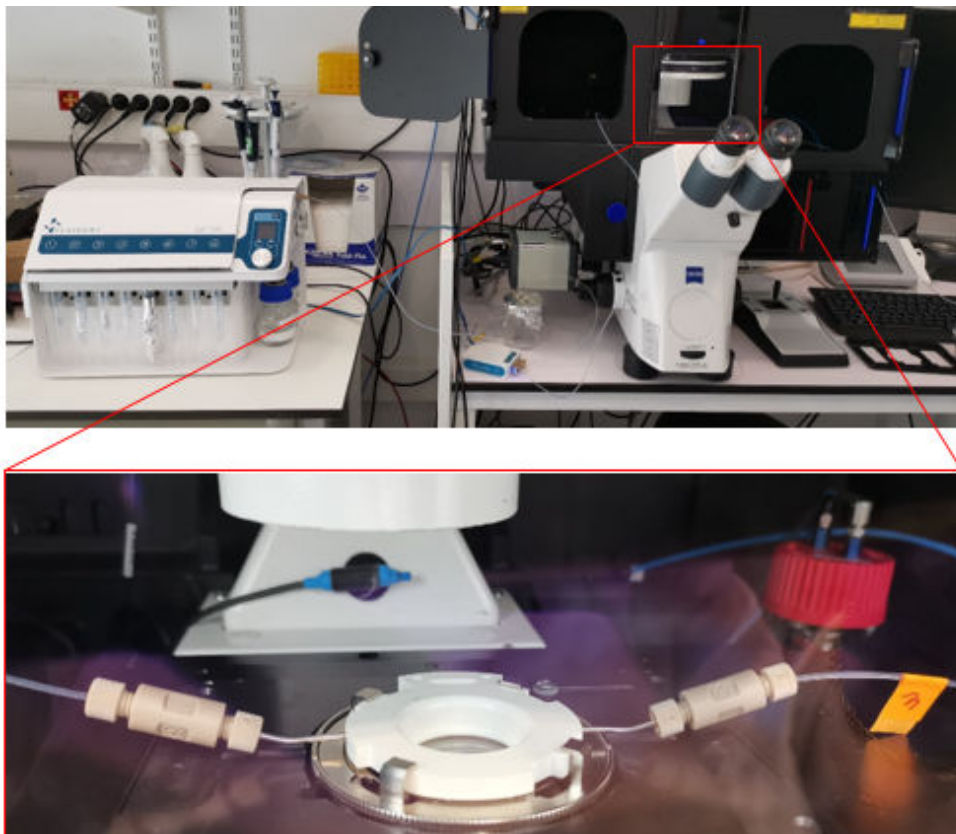


# AUTOMATED IMMUNOFLUORESCENCE PROTOCOL USING ARIA

## APPLICATION NOTE



## INTRODUCTION

Immunofluorescence (IF) is a widely used laboratory technique in cell and molecular biology that provides detailed information about the localization and distribution of specific proteins within a cell, tissue, or organism. It involves the use of antibodies, either fluorescently conjugated or revealed through the use of fluorescent secondary antibodies, to bind to the target proteins and enable visualization using fluorescent microscopy.

IF is a multi-step process that includes cell fixation, cell permeabilization, unspecific site blocking, and direct or indirect staining, followed by several washing steps. Traditionally, these steps are performed through manual pipetting. However, the Automated Immunofluorescence Protocol reduces the time-consuming and error-prone manual handling involved in these steps (inaccurate volume and flow rate of solution delivered at each step).

In recent years, there has been an increased focus on automating laboratory protocols to improve the efficiency, accuracy, and reproducibility of results. Microfluidics, the science and technology of handling and manipulating small volumes of fluid, has played a significant role in this trend by offering precise control of flow and volume in complex experiments such as IF.

In this application note, we propose an **Automated Immunofluorescence Protocol** through the use of the automated sequential injection system, ARIA, combined with the imaging chamber FCS2 from Biopetechs. This approach **enhances the accuracy and reproducibility** of the data generated compared to manual pipetting, and provides greater efficiency in the process. Additionally, the fluid delivery procedure is coupled with a Zeiss Axio Observer for imaging, although it can be adapted to any imaging system.

This application note has been produced in collaboration with Samy GOBAA (Head of the Biomaterials and Microfluidics core facility at Institut Pasteur) and Heloïse Mary (Research Engineer at BMcf).

We thank them for hosting, providing labware, and providing Fluigent with research and guidelines.



