Chromeo™ STED Immunofluorescence System

(version A1)

Catalog No. 15260

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Introduction

Fluorescent Microscopy is a widely used technique to detect specific proteins in their cellular environment and to answer questions regarding their localization, modification, interactions and life cycle. The most common immunofluorescence (IF) technique is indirect immunofluorescence, which is the labeling of a cellular protein with a primary antibody and the subsequent detection of the primary antibody with a fluorescent-conjugated secondary antibody. By using primary antibodies from different species (rabbit, goat, mouse, *etc.*), several different proteins can be visualized simultaneously with secondary antibodies conjugated to different color fluorophores (the fluorescent molecule conjugated to the secondary antibody). While in some cases it is advantageous to use primary antibodies directly conjugated to fluorophores, the use of secondary antibodies results in signal amplification and, consequently, greater sensitivity.

Immunofluorescence experiments attempting to describe the different properties of proteins have been limited by a very specific physical property, the Abbe Law of Limiting Resolution. The Abbe limit restricts the ability of the observer to visually resolve objects separated by less than ~200 nm. However, recent advances in super-resolution techniques such as STED (STimulated Emission Depletion) in combination with confocal scanning enable the observer to exceed the Abbe limit and to resolve details as small as 20 nanometers. This facilitates the imaging of sub-cellular structures that previously could not be visualized.

For super-resolution microscopy to yield clear, conclusive high-resolution images, it is extremely important to optimize the techniques and reagents used for sample preparation. Proper sample preparation is the most significant factor for obtaining high-quality images. The Chromeo ™ STED Immunofluorescence System takes the guesswork and challenge out of your immunofluorescence experiments by providing you with a complete set of proven, QC-tested IF reagents and an optimized protocol.

The Chromeo STED Immunofluorescence System contains enough reagents for preparing 24 immunofluorescence slides. It is recommended that you only use primary antibodies that have performed well in other IF techniques.

product	format	catalog no.
Chromeo™ STED Immunofluorescence System	1 kit	15260

The Chromeo™ STED Immunofluorescence System is for research use only. Not for use in diagnostic procedures.

Kit Performance

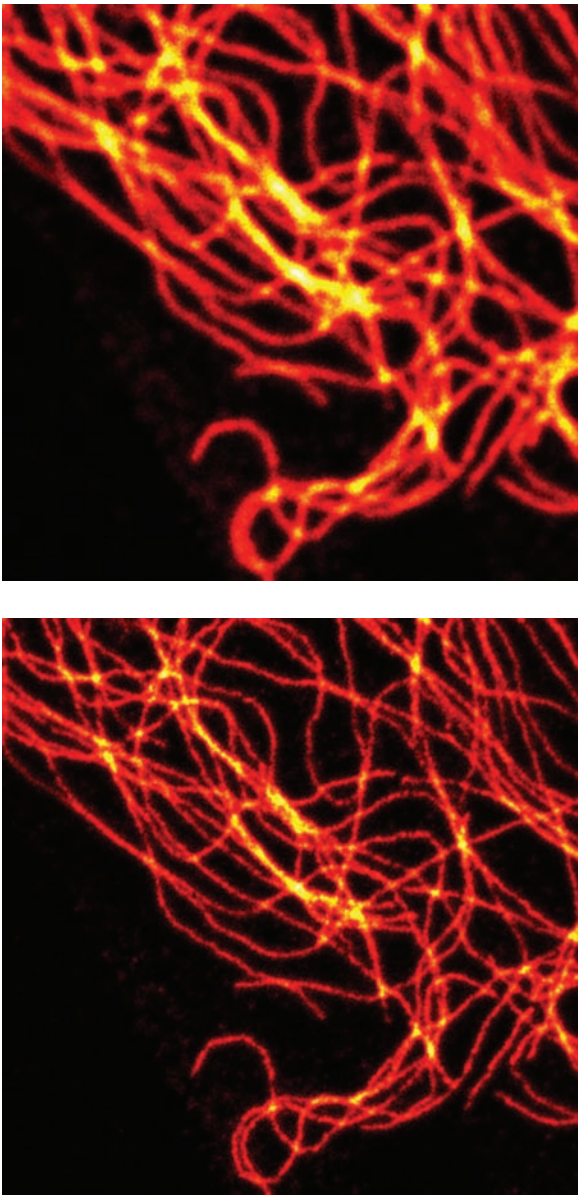

Figure 1: STED microscopy overcomes the Abbe limit, enabling much higher resolution than confocal microscopy. Microtubulin was stained with a primary polyclonal rabbit antibody and Chromeo™ 488 Goat anti-rabbit IgG (Catalog No. 15041) secondary antibody. The top image was prepared using a confocal microscope, while that on the bottom was produced using the Leica TCS STED CW microscope. Images courtesy of Leica Microsystems, Germany.

Kit Components and Storage

Please store each component at the temperature indicated in the table below and protect from light, if indicated.

