GE Healthcare

IN Cell Analyzer

Water immersion optics

In high-content imaging, air objective lenses have been the standard due to their long working distance, which enables imaging of any sample type, and extended depth of focus, which captures more information in a single image. Additionally, their simple implementation makes them ideal for automated imaging. Air objectives, however, cannot have a numerical aperture (NA) greater than 1.0, limiting their light gathering ability and resolving power. When higher NA is required, an immersion medium must be used between the front lens of the objective and the sample. Water, a common immersion medium, enables imaging with objectives with an NA up to 1.2. Importantly, water immersion lenses also minimize spherical aberration when imaging thick biological samples in aqueous medium.

How does water immersion work?

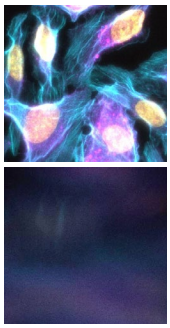
Water immersion optics are available as an option on IN Cell Analyzer 2200, 2500*HS*, 6000, and 6500*HS* systems. The Liquid Handling module must be installed to enable water immersion imaging as it is used to apply and remove water from the objective lens. These actions can be triggered automatically (see chart at right), while other times they are triggered by user-defined parameters in an experimental protocol.

Water *applied* to objective	Water *removed* from objective
Sample is loaded	Sample is ejected
Immersion objective selected	Active objective is changed from water immersion to any other objective
	Prior to water reapplication

There are many factors that affect how frequently water must be reapplied during a protocol, and optimizing these parameters requires some trial and error. During a protocol, water can be easily reapplied, enabling the flexibility required to ensure smooth and robust collection of images. However, the main disadvantage of frequent water reapplication is that it adds additional time to the acquisition overall, about 35 seconds per reapplication. Therefore, to optimize scan times, there are a few factors to understand when determining reapplication frequency during a protocol:

When should I reapply water?

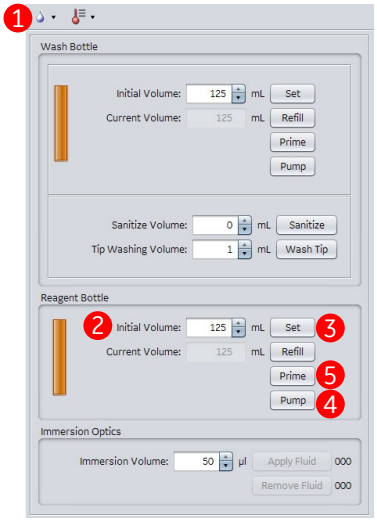
The best indicator that water should be reapplied is image quality. Once there is not enough water to seamlessly connect the gap between the front lens of the objective and the bottom of the sample, images will be very out-of-focus or only background will be visible.



- **Acquisition time.** The longer the experiment, the more water must be reapplied. As a general rule, water must be reapplied every 30 minutes during acquisition.
- **2D vs. 3D.** Acquisition of Z stacks requires increased frequency of water reapplication due to an increase in the number of images acquired, as well as overall acquisition time.
- **Plate material.** Plastic bottom plates require more frequent water application than glass because water is spread across the plastic surface more readily. Additionally, treatment of the plastic surface may alter the hydrophobicity of the plate requiring more frequent reapplication.
- **Sample temperature.** Higher temperatures cause water to evaporate more quickly, requiring more frequent water application.

Before you begin

1. Fill the reagent bottle.
   a. Open the door on the right side of the IN Cell Analyzer system.
   b. Remove the reagent bottle and fill to ~200 ml with sterile, deionized water.
   c. Replace the reagent bottle. Click the Liquid Handling (LH) ❶ icon and enter a value in the **Initial Volume** ❷ field. Click **Set** ❸.
2. Empty the waste bottle. Levels in the waste bottle must be monitored to make sure it's not full. Maximum levels should not exceed 1.5 L within a supplied 2 L bottle.
3. Click the LH ❶ icon and prepare the LH system for use:
   - If it has been less than a month since the LH system was used, click **Pump** ❹. The pump process will take ~20 seconds and will fill the dispense tube with water.
   - If it has been more than a month since the LH system was used, click **Prime** ❺. The prime process will take ~ 2 minutes and will fill tubing from the reagent bottle to the dispenser with water.



Load sample and verify plate map

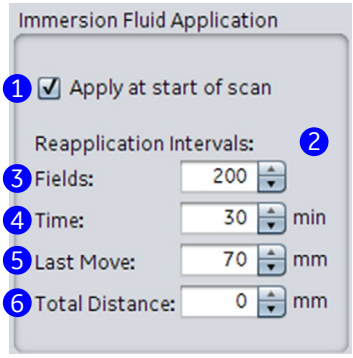
1. Load the sample and verify the plate map as described in the "Start-up and basic acquisition" Application Guide (29258710AA).
2. From the Dashboard of the IN Cell Analyzer acquisition software, select a water immersion objective from the **Objective** drop-down list.
3. When prompted, click **Yes** to apply water to the objective.
   Note: Click the LH icon and use the **Apply Fluid** and/or **Remove Fluid** controls at the bottom of the window to apply or remove water at any time.

Sample considerations

- Water immersion optics work best with low-skirt plates optimized for high-content imaging due to the smaller working distance of water immersion lenses. For example, imaging a plate with a 2.5 mm skirt height prevents imaging of small portions of the top row and left column of the plate.
- Samples should be in aqueous media to minimize spherical aberration.

Create protocol

1. Set up the protocol including Channels, Fields, Z-sectioning, Laser/Software Autofocus, etc., as usual.
   Note: Water immersion objectives have smaller depth of focus than lower NA air objectives so adjust Z section spacing accordingly.
2. In the Liquid Handling tab, click the **Apply at start of scan** ❶ check box to apply water at the beginning of the protocol.
3. In the Reapplication intervals ❷ section, input Reapplication Intervals according to the guidelines below. For more information on the functionality of the Reapplication Interval triggers, see "Immersion fluid reapplication tips".
   - **Fields** ❸**.** When a non-zero value is entered, water will be reapplied to the objective after the specified number of fields have been imaged.
     - For a 2D experiment, set **Fields** to ~200.
     - For a small 3D experiment with ~50 images in each Z stack (number of Z sections × number of channels) set **Fields** to ~150.
     - For a large 3D experiment with ~500 images in each Z stack (number of Z sections × number of channels) set **Fields** to ~15.
   - **Time** ❹**.** When a non-zero value is entered, water will be reapplied to the objective after the specified number of minutes have elapsed. For most experiment types, a **Time** value of 30 min is appropriate.
   - **Last Move** ❺**.** When a non-zero value is entered, water will be reapplied to the objective if the last move (i.e., move from well H12 to A1) exceeds the specified distance. For most plate scanning experiments, a **Last Move** value of 70 mm is appropriate.



   - **Total Distance** ❻**.** When a non-zero value is entered, water will be reapplied to the objective after the total distance traveled during the experiment exceeds the specified distance.
     - For plate scanning experiments, set **Total Distance** to 0. The Total Distance reapplication trigger is less useful for typical plate experiments when the other triggers (Fields, Time, and Last Move) are used as described.
     - When imaging many points over time on a non-SBS format sample type (35 mm dish, slides, etc.) enter a **Total Distance** value of ~1000 mm.
4. Select wells to be imaged, save the protocol, and click **Scan**.

Immersion fluid reapplication tips

The Reapplication Intervals triggers were implemented to ensure sufficient fluid is present at all times during an experiment without significantly increasing imaging time by adding unnecessary reapplication steps. Use the tips below to optimize Reapplication Interval parameters for your experiment.

- If a Reapplication Interval value is set to 0, that particular parameter will be ignored.
- Water will never be reapplied during acquisition of an FOV. Reapplication will occur after acquisition of an FOV is complete.
- Multiple reapplication triggers may be used during a single protocol. When one trigger is activated, all others will be reset. This helps reduce the overall number of reapplication events, and therefore acquisition time, of a single experiment. For example, if **Fields** is set to 200 and **Time** is set to 30 mins and imaging of 10 fields takes 30 minutes, water will be reapplied. After reapplication, the counter for **Fields** and **Time** will start again at 0.

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