

FilmTracer™ Calcein Biofilm Stains

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
FilmTracer™ calcein red-orange biofilm stain	20 × 50 µg	<ul style="list-style-type: none"> • ≤-20°C • Desiccate • Protect from light 	When stored as directed, the product is stable for at least 6 months.
FilmTracer™ calcein violet biofilm stain	20 × 25 µg		
FilmTracer™ calcein green biofilm stain	20 × 50 µg		

Approximate fluorescence excitation/emission maxima: FilmTracer™ calcein red-orange biofilm stain: 576/590 nm; FilmTracer™ calcein violet biofilm stain: 400/450 nm; FilmTracer™ calcein green biofilm stain: 494/514 nm.

Introduction

Biofilms present a unique set of challenges for fluorescent staining and subsequent imaging. A typical biofilm not only exhibits heterogeneous thickness throughout the surface, placing stringent restrictions on stain penetration, but also contains regions of widely varying environmental conditions. Evidence suggests that bacterial cells exist in various physiological states within these biofilm microenvironments. Furthermore, biofilms contain many undefined components (*e.g.*, the extracellular polymeric matrix) that differ with species and conditions.

FilmTracer™ calcein red-orange, FilmTracer™ calcein violet, and FilmTracer™ calcein green stains are acetoxymethyl (AM) ester derivatives of fluorescent indicators and chelators that make up one of the most useful groups of compounds for the study of live cells. Modification of carboxylic acids with AM ester groups results in an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a charged form that leaks out of cells far more slowly than its parent compound. Frequently, hydrolysis of the esterified groups is essential for binding of the target ion. In most cases (*e.g.*, calcein green), the AM ester is colorless and nonfluorescent until hydrolyzed. This property is useful in diagnosing spontaneous hydrolysis during storage. The calcein red-orange version is fluorescent prior to cleavage; however, the intracellular fluorescence is much brighter than background fluorescence after rinsing.

The FilmTracer™ calcein biofilm stains are proving to be excellent, reliable indicators of esterase activity or cell viability in staphylococci sp. In a flow-cell grown *Staphylococcus epidermidis* biofilm, the antimicrobial action of alkyl dimethyl benzyl ammonium chloride (ADBAC) was visualized with calcein green. The time-lapse confocal scanning laser microscopy examination of fluorescence showed permeabilization of cells by the biocide.^{1,2} Calcein AM green stain has also been used to observe biofilm permeabilization effects with time-lapse fluorescence microscopy as bacteria alighted on chitosan-coated surfaces.³

FilmTracer™ calcein red-orange biofilm stain (excitation/emission maxima of ~576/590 nm; Figure 1) and FilmTracer™ calcein violet biofilm stain (excitation/emission maxima of ~400/450 nm; Figure 2) may be useful in these applications, or in combination with spectrally distinct fluorescent proteins or other fluorophores.

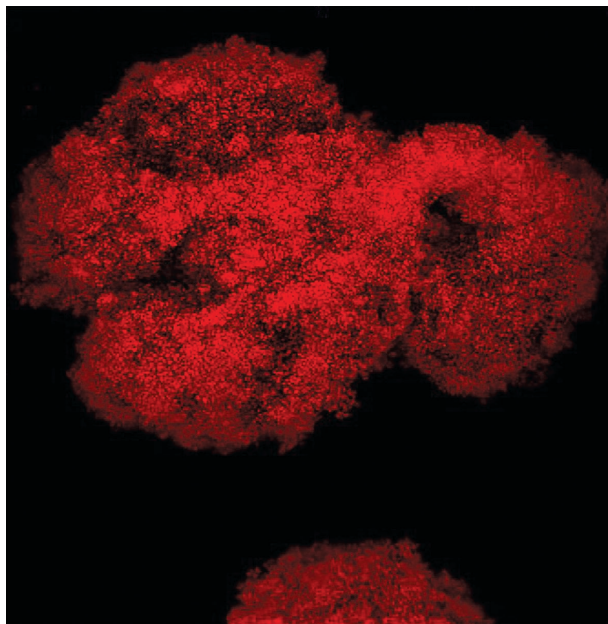


Figure 1. FilmTracer™ calcein red-orange AM applied to a *Staphylococcus epidermidis* biofilm. The esterase substrate FilmTracer™ calcein red-orange AM appears to stain all of the bacteria in the biofilm, suggesting that the bacteria are all actively producing esterase. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63X/0.9 NA water immersion objective.