Reagents	Quantity	Storage / Stability
MAX Stain™ Coverslips	50 coverslips	Room temperature
MAX Stain™ Slides	24 clean slides	Room temperature
MAXblock™ Blocking Medium	24 ml	4°C for 6 months
MAXbind™ Staining Medium	100 ml	4°C for 6 months
MAXwash™ Washing Medium	440 ml	4°C for 6 months
MAXfluor™ Mounting Medium S	650 µl	4°C for 6 months

Additional materials required

- Adherent cells of interest
- Cell culture plates
- Sterile growth medium
- Sterile tissue culture-grade 1X PBS
- Fixative of choice
- Reagents for Coverslip Preparation (see Protocol A for details)
- Reagents for Fixation (see Protocol C for details)
- Sterile tissue culture pipettes
- Clear nail polish

Required equipment

- CO_2 incubator or slide warmer
- Flat-edged forceps
- 2 glass beakers
- Rotating platform
- Microscope with appropriate filter sets

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

A. Preparation of Acid-etched Coverslips

Several protocols exist for coverslip preparation, especially as regards adhesion requirements of some cell lines. This method represents one option that provides a clean surface, which increases adhesion of the cells. Other methods to prepare the coverslips are also compatible with the kit.

Materials required

- 1 M HCl
- ddH_2O
- 2 glass beakers
- 95% ethanol
- Tweezers

1. Separate the coverslips and place them in a glass beaker.
2. Add 1 M HCl and heat at approximately 50°C for 6-12 hours.
3. Cool to room temperature.
4. Decant the HCl solution carefully and rinse the coverslips with ddH_2O.
5. Transfer the coverslips into a second glass beaker and fill with ddH_2O.
6. Sonicate in a water bath for 30 minutes.
7. Decant the solution, then add fresh ddH_2O and repeat the sonication step.
8. Decant the solution, then fill the beaker with 50% ethanol and 50% ddH_2O and sonicate in a water bath for 30 minutes.
9. Decant the solution, then fill the beaker with 70% ethanol and 30% ddH_2O and sonicate in a water bath for 30 minutes.
10. Decant the solution, then fill the beaker with 95% ethanol and sonicate in a water bath for 30 minutes.
11. Decant the solution, then fill the beaker with 95% ethanol.
12. Place a coverslip into the well of a 6-well cell culture plate with tweezers, then allow the ethanol to evaporate.

B. Cell Preparation

1. Grow the cells of interest in growth media to the desired confluence at 37°C in a CO_2 incubator. It is recommend that cells be grown to 50-90% confluence in a 10 cm plate.

2. Collect cells by pipette washing (or scraping) into a 15 ml conical tube. Pellet cells by spinning at 1000 x *g* for 5 minutes at 4°C, then aspirate the growth media. Gently resuspended the cells in 3 ml fresh growth media.
3. Add 2.5 ml of growth media to each well of the 6-well plate with an acid-etched ethanol-sterilized coverslip, then add 500 µl of the cell suspension from No. 2 above. Grow to ~50% confluence (approximately 20-24 hours).
4. Aspirate the growth medium and proceed to Protocol C.

C. Fixation

Please note that some antibodies, cellular structures and samples will respond very differently to different fixation methods. To stain microtubules, fixation in ice-cold methanol produces the best results. For actin staining, formaldehyde fixation is required.

C1. Methanol fixation

Materials required

- Methanol (pre-chilled to -20°C)

1. Add 3 ml methanol (pre-chilled to -20°C) to each well. Immediately place the entire plate in a -20°C freezer for exactly 7 minutes.

   Optional: Cells can be fixed overnight at 4°C.
2. Remove the plate from the freezer and carefully aspirate the methanol from the well.
3. Wash the cells 1X with 1 ml MAXwash Washing Medium. Aspirate to remove the MAXwash.

C2. Formaldehyde fixation and permeabilization

Materials required

- 4% Formaldehyde in PBS
- PBS
- 1% Triton X-100 in PBS

1. Add 4% Formaldehyde in PBS and incubate for 20 minutes at room temperature.
2. Wash the cells 2 x 5 minutes with PBS.
3. Add 1% Triton in PBS and incubate for 10 minutes at room temperature.
4. Wash the cells 2 x 5 minutes with PBS.
5. Wash the cells 1X with 1 ml MAXwash Washing Medium. Aspirate to remove the MAXwash.

D. Blocking

1. Add 1 ml MAXblock Blocking Medium to each well of fixed cells.
2. Incubate the 6-well plate for 1 hour at 37°C in a humid environment, *e.g.* an incubator or slide warmer.
3. After the incubation, aspirate the MAXblock, then add 1 ml MAXwash. Rock the plate for 10 minutes on a rotating platform. During the wash, prepare the Primary Antibody Dilution Mix.