## MATERIALS & METHODS: AUTOMATED IMMUNOFLUORESCENCE PROTOCOL

### 1. Materials

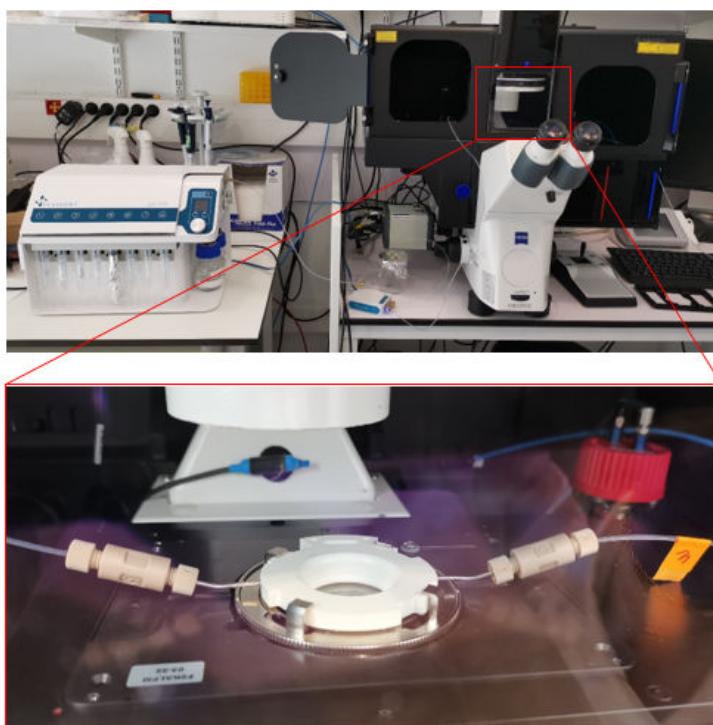
#### ARIA single output:

- Aria is an automated sequential injection system for cellular perfusion or timed injection protocols. It enables automation of delivery of **up to 10 different solutions** into a chamber or microfluidic chip by following user-defined protocols.
- The single output version is ideal for **high-quality multiplexed imaging experiments**. It offers a compromise between manual pipetting and all-in-one systems that are restricted to a specific application, integrated microscope, specific chip type, and set of solutions. The Aria can **automate any protocol with multiple solution delivery**, saving time and reducing variability between experiments compared to manual methods.

#### Biopetechs Live Cell Imaging Chamber (FCS2):

- The Focht Chamber System 2 (FCS2®) is a closed system, live-cell micro-observation chamber that utilizes several patented technologies. In addition to its uniform temperature control and user-definable perfusion capability, it is fully compatible with all modes of microscopy. The chamber is adaptable to the protocol instead of having to adapt the protocol to the chamber.

#### Widefield Microscope (in this app note we used a Zeiss Axio Observer)



*Figure 1. Automated Immunofluorescence Protocol Setup.*

## 2. Methods

To perform the immunofluorescence process, the preliminary steps of cell preparation and FCS2 chamber preparation are necessary.

### Step 1: Cells & coverslip preparation (performed manually)

#### Coverslip cleaning and coating:

Round coverslips (ø 40mm) were washed in a solution of EtOH 70% and sonicated for 30 seconds, rinsed in distilled H<sub>2</sub>O and dried with compressed air. Coverslips were then activated with plasma for 1min and coated with collagen at 50µg/ml. Coverslips were incubated for 1h @ 37°C to promote collagen coating, and washed with PBS 1X.

#### Cell seeding:

Primary human endothelial cells (HUVECs from Lonza) were seeded onto collagen-coated coverslips in EGM-2 culture medium (Lonza). For ø 40mm, the ideal cell density was 100,000 cells in 500ml per coverslip. Cells were left in the incubator for 1-2 hours to adhere properly onto the coverslip.

### Step 2: Cell fixation (performed manually or included in the automated procedure)

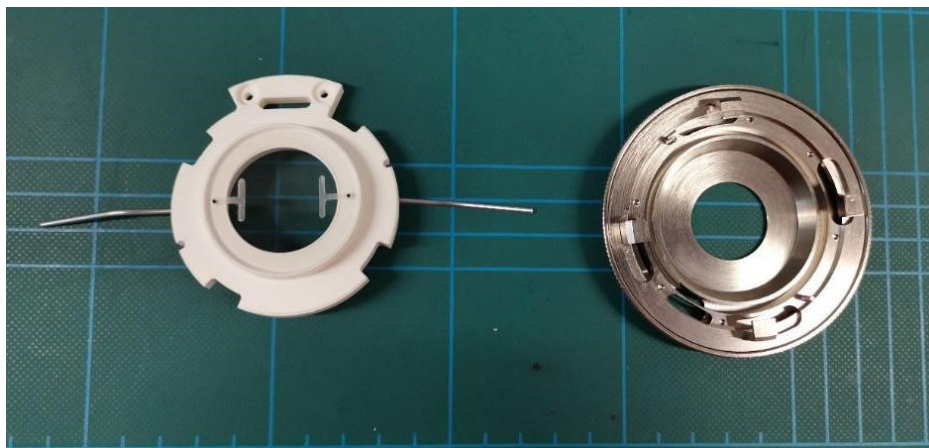
In the steps prior to the Automated Immunofluorescence Protocol, a cell fixation must be performed.

Prior to fixation, cells were washed twice with PBS 1X. The fixation was performed using 4% PFA in PBS 1X under a chemical hood for 20 minutes.

