.

Appendix A

Agilent BioTek Gen5 and Cytation 5 Setup — Centering the BCG in the Field of View

For 384-well imaging, the Agilent BioTek Cytation 5 was equipped with a 2.5x objective and GFP LED/Filter Set modules (Table 1). The plate dimensions were determined using the Auto Map feature in Gen5. Exposure was set to Auto using the appropriate well for determination of optimal exposure using default image acquisition settings. The Agilent BioTek Gen5 statistical data analysis tool uses a mask to define the cell-free detection zone for fluorescent intensity analysis of images. It is critical to ensure that the well location of the cell-free zone is centered during imaging of each well in order to properly define the mask characteristics such as the plug shape and size. Any required horizontal or vertical offsets from center of well were determined using the available real-time Manual Mode imaging function using brightfield digital microscopy of the BCG prior to cell seeding (Figure 6). Wells at the corner of the 384-well plate, A1 and P24, were used to define the vertical and horizontal offsets by visualization of the BCG (Figure 6A). Additional wells across the plate were also checked to ensure the cells free zones resulting from cell seeding and degradation of the BCG will remain in the field of view for all wells during imaging. A Vertical offset of −406 µm was adequate for this application and the value transferred to the Read step in the Gen5 protocol (Figure 6B).
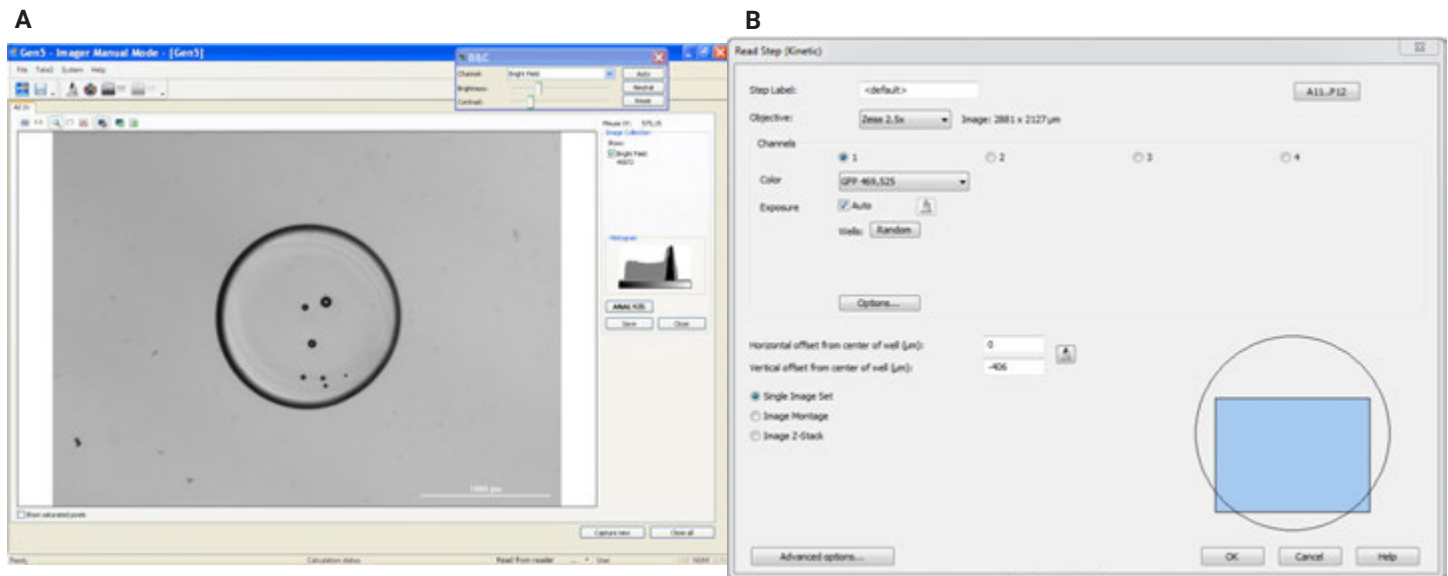

A   **B**

Figure 6. Microplate and protocol definitions. A) Image of BCG in microplate well. The Agilent BioTek Cytation 5 cell imaging multimode reader was used in Manual Mode using brightfield digital microscopy to determine the horizontal and vertical offsets required to ensure the cell-free zone left after dissolution of BCG would be centered during image acquisition. B) Agilent BioTek Gen5 microplate reader and imager software read step setup. In the Gen5 read step of the protocol a 2.5x objective was selected and options left at default settings. Horizontal and vertical offset from center of well values were defined based on manual mode imaging determinants.