E. Primary Antibody Incubation

1. Prepare Primary Antibody Dilution Mix by diluting the primary antibody of interest in 1 ml per well of MAXbind Staining Medium to your desired dilution. For example, to prepare 1 well of a 1:500 dilution, add 2 µl primary antibody to 1 ml MAXbind Staining Medium.
2. Aspirate the MAXwash and add 1 ml Primary Antibody Dilution Mix to each well.
3. Incubate for 1 hour at 37°C in a humid environment, *e.g.* an incubator or slide warmer.
4. Aspirate the Primary Antibody Dilution Mix from the cells and add 1 ml MAXwash, then rock the plate for 10 minutes on a rotating platform. Aspirate the MAXwash and repeat this wash step 2 more times for a total of 3 washes. During the last wash, prepare the Secondary Antibody Dilution Mix.

F. Secondary Antibody Incubation

Important: At this stage it is important to limit the amount of light exposure to any dye. Premature excitation of the dye will result in less than desirable imaging intensity. We strongly recommend wrapping the 6-well plates in aluminum foil, or similar, while working through the remainder of the protocol.

1. Prepare Secondary Antibody Dilution Mix by diluting the secondary antibody to the appropriate dilution in 1 ml MAXbind Staining Medium for each well. For example, to prepare 1 well of a 1:500 dilution, add 2 µl secondary antibody to 1 ml MAXbind Staining Medium. Most secondary antibodies can be used at dilutions between 1:500 and 1:2,000, though the optimal dilution for your cell line, primary and secondary antibodies will need to be determined empirically.
2. Aspirate the MAXwash and add 1 ml of the Secondary Antibody Dilution Mix to each well.
3. Incubate 1 hour at 37°C in a darkened, humid environment, *e.g.* an incubator or slide warmer.
4. Aspirate the Secondary Antibody Dilution Mix from the cells and add 1 ml MAXwash. Rock the plate for 10 minutes on a rotating platform. Aspirate the MAXwash and repeat this wash step 3 more times for a total of 4 washes.
5. For a single staining experiment, proceed to Step 7 below.

6. For a double staining experiment, perform the 2nd primary and secondary stainings by repeating the steps in Protocol E, and in Protocol F – Steps 1-4.
7. After the last wash, carefully remove the coverslip from the well using flat-edged forceps. In some cases, the coverslip is slightly stuck to the bottom of the well and may need to be dislodged using a beveled needle. Using the needle, carefully stand up the coverslip in the well and grab it with forceps.
8. Dry the coverslip to remove excess MAXwash. Hold a Kimwipe to the corner of the coverslip to wick away any excess MAXwash from the coverslip. Remember to limit light exposure as much as possible.

G. Slide Mounting

1. Perform mounting in indirect light. Mount the cells by adding 15 µl MAXfluor Mounting Medium S onto the coverslip, and then placing the coverslip face down onto the slide. A very light tap on the coverslip with a plastic pipette tip is the maximum force that is ever required, and the presence of bubbles under the coverslip is not a concern. Attempting to eliminate bubbles is likely to cause damage to the coverslip or the sample.
2. Gently wipe away any excess mounting medium that leaks out of the sides of the coverslip, then seal the coverslip to the slide with clear nail polish by applying it around the edges, and allow it to cure.
3. Image your slides using your preferred protocol.

Appendix

Section A. Related Products

Fluorescent Cell Stains	Format	Catalog No.
LavaCell™ Live Cell Membrane Staining Kit	200 µg	15004
Chromeo™ Live Cell Mitochondrial Staining Kit	1 kit	15005
Chromeo™ Red Fluorescent Fixed Cell Staining Kit	1 kit	15006

Fluorescent Secondary Antibodies	Excitation / Emission (nm)	Format	Catalog No.
Chromeo™ 488 Goat anti-Mouse IgG	488 / 517	1 mg	15031
Chromeo™ 488 Goat anti-Rabbit IgG	488 / 517	1 mg	15041
Chromeo™ 494 Goat anti-Rabbit IgG	494 / 628	1 mg	15042
Chromeo™ 546 Goat anti-Mouse IgG	545 / 561	1 mg	15033
Chromeo™ 546 Goat anti-Rabbit IgG	545 / 561	1 mg	15043
Chromeo™ 642 Goat anti-Mouse IgG	642 / 660	1 mg	15034
Chromeo™ 642 Goat anti-Rabbit IgG	642 / 660	1 mg	15044
ATTO 594 Goat anti-Mouse IgG	601 / 660	250 µl	15037
ATTO 594 Goat anti-Rabbit IgG	601 / 660	250 µl	15047
ATTO 647N (STED) Goat anti-Mouse IgG	644 / 669	250 µl	15038
ATTO 647N (STED) Goat anti-Rabbit IgG	644 / 669	250 µl	15048
ATTO 655N (STED) Goat anti-Mouse IgG	663 / 684	250 µl	15039
ATTO 655N (STED) Goat anti-Rabbit IgG	663 / 684	250 µl	15049

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