This step can be included in the automated sequence. Make sure to rinse abundantly with PBS 1X after this step to remove PFA from the ARIA device.

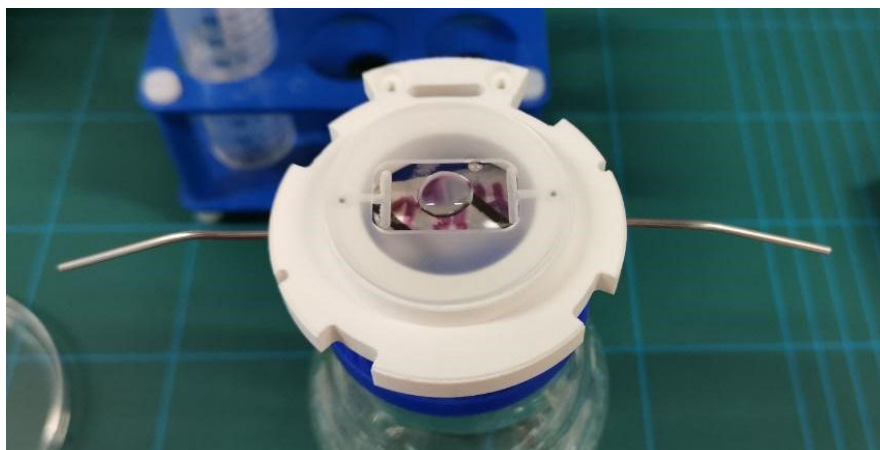
### Step 3: Coverslip assembly onto the FCS2 chamber

1. Mount your coverslip (covered with adherent cells) onto the white part (perform this step with the chamber upside down).



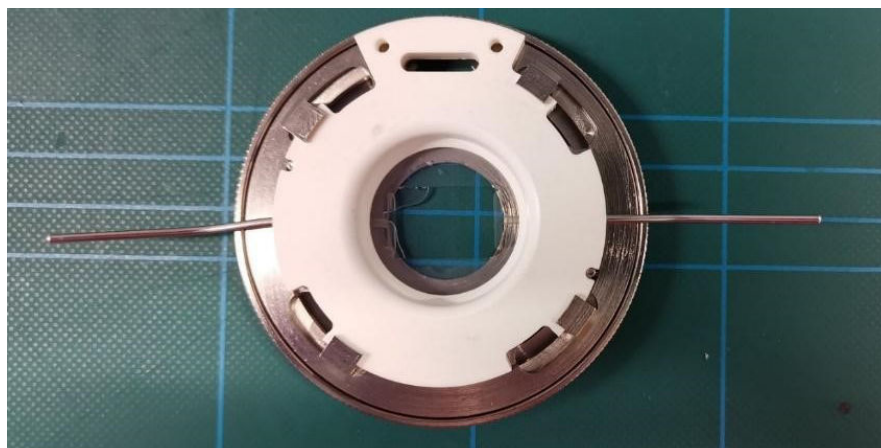
**Figure 2.** Coverslip assembly: Upper half and Perfusion tubes (left); Self-locking base (right).

2. Hydrate cells with a drop of PBS 1X



**Figure 3.** Coverslip assembly: Upper half and Perfusion tubes with coverslip.

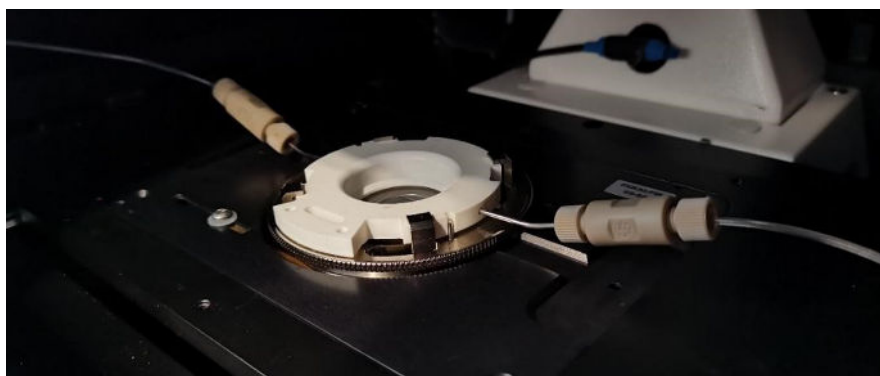
3. Close the chamber (turn until ring is closed)



**Figure 4.** Coverslip assembled.



4. Place the FCS2 chamber onto the microscope stage (use the stage adaptor provided by Bioprotechs fitted to your stage brand and model)



*Figure 5. FCS2 chamber placement on the microscope stage.*

If you need more information about the FCS2 chamber (how to assemble it, available configurations, stage adaptors, etc), please visit [the dedicated webpage](#).

## Step 4: Automated Immunofluorescence Protocol (Permeabilization, blocking, and immunostaining)

In this application note, we followed a typical IF protocol:

- Cell permeabilization with 0,1% Triton 100X in PBS 1X for 15min
- PBS wash
- Blocking with PBS 1X + 2% BSA for 20min
- PBS wash
- Cell staining with a cocktail of fluorescently coupled antibodies and dyes in PBS + 2% BSA (DAPI for nucleus staining diluted at 1/1000, Phalloidin-AF488 for F-actin staining diluted at 1/200 and UEA-1-lectin DyLight 647 for membrane staining of endothelial cells diluted at 1/200)

### ARIA unit preparation

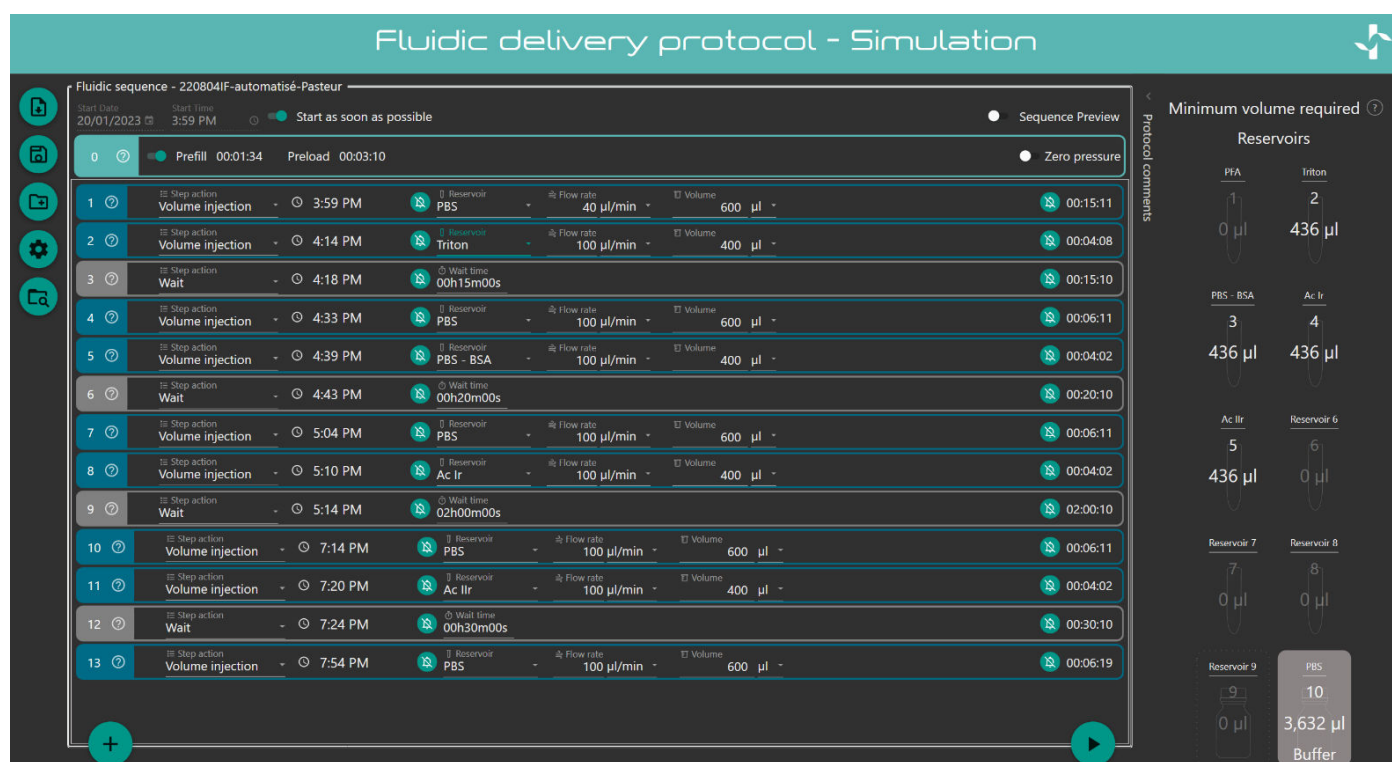
To start the protocol, plug the ARIA unit into a pressure source (either an [FLPG](#) unit or directly on the wall pressure source when available in your lab). Please ensure a minimum of 2.2 bar is reached before switching on the ARIA unit power supply. Connect your ARIA to a computer using the USB cable provided and open the **ARIA Software**.

Perform the [calibration of the ARIA](#) unit by following the steps indicated on the software. Create a custom ARIA program according to your preferred IF protocol.

- Create your Automated Immunofluorescence Protocol by adding steps (click on the “plus” button)



- To inject a solution into your coverslip, choose “Volume injection” and select the corresponding reservoir (example: Reservoir 2 “Triton” for permeabilization).
- Set the fluid delivery parameters (flow rate and volume) (example: 400µl at 100µl/min).
- To incubate a solution on your coverslip, include a “wait” step after the “volume injection” step and set the required timing (example: 15min for the permeabilization step).
- Continue as described above for the entire IF protocol. An example of the entire ARIA fluid delivery protocol is below:



**Figure 6.** Automated immunofluorescence protocol using Aria Automation Software

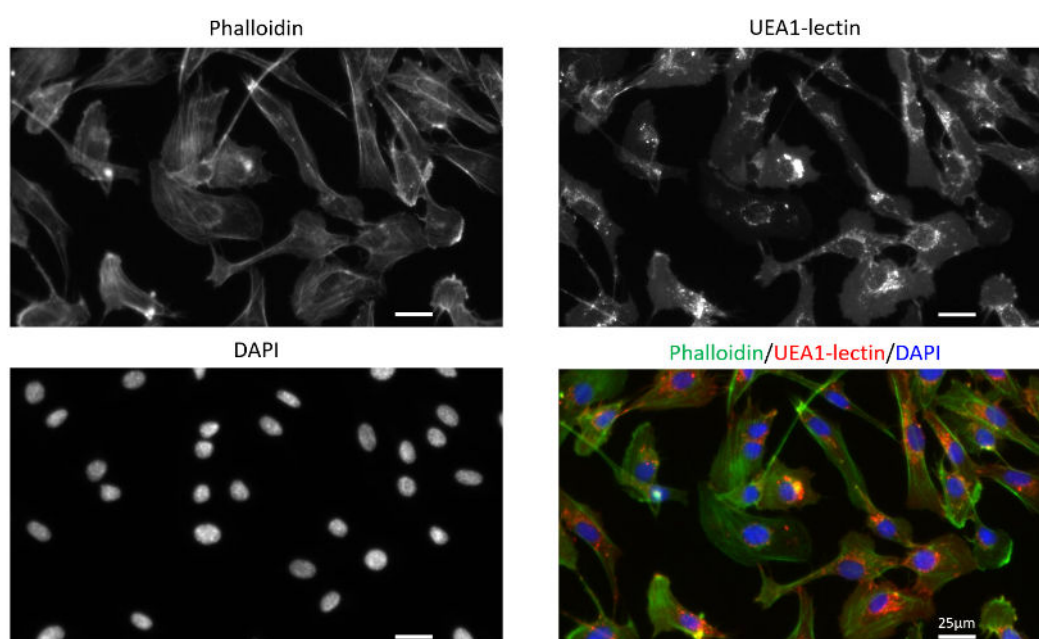
On the ARIA device, place your solution in the reservoirs specified in the ARIA software. In our example, the Permeabilization solution (containing 0,1% Triton) is placed in position “Reservoir 2”, and the Blocking solution (containing 2% BSA) is placed in position “Reservoir 3”, etc.



**Figure 7.** The ARIA automated sequential injection system.

## RESULTS: IMAGE ACQUISITION

To acquire our images, we used a Zeiss Axio Observer (inverted widefield microscope). Please note that any microscope can be used to acquire your images as for any IF experiment.



**Figure 8.** Automated immunofluorescence of primary human endothelial cells (HUVECs) stained with Phalloidin-AF488 for F-actin visualization, UEA1-lectin-DyLight as a membrane marker of endothelial cells and DAPI for nuclei staining. Images were acquired on a Zeiss widefield microscopy at 40X magnification.



## CONCLUSION:

The overall automated immunofluorescence protocol takes around 4 hours and 30 minutes (this timing directly depends on the incubation time of the primary and secondary antibodies). In comparison to manual pipetting, this represents a gain of time of at least 1 hour and 30 minutes. The automated completion of this procedure enables the user to perform other tasks while the ARIA program is running.

In this application note, we applied our automated immunofluorescence procedure to cells seeded on coverslips and placed in an imaging chamber. However, this protocol is also relevant for cells or tissues cultured in [microfluidic chips](#).

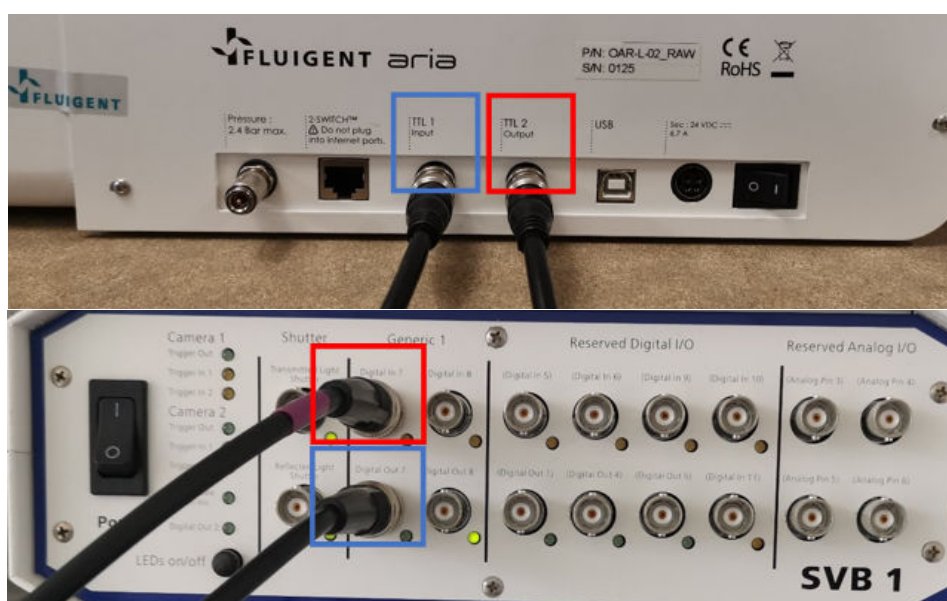
## ANNEX – Fully Automated TTL Signal Connection Protocol for Fluid Delivery and Image Acquisition Cycles.

The ARIA can be seamlessly integrated with a microscope to perform multiple cycles of fluid delivery and image acquisition automatically, including the implementation of an automated immunofluorescence protocol. This simple workflow enables the device to automate and accelerate data collection. This can be particularly useful performing multiplexed imaging. This protocol has been [published in Nature protocols](#).

The main advantage of this procedure is the ability to program a series of solution delivery orders coupled to automatic triggering of image acquisition. This is achieved through a TTL signal sent by the ARIA to the microscope to initiate a sequence of image acquisition after a chosen fluidic step. Once the images have been acquired, another TTL signal is sent by the microscope to the ARIA unit to restart the fluidic delivery sequence.

### Hardware setting:

- Connect a BNC cable between the TTL output port of the ARIA unit and the TTL input of the microscope (as illustrated on picture – red boxes). This will allow ARIA to send a signal to the microscope.
- Connect another BNC cable in between the microscope TTL output port and the TTL input port of the ARIA unit (as illustrated on picture – blue boxes).

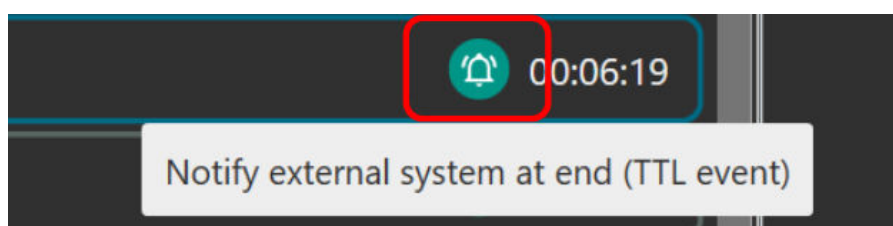
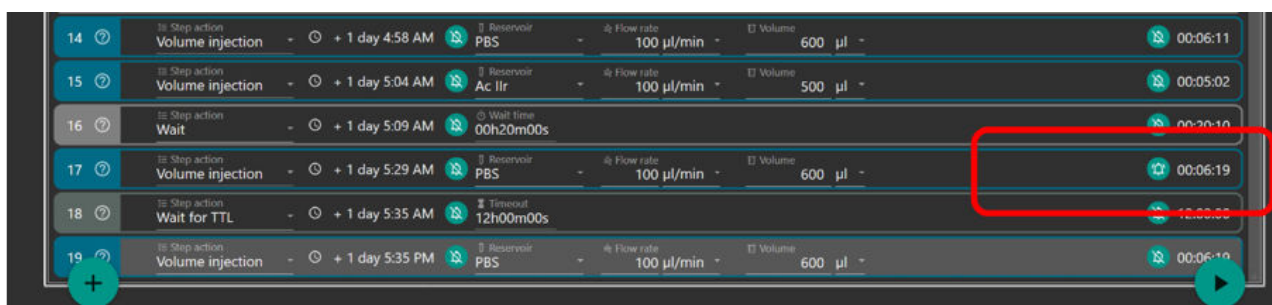


**Figure 1.** Red boxes: TTL output port of the ARIA unit; TTL input of the microscope. Blue boxes: TTL output port of the microscope; TTL input port of the ARIA unit.

Here we explain how to set up the TTL signal for the automated immunofluorescence protocol when working with a Zen-controlled Zeiss microscope. The procedure must be adapted to your microscope (in terms of how TTL signals are received and sent by your microscope). However, this does not affect the way ARIA works.

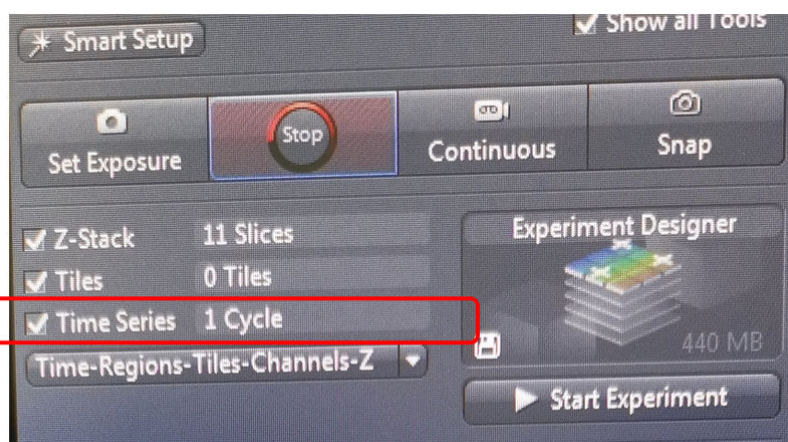
To launch an image acquisition order at the end of a specific fluid delivery step:

- On the **ARIA software**, choose “Notify external system at end (TTL event)” by ticking the “Bell” at the end of the fluidic step (on the right-hand side). This allows the system to send a TTL signal to the microscope right after the completion of the volume injection step.



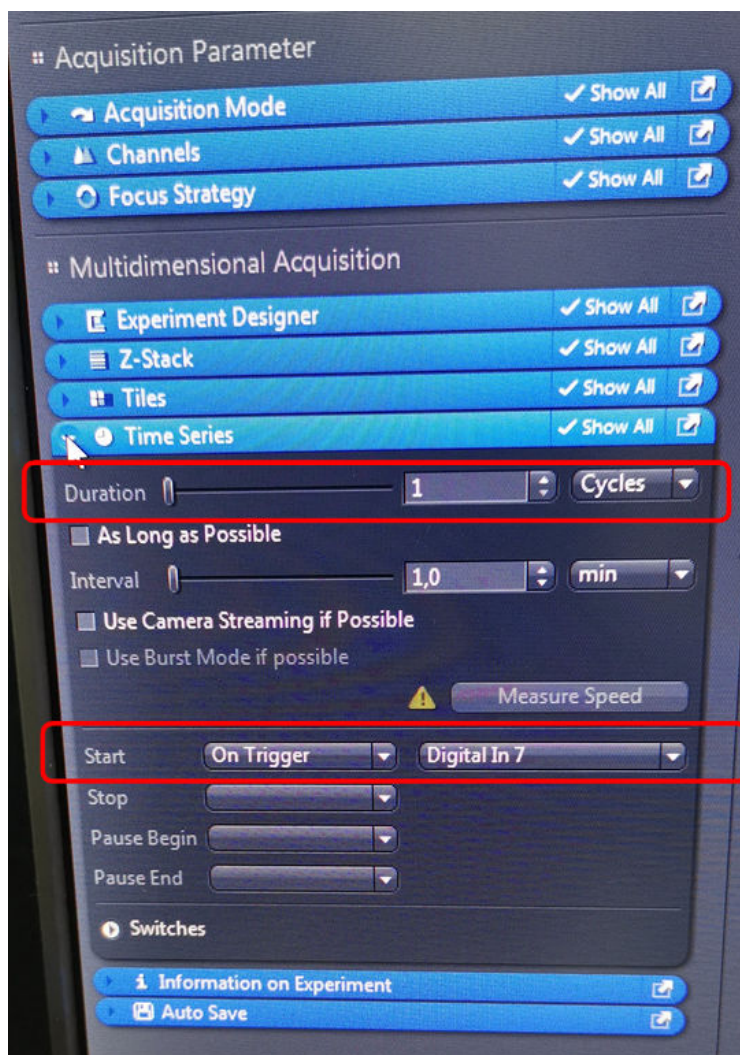
**Figure 2.** Sequential injection automated protocol using Aria Automation Software

- On the **Zen software**, in the Acquisition settings, check “Time Series” (even though you do not wish to perform time lapse experiments).



**Figure 3.** Zen Software; Acquisition settings.

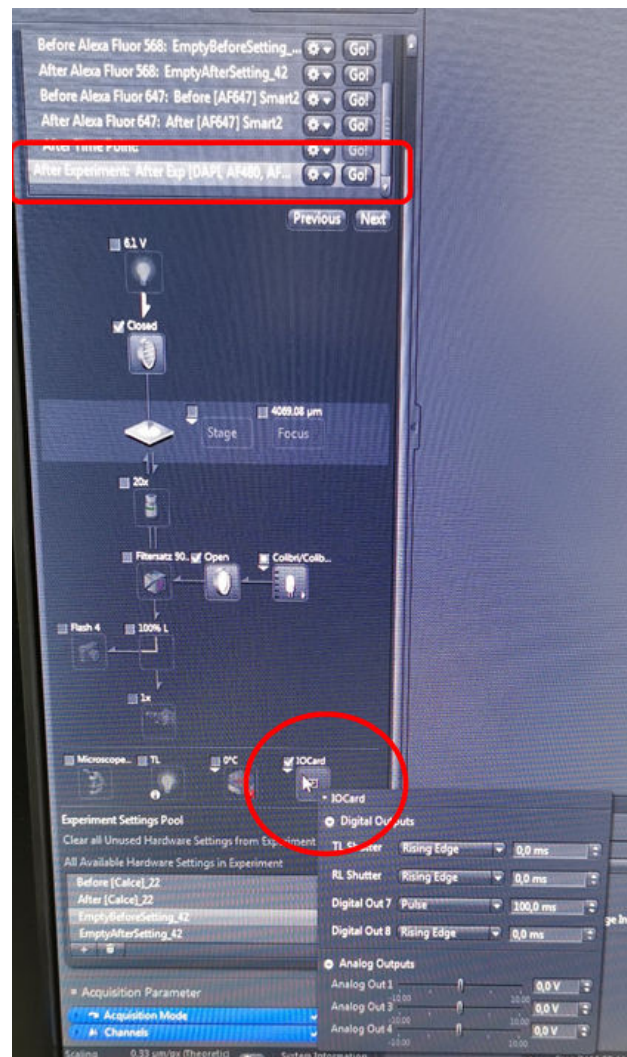
- In the Time Series tab, Set Duration 1 cycle and define Start “On Trigger”. Select the right TTL input port (here Digital In 7).



**Figure 4.** Zen Software; Acquisition settings.

- Proceed as usual for the other acquisition settings (channels, exposure time, z-stacks, etc)
- To resume your fluidic sequence, trigger a TTL signal output on Zen. In the “Light Path Setting” tab, in the option “After Experiment” tick “IO Card”.





*Figure 5. Zen Software; Acquisition settings.*

- Choose your TTL output port, here “Digital Output 7”. Please set the “Pulse” to “100ms”. This is **the parameter of the TTL signal ARIA is able to receive.**



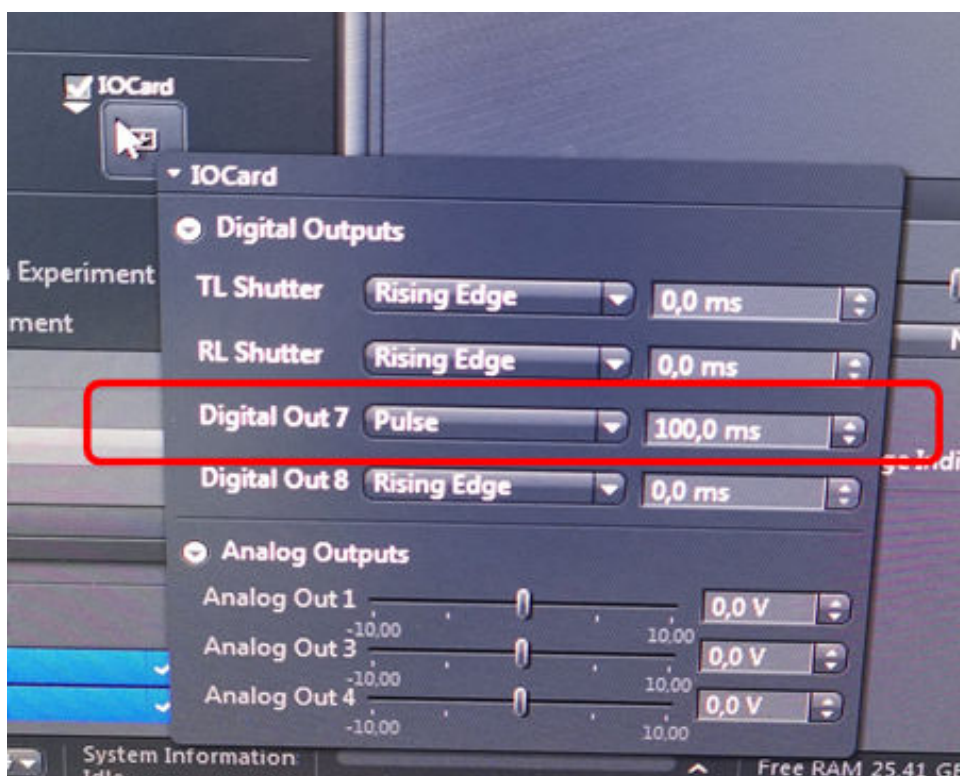


Figure 6. Zen Software; Digital Outputs.

- On the ARIA software, please select the step action “Wait for TTL” and set an overestimated timeout to be sure that ARIA receives the signal. The signal sent by the microscope should trigger the activation of the next step action you have selected.

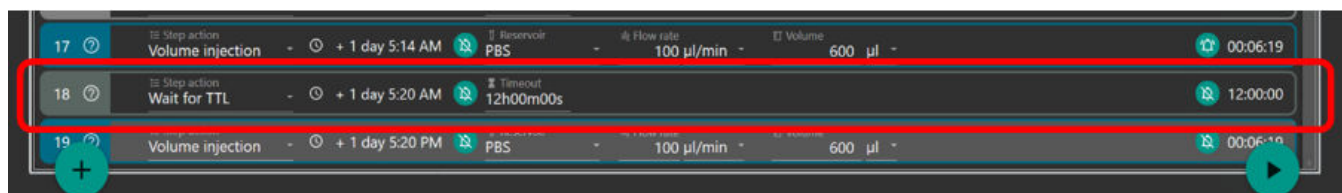


Figure 7. Sequential injection automated protocol using Aria Automation Software