Percent confluence determination using Gen5 Image+

The percent confluence analysis was defined using one of two methods: 1) addition of a Data Reduction step by selecting Statistics under Image Analysis tools or 2) defined post-image acquisition using the Analyze Tool upon selection of an individual image (Figure 7).
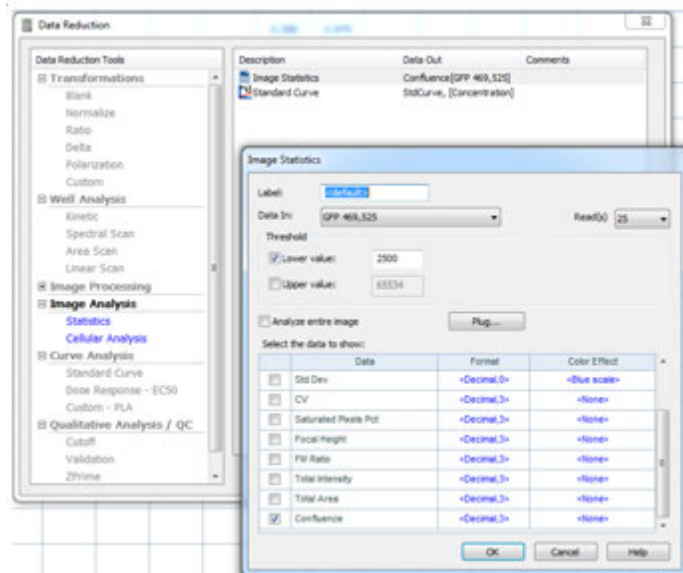
Data reduction step

A Data Reduction step can be added to a Gen5 protocol prior to running an experiment in Gen5 (Figure 7A). An Image Analysis: Statistics data reduction step was added to allow threshold values to be set as well as mask plug shape and size. Selection of the statistical parameters to be output is selected at this point as well.

Analyze tool: The Analyze tool allows real-time analysis of a post-acquisition image. The tool is especially useful during assay development and optimization to determine threshold values and plug size and position for use in the data reduction step. The Analyze tool was accessed by selecting an image from the plate matrix window (Figure 7B) and selecting the Analyze button. Within the Analyze tool window the mask shape and size were defined. The Line tool can be used to determine background signal levels to define threshold values to be used for analysis. Real-time statistical analysis is initiated by selecting the Start button. The data output is shown and was selected by editing the data reduction step as described above.

Visualization of a representative sampling of images was preformed, regardless of the method employed above, to verify adequate masking of the nonmigratory cell layer in control wells. A plate layout was added in Gen5 and the percent confluence values provided by the statistical analysis were used for data analysis by Gen5 or by exportation for use with Microsoft Excel or GraphPad Prism.
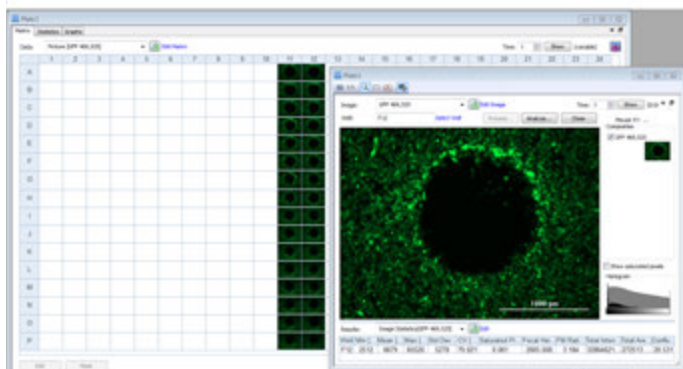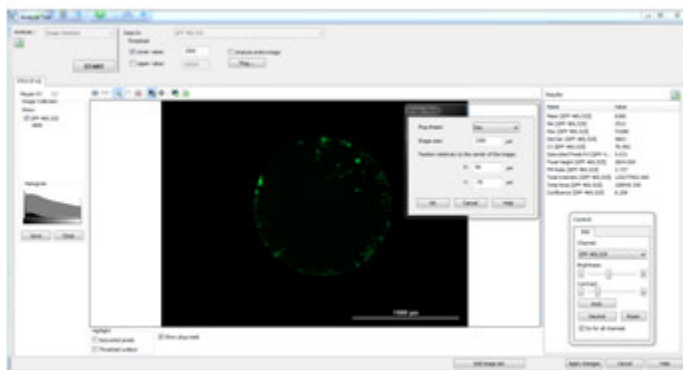
Figure 7. Agilent BioTek Gen5 statistical analysis. A) Data reduction step. Addition of an Image statistics data reduction step was added to allow Mask plug shape and size to be defined as well as selection of the statistical parameters to be output. Confluence is selected. B) Analyze tool activation. The Analyze tool was opened by selecting an image from the plate matrix window. C) Analyze tool setup. Within the Analyze tool window the mask shape and size were defined, threshold values set and statistical analysis initiated by selecting the Start button.

Percent closure

Cell migration analysis has historically relied on the use of calculations based on the percent closure of the area of detection or cell-free zone post-migration as defined below.[2]

$$\left(\frac{(Pre-migration)_{area} - (Migration)_{area}}{(Pre-migration)_{area}}\right)X100$$

A simplified determination relies on the use of the percent confluency statistic automatically calculated in the Gen5 software. The percent closure is then calculated by comparison of sample wells to control wells in which migration was prevented by a known inhibitor. Percent closure was calculated as follows:

$$(Migration)_{confluence} - (Pre-Migration)_{confluence}$$

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