heliX cyto
User Manual

BRUKER
Biosensors

QUICK GUIDE

for **single-cell Interaction Cytometry** (scIC)

Dynamic Biosensors GmbH
v1.0

Table of Contents

This guide is meant as a short overview of the **heliX**cyto system and its capabilities. All the chapters are expanded upon in the main **heliX**cyto guide found at ***https://www.dynamic-biosensors.com/helios-download-latest/***

heliXcyto Terminology

single-cell Interaction Cytometry

single-cell Interaction Cytometry (**scIC**) is a technology that measures the binding kinetics of a fluorescently labeled molecule (analyte) to a target (ligand) on the cell surface by recording fluorescent signal in a time resolved manner.

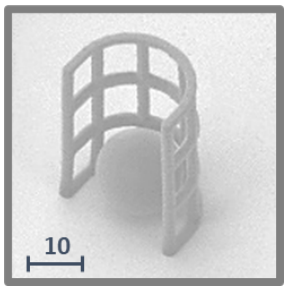
Analyte

Analyte is a fluorescently labeled molecule in a mobile phase that can bind to a target on the surface of a cell.

Ligand

"Ligand" is the name used in the heliOS software to describe a target on the cell surface to which an analyte can bind. This may be a receptor molecule, a lipid, a sugar or another cell-surface molecule.

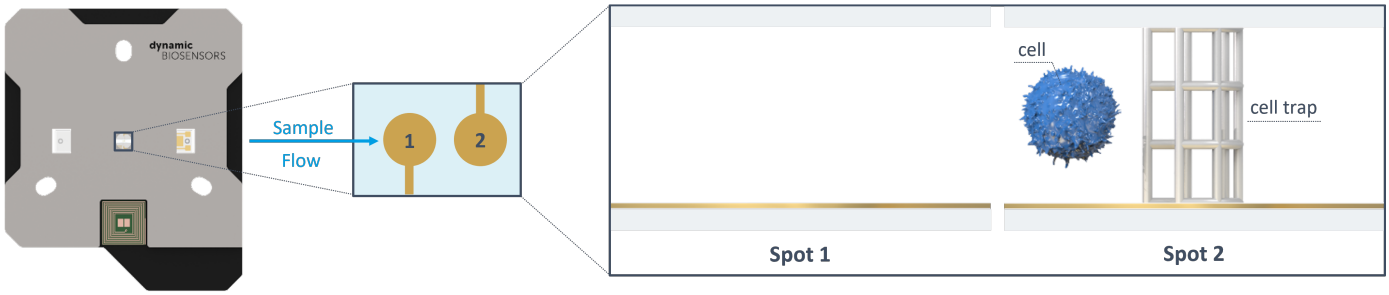
Cell Trap



Cell traps are flow-permeable, biocompatible polymer cages on a **heliX**cyto chip. They are designed to capture and immobilize differently sized cells from about 6 to 25 µm in the flow channel. Cell traps are available in 3 different sizes: small, medium and large.

heliXcyto Chip

heliXcyto chips contain a single microfluidic channel with openings on each side. Two golden electrode spots are located in the middle of the channel. One spot serves as a reference spot, and the second spot carries 3D biocompatible polymer cages for cell capture and serves as a measurement spot. The measurement spot can contain 1 or 5 traps of the same type. The reference spot allows real-time referencing of the collected fluorescent signal. The **heliX**cyto chip is reusable and disposable.



Maintenance Chip

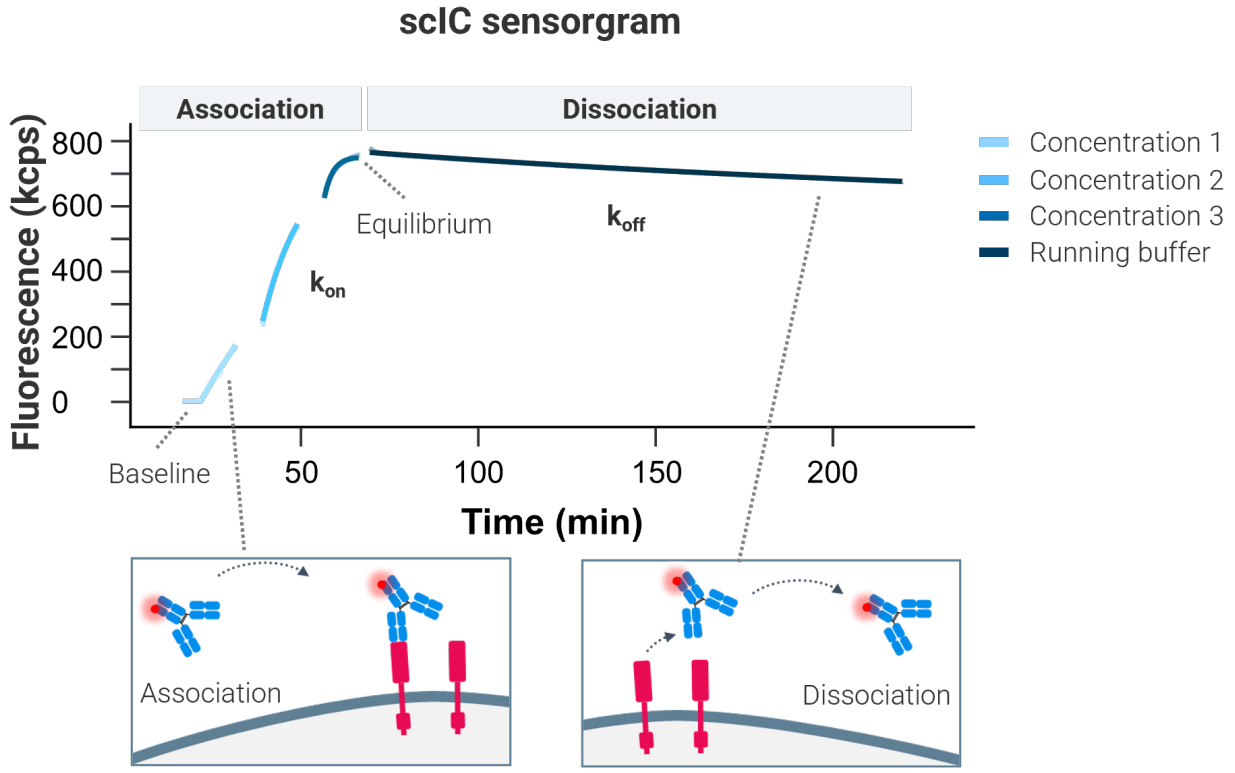
The **Maintenance Chip** is a single channel microfluidic chip used for all cleaning, testing and maintenance operations. These operations include **Clean & Sleep** routine, **Wake Up and Prime** routine, **System wash** and the **Fluidics test**. This chip should not be used for actual experiments with cell/protein solutions to prevent further contamination of the chip.

Running Buffer

Running buffer is the buffer used in the **heliX**cyto during the measurement. The use of Running Buffer 1 (**RB 1**) is generally recommended
Please refer to scIC compatibility sheet found in the main guide for more information.

Sensorgram

An **scIC** sensorgram is a plot of fluorescence response in counts per second (cps) over time, illustrating the progression of an interaction on or in cells. This curve can be viewed in real time in **heliOS** during the measurement. When analyzing sensorgrams, the best fitting exponential to the observed fluorescent traces are determined and kinetic rates (k_{on}, k_{off}) extracted.



Passivation

Passivation is an optional step in the run, where a passivation reagent is injected onto the chip and incubated to block surfaces. This can help preventing adsorption of analytes to the chip materials.

Normalization

A normalization step is employed to account for slight differences in signals due to signal collection from each measurement spot by a separate detector. A fluorescent dye of a defined concentration (based on the highest analyte concentration and degree of analyte labeling) is injected at the start of an assay. This normalization step is automatically included in the measurement method, and the measured normalization peak is used to automatically correct for differences between detectors during data analysis, prior to real-time referencing.

scIC Measurement Principles

scIC Applications

single-cell Interaction Cytometry (**scIC**) measures the kinetic rates and affinity of a fluorescently labeled analyte binding to and unbinding from its target directly on living cells. Detection of analytes in **scIC** is size-independent and therefore pertinent to any molecule from sub-nm to > 100 nm and relevant to all molecule classes from small molecules, peptides, aptamers, nanobodies, affibodies, antibodies, bispecific antibody formats, proteins, protein complexes, up to vesicles (exosomes), lipid nanoparticles, and viruses.

scIC *technology can address the following application areas:*

- measurement of signal amplitudes in binding screens
- kinetic analysis of k_{on} and k_{off} rates
- affinity and avidity evaluation through k_{off} and biphasic fit models
- specificity tests
- relative quantification of membrane proteins on the cell surface
- epitope binning and competition studies
- inhibition assays
- assessment of internalization of targeted proteins

heliX^{cyto} Hardware Capabilities

Fluidic System

The fluidic system of the **heliX**^{cyto} is fed by up to 3 different buffer bottles, enabling variations in running and maintenance buffer. Pumps in the **heliX**^{cyto} can be set to flow rates between 20 and 500 µL/min, catering to different sample and assay requirements. The flow channel of the chip connects to the fluidic system through two openings. The left-side opening is connected to the sample, buffer, and wash lines, while the right-side opening is linked to the regeneration line. This two-way fluidic system enables stable cell capture while running different steps of a measurement and eventually chip regeneration for in-assay chip reusability.

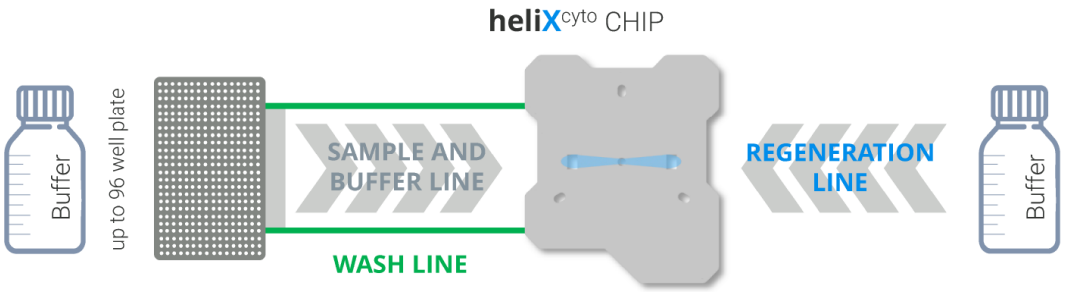


Figure 1. Chip integration in Fluidic system

Optical System

The fluorescence signal is excited by red and green light-emitting diodes (LEDs) and detected by four single-photon counters, which collect red and green signals from each spot throughout the full depth of the channel. Two independent signals can be monitored simultaneously on the same measurement spot, allowing two interactions to be measured at once in a dual-color assay setup.

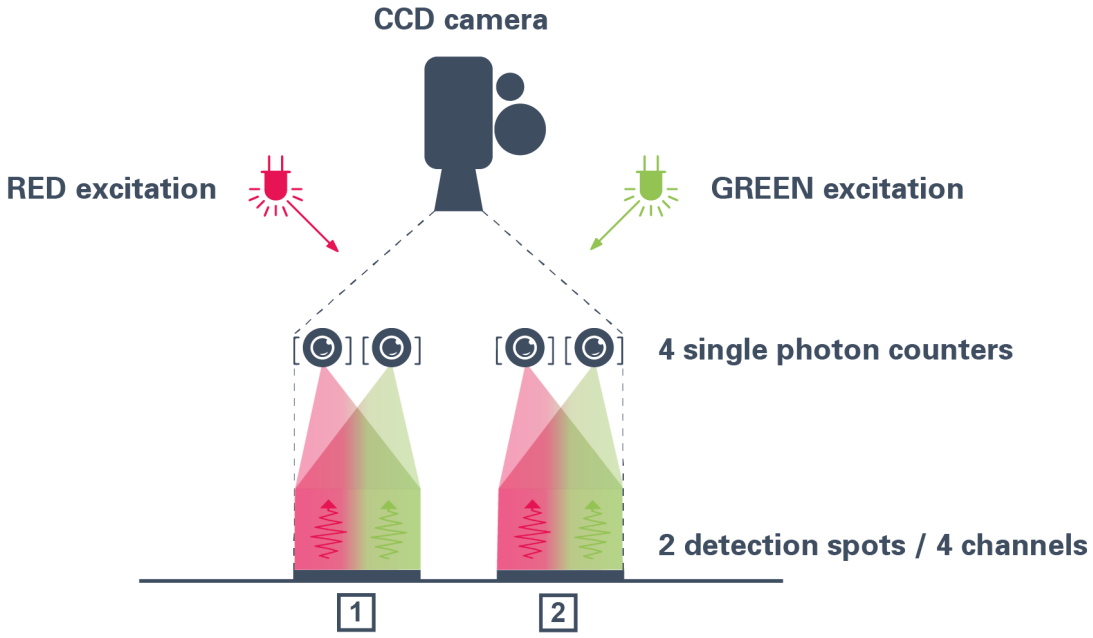


Figure 2. Optics setup

Signal collection from each measurement spot is handled by a separate detector, which may result in slight differences in the raw counts. To account for this, a normalization step is automatically included in data generating assays.

In addition to the single-photon counters, the instrument is equipped with a CCD camera and a reflected light microscope. These tools allow for real-time cell imaging, enabling the assessment of cell integrity and analyte quality throughout the measurement. This combined setup ensures both the interactions and the biological conditions are tracked, providing a comprehensive evaluation of the experiment.

scIC workflow

The scIC Measurement Process can be broken down into 3 key steps

1. **Experimental Design in heliOS**: Each measurement begins with designing the experiment in the **heliOS** software. The assay workflow is set up by selecting predefined methods and adjusting experimental parameters, such as the used cell line, analyte concentration, and buffer conditions. **heliOS** automatically calculates the precise quantities of cells, analytes, buffers, and other solutions necessary for the measurement, streamlining the preparation process.
2. **Preparation of buffers, analytes, and cells**: After the experimental design is finalized, the required materials are prepared according to the specifications provided by **heliOS**. Buffers, analytes, and cells are loaded into the instrument autosampler.
3. **Measurement and Data Analysis**: The system performs the scheduled series of automated real-time interaction measurements requiring no further intervention from the user. Data can be analyzed while the measurement is still running to get immediate insights into the results.

scIC Assays in heliOS

In addition to data-generating assays, the software also provides methods for chip testing, cleaning and instrument maintenance.

Table 1. Verified Data-generating assays

scIC Kinetics	Measures full kinetics on cells with analyte association adjustable between 1 and 5 concentrations, followed with a single dissociation after the highest concentration. Either green or red channel. Contains option for Analyte only test.
scIC Kinetics - Dual Color	Measures full kinetics on cells with analyte association adjustable between 1 and 5 concentrations, followed with a single dissociation after the highest concentration. Both green and red channel enabled simultaneously. Contains option for Analyte only test.
scIC Dissociation - Dual Color	Measures the dissociation of an analyte from cells that have been pre-incubated with a fluorescently labeled analyte with green and/or red channel.

Preparations and Assay Development for scIC Measurement

There are a few points to consider before setting up an scIC measurement

- Analyte concentration, Degree of Labeling, properties, and quality.
- Normalization solution and excitation power.
- Cell quality and handling.

Analyte Concentration and Degree of Labeling

As per standard kinetic measurement practices, we recommend selecting an analyte molar concentration range of 0.1x to 10x the expected equilibrium dissociation constant (K_d) for multiple concentration kinetics. For single-concentration kinetics or dissociation measurements, the analyte concentration should range between 1x and 10x the expected K_d. The required analyte volume calculated for a 10-minute association time at a flow rate of 25 µL/min totals approximately 300 µL.

The Degree of Labeling (**DOL**) for an analyte should ideally be 1, though **DOL** values of 2 or 3 are still acceptable. However, be aware that a higher number of labels on an analyte may impact its binding properties, and may increase the likelihood of aggregation or unspecific binding. To achieve an optimal DOL, follow the protocol provided with the Labeling Kits containing red or green dye from the **heliX**^{cyto} reagents line.

Normalization

Normalization is the first step in all data-generating methods. It compensates for the slight signal variations caused by using separate detectors for each measurement spot. In this step, a fluorescent dye at a user-defined concentration is injected at the start of the assay.

The concentration of the ***Normalization Solution*** is calculated by multiplying the highest fluorescently labeled analyte concentration used in the measurement by the analyte's **Degree of Labeling (DOL)**. This concentration guides the LED excitation power applied during the measurement. The goal is for the Normalization Solution peak to reach approximately the same fluorescence signal amplitude as the highest analyte concentration.

Refer to the Normalization solution concentration chart in the main guide to set the initial values. Note that the chart provides guidance for initial settings, but LED excitation power can be further adjusted during assay development.

Cell Quality and Preparation

Cell viability should be above 95%, and cells should generally be in a good condition. For adherent cells, we recommend working with cells that are 50-70% confluent. For suspension cells, we recommend harvesting at the mid-log phase of growth. 50 µl of cell suspension at 1E+06 cells/mL is needed per run.

Suspension cells harvesting

1. Centrifuge approx. 2E+06 cells at 300 g for 7 minutes to remove cell culture media. Discard the supernatant.
2. Wash the cells once by resuspending the cell pellet in approx. 1 mL of DPBS. Centrifuge the suspension at 400 g for 5 minutes to pellet the living cells. Carefully discard the supernatant to remove fragments and media.
3. Gently resuspend in 1 mL of DPBS, transfer the suspension on top of a cell strainer and strain the suspension by gravity flow.
4. Measure cell concentration of the strained suspension and adjust to 1E+06 cells/mL.

TIP | *Some suspension cells tend to form clusters. Gently resuspend clusters and include a straining step before the first centrifugation step. Use wide-bore pipette tips when handling cell suspensions to avoid high shear forces during transfer or resuspension.*

Adherent cells harvesting

1. Wash cells with sterile DPBS.
2. Dissociate cells from the culturing flask using your preferred dissociation reagent (e.g. TrypLE).
3. Resuspend the dissociated cells to the preferred volume of media and filter with a 30 nm cell strainer.
4. Optionally, add EDTA in case of strongly adherent cells (5 mM final concentration).
5. Centrifuge the cells at 300 g for 7 minutes to remove cell culture media. Discard the supernatant.
6. Resuspend the cells to 1 mL of DPBS.
7. Measure cell concentration and adjust to 1E+06 cells/mL.

TIP | *Cell media diluted 1:4 with DPBS can be used to add nutrients to cell samples in case of sensitive cells or long assay workflow.*

Cell Fixation

1. Centrifuge up to 10E+06 cells at 300 g for 7 minutes to remove cell culture media. Discard the supernatant.
2. Resuspend the cell pellet in 1 mL PBS, centrifuge and discard the supernatant.
3. Resuspend the cell pellet in 500 µL PBS/2% PFA (62.5 µL of 16% PFA solution + 437.5 µL PBS).
4. Incubate the cells at room temperature for 10 minutes (with intermittent gentle shaking).
5. Add 500 µL of PBS and centrifuge the suspension at 400 g for 5 minutes to remove the PFA.
6. Discard the supernatant.
7. Resuspend the cells in 1 mL PBS, filter through a 30 µm cell strainer, centrifuge (400 g, 5 min) and discard supernatant.
8. Resuspend the cells in about 1 mL of PBS (optional: adjust to a final concentration of 5E+06 cells/mL).
9. Store fixed cells at 2-8°C, use within a month.

NOTE

- *If more cells should be fixed, please scale up all amounts accordingly.*
- *Centrifugation speed during harvesting of cells can be adjusted to cell type, if cells are sensitive and lower g-forces are commonly used.*
- *PFA concentration may be varied between 0.5 and 4%.*

scIC Assay setup

The following **scIC** assays should be included in assay development and can be repeated within assay workflows later on. The assay development workflow is divided into 3 distinct consecutive steps:

1. Cyto chip test
2. Analyte only test
3. Kinetics assay

Cyto Chip Test

The quality of a new chip needs to be evaluated before using a cyto chip in a measurement. Begin by running a **Cyto Chip Test** separately. See **Helix cyto chip** chapter in the main guide on how to set up and assess a chip test.

Analyte Only Test

The development of an **scIC** assay begins with an **Analyte Only Test**, which evaluates the behavior of fluorescently labeled analytes on a fresh chip surface without cells. This test can also be repeated periodically to assess the stability of labeled analytes. **scIC Analyte Only Test** can be configured in Kinetics assays by enabling the ***Analyte only*** checkbox under Cell settings. For analyte quality assessment, the highest used concentration is sufficient and can be configured by disabling all but one of the associations in the assay.

Kinetics Assay Workflow Setup

The kinetics assay is normally used within an automated workflow that contains both data-generating assays as well as maintenance elements. Once the analyte has passed the quality control Analyte only test, it can be used in a measurement on cells. In order to quantify kinetic rates reliably, the signal response during association should be concentration-dependent, increasing with higher analyte concentrations. The highest concentration should reach a plateau, which indicates that binding steady-state has been achieved. The dissociation needs to be run long enough to unbind a significant portion of bound analyte (more than 5%) to enable reliable fitting of the curve. Use the default parameters in the assay conditions as a starting point and adjust further depending on your results.

Example 1. Recommended assay workflow

The assay workflow allows users to queue measurement and maintenance methods based on their specific experimental needs. An assay workflow may include the following steps in the indicated order:

1. **Prime** (Maintenance chip)
2. **scIC Kinetics** (Cells A, Analyte A, Cyto chip)
3. **System Wash** (Maintenance chip)
4. **scIC Kinetics** (Cells B, Analyte A, Cyto chip)
5. **System Wash** (Maintenance chip)
6. Analyte only configured **scIC** Kinetics (Analyte A, Cyto chip)

Prime and System Wash steps are performed on a Maintenance Chip. A System Wash is included when switching to a different cell line and before conducting the Analyte Only Test at the end of the workflow. For more details, refer to the section on Cyto system washing in the main guide.

When queuing kinetics assays on cells, consider cell viability, which is dependent on the cell line. For sensitive cell lines, it is recommended to run only 1-2 assays per assay workflow. For more resilient cell lines, additional assays can be queued.

NOTE | *For additional assays, prepare cell solution in separate vials by naming the cells differently.*

Ensure all reagents are fresh and filtered through a 0.22 µm filter. Finally, place the prepared reagents into the instrument as instructed by **heliOS**. The run can be started by clicking the blue arrow "Run" icon and following the steps in the Assay Start Wizard.

Once the run has started, the device operates independently until the entire assay workflow is completed.

Analysis of scIC experiments

scIC Analysis Workflow

scIC data can differ from standard biosensor data due to the natural complexity of events occurring on cells captured on the **heliX**^{cyto} chip surface, which may result in sensorgram irregularities. Therefore, quality control is emphasized for images captured at each measurement step and for the generated sensorgrams in the landing page of the automated analysis. Additionally, **heliOS** provides tools to address sensorgram irregularities during **scIC** manual data analysis.

scIC data analysis process:

1. **Image Inspection:**
   Inspect all images captured throughout the measurement (*Images* tab in the experiment window).
   - How many cells remained stably in the traps throughout the entire measurement?
   - What is the state of the cells? Check for granularity and cell integrity.
   - Are the traps clean after the regeneration step?
2. **Raw data check:**
   Examine the raw data for reference spot 1 and measurement spot 2
   - The signal of the normalization peak should be close to or higher than the highest raw data signal.
   - Does the signal return to the baseline level on both spots, or is there evidence of unspecific binding?
3. **Normalized Data Check:**
   - Are the injection jumps for spot 1 and spot 2 overlaying properly?
4. **Referenced Data Check:**
   - Does the regeneration step bring the signal back to baseline? If not, check if there are cells or debris in the traps after regeneration by reviewing the images.
   - Are there spikes, outliers, or other irregularities in the referenced curves? If so, review the images to identify potential causes and consider manual adjustments.
5. **Data Fit Check:**
   Performing a visual quality assessment
   - Does the fit accurately describe the behavior of the curves, or does the fit line cross the sensorgram?
   - Does the fit appropriately describe the curvature of the data?
   - Is the amplitude consistent with the referenced data?

scIC Automated Data Analysis

The **scIC** automated analysis processes raw data to quality control plots and fitted data in just a few clicks.

1. Open an **scIC** experiment by double-clicking on the selected experiment in the *Experiment List*
2. Click the large blue *Analyze* button at the bottom of the experiment tab.

3. From the experiment workflow, select the assay you would like to analyze.
4. Choose *Kinetics* **scIC** from the available analysis types and click *Next*.

NOTE	*If the experiment is still running, only completed assays will be shown in the list. Once an assay is selected, the next window will display all available analysis types specific to the method/assay used.*

5. If needed, configure the analysis in the following window. Default fit model is "Kinetics - Free end level" with the checkbox "Force fit end level to Zero" activated. Deviate from this model only if biology or data requires a more complex description.
6. Once the configuration is finalized, click *Analyze* to see all derived snapshots, raw and processed sensorgrams, and kinetics values.

Due to inherent complexity of the samples used for **scIC** assays, resulting sensorgrams can have irregularities. To address this, quality control of images and sensorgrams is emphasized in the automated analysis. If manual corrections or more in-depth analysis is required, tools for artifact corrections are provided within the manual analysis *Scratchpad*.

heliX^{cyto} Maintenance and Decontamination

heliX^{cyto} maintenance is essential to ensure the instrument's optimal performance and longevity. Regular upkeep includes cleaning procedures that prevent contamination and maintain the integrity of the system, enabling accurate results and smooth operation. Consistent care is critical for the instrument's reliability and the quality of experimental outcomes.

heliX^{cyto} maintenance entails two key procedures: **Cyto System Wash**, which can be integrated directly into an assay workflow, and **Clean & Sleep**, which is performed as a separate routine to maintain the instrument right before use after a previous longer overnight measurement or when not in use for more than 2 days.

Both **System Wash** and **Clean & Sleep** require the presence of a **heliX**® **Maintenance Chip** in the chip tray.

Clean & Sleep Routine

The **Clean & Sleep** routine is meant for cleaning and device shut-down/storage. The method rinses the fluidic tubing of a **heliX**® device first with ultrapure water and then with 70% ethanol. Finally, all tubes are vented with air. This cleaning procedure is fully automated and takes approximately 40 minutes. During the procedure, the ethanol is ejected into the water bottle, so the contents of the bottle should be discarded afterward. After a **Clean & Sleep**, the instrument enters a sleep mode and cannot be used until a **Wake Up & Prime** is performed. A **heliX**® **Maintenance Chip** is required for both runs.

IMPORTANT	*To prevent tube contamination, be sure to* **discard the contents of the water bottle** *(water/ethanol mixture) and* **empty the waste container** *after the cleaning procedure.*

Shut-down and long-term storage

For **extended periods of non-use, please remove the bottle** containing water and ethanol after the Clean & Sleep procedure from the buffer tray and **leave the tubing exposed to air**. Also remove the **heliX**® **Maintenance Chip**, and store in its original bag. This prevents backflow and contamination. **Power off device**.

Cyto system wash

heliX^{cyto} System Wash extensively cleans the microfluidic system using ***Cleaning Solution 3 (CS3)*** in the course of about 45 minutes. ***CS3*** is picked up in two stages. First needle and sample loop are filled and incubated to remove any contaminants.

NOTE	*Run a* **Cyto System Wash at least daily** *when the device is in use. We recommend to add the* **Cyto**

> **System Wash** *method to an assay workflow either after each assay (when switching to a new cell line or if sample quality is suboptimal) or to the end of an assay workflow. Exchange* **maintenance chip** *after a maximum of* **50 Cyto System Washes** *shown as Regenerations count in the Chip tray view of the Device control.*

scIC FAQs

1. ***Q: How often should I clean the device?***

   A: The fluidic system of **heliX**^{cyto} is kept clean with Cyto System Wash and Clean&Sleep methods. The microfluidic system is recommended to be cleaned via Cyto System Wash on a Maintenance Chip after each assay (especially when changing cell type) or latest at the end of an assay workflow. The Clean&Sleep procedure is to be applied right before use after a previous long measurement (e.g. in the morning after an overnight experiment) or when the **heliX**^{cyto} will not be in use for more than 2 days (shutdown).

2. ***Q: How long can the solutions: RB 1 / water/ CS 1 / CS 3 / Normalization solution be stored in the device?***

   A: Generally, before each measurement, all solutions are to be examined for the remaining volume and for potential turbidity or precipitation. In such a case the solution needs to be exchanged immediately. Water and RB 1 are to be replaced daily and RB 1 is to be filtered before each measurement. Diluted Normalization solution can be used for 2 days in a row. Cleaning Solutions are stable for approximately 3 days.

3. ***Q: How long can I use the* heliX^{cyto} *chip?***

   A: The expiry date of the **heliX**^{cyto} chip can be checked in the Chip tray tab in the Device section in **heliOS**. No guarantee for chip integrity can be given after the expiration date. Nevertheless, as long as the traps remain stable, the surface is clean, and the fluorescence background is below the cut-off indicated in the Chip Test, the chip is considered usable for measurements. Regular external chip cleaning (***Chip Cleaning kit*** CCK-1-1) is recommended to be performed after chip usage to significantly prolong chip lifetime.

4. ***Q: How long can I use the Maintenance chip?***

   A: The Maintenance chip is required to be replaced after 50 System washes (counted as regenerations in the Chip tray tab of the Device control in **heliOS**).

5. ***Q: How often should the* heliX^{cyto} *Chip Test be performed?***

   A: The Chip Test collects information about fluorescence background, cleanliness, and integrity of the chip before starting a new experiment. It is to be performed at least once when a new chip is being used, but is recommended to be performed before or at the beginning of each experiment to check the status of the chip. This enables measurement conditions to be adjusted and abnormalities in the sensorgrams to be traced to the chip or device.

6. ***Q: What is the analyte labeling chemistry?***

   A: A red or green fluorescent dye, respectively, is coupled by our labeling kits via NHS-esters to primary amines in protein analytes (e.g. lysines or N-terminus). Details and a troubleshooting section can be found in our **heliX**^{cyto} Labeling Kit red dye (***Labeling Kit red dye 2*** CY-LK-R2-1) and **heliX**^{cyto} Labeling Kit green dye (***Labeling Kit green dye*** CY-LK-G1-1) manuals from our webshop.

7. ***Q: Where to find the* heliOS *credentials and licence information?***

   A: The process of entering credentials and licence information is described in the **heliOS** Installation section in the **heliX**^{cyto} guide from our website (**heliX**^{cyto} ***guide***). Your licence information should be saved in the **scIC** folder on the **heliX**^{cyto} PC Desktop, which was created by our Application Specialist during training.

8. ***Q: What are the excitation/emission ranges of the* heliX^{cyto} *?***

   A: Excitation in red is 605-625 nm, emission (detection) is 655-685 nm. Excitation in green 490-510 nm, emission in green is 525-575 nm.

9. ***Q: How many times do I have to measure the same experiment for statistical reliability?***

   A: To achieve reliable average kinetic rates, 3-4 repetitions of 3-concentration Kinetics measurements are recommended to be performed in a homogeneous cell population. In a consistent data set, association and dissociation rates of replicates should be within a 2-4 times window.

10. ***Q: What is the sensitivity of* scIC *measurements?***

    A: The lower detection limit in terms of target expression depends on the analyte labeling, but typically **scIC** can

already measure kinetics on as few as 1000-10000 molecules per cell. To increase sensitivity, at least 5 cells are recommended to be captured, and analyte DOL of 5-10 and LED power are to be balanced to achieve maximum fluorescence counts.

11. ***Q: What are the detection limits of the* heliX^{cyto} *in terms of kinetic rates?***

    A: Please refer to the Technical specifications of the **heliX^{cyto}** for the most up-to-date technical limits, which can be found in the **heliX^{cyto}** guide (**heliX^{cyto}** ***guide***).

    - Dissociation constant K_d: 10 pM to 1 mM
    - Association rate constant k_{on}: 1E3 to 1E7 M-1s-1
    - Dissociation rate constant k_{off}: 1E-6 to 1 s-1

For detailed information please check our **heliX^{cyto}** guide from our website!

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Instruments and chips are engineered and manufactured in Germany.

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