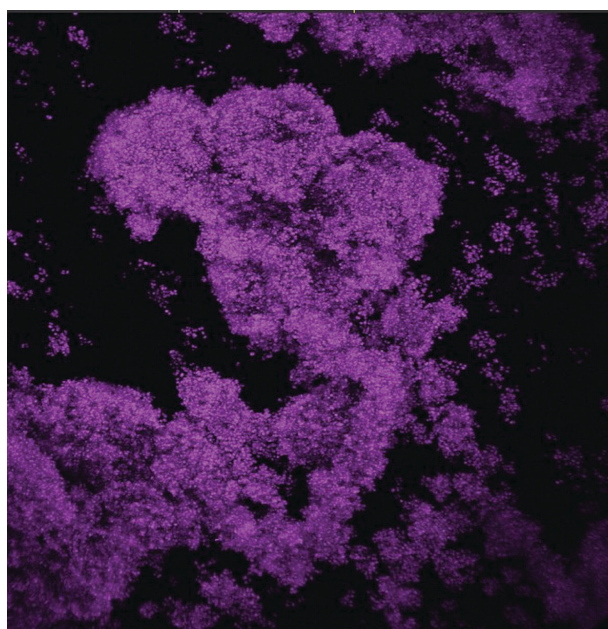


Figure 2. FilmTracer™ calcein violet AM applied to a *Staphylococcus epidermidis* biofilm. The esterase substrate FilmTracer™ calcein violet AM appears to stain all of the bacteria in the biofilm, suggesting that the bacteria are all actively producing esterase. The image was obtained using a Leica TCS-SP2 AOBS confocal microscope with a 63X/0.9 NA water immersion objective, and a Spectra-Physics Mai Tai® two-photon confocal microscope.

Before You Begin

Materials Required but Not Provided

- Biofilm samples: Biofilms may be grown on coupons in a biofilm reactor, as colony biofilms, in flow-cell system, or in drip-flow reactors. This protocol describes staining biofilms grown on glass coupons in a CDC reactor. For more information on the CDC reactor or other reactor types, refer to the BioSurface Technologies website (www.imt.net/~mitbst/Products.html), contact Center for Biofilm Engineering, Montana State University, Bozeman, Montana, or refer to the standard protocols outlined in the following ASTM methods: ASTM E2647, ASTM E2562, ASTM E2196.
- Fluorescence microscope with appropriate excitation/emission filters
- 0.2 μm filter-sterilized water
- Staining dishes (*e.g.*, 60-mm dish, 6-well plate, etc.)
- Anhydrous dimethylsulfoxide (DMSO)

Preparing Reagents

Reconstitute AM esters only as required using high-quality, anhydrous dimethylsulfoxide (DMSO). Store reagent-grade DMSO well sealed under argon or nitrogen, and desiccated; you may use desiccant beads (*e.g.*, molecular sieves) for short-term storage. Dissolution of the pure AM esters in DMSO may be slow. Once prepared, use the DMSO stock solutions of AM esters within a short time period for one series of experiments. Keep DMSO stock solutions anhydrous, because the solvent readily takes up moisture, leading to decomposition of the dye. Store stock solutions well sealed, frozen, and desiccated.

Keep the AM esters in as concentrated a stock as possible so that minimal amounts (ideally $\leq 0.1\%$) of DMSO are present in the loading solutions.

FilmTracer™ calcein red-orange biofilm stain: Prepare a stock solution by adding 150 μL DMSO to one vial. Mix thoroughly until contents are completely dissolved.

FilmTracer™ calcein violet biofilm stain: Prepare a stock solution by adding 50 μL DMSO to one vial. Mix thoroughly until contents are completely dissolved.

FilmTracer™ calcein green biofilm stain: Prepare a stock solution by adding 100 μL DMSO to one vial. Mix thoroughly until contents are completely dissolved.

Caution

- FilmTracer™ calcein stains are potentially harmful, and may cause sensitization by inhalation. FilmTracer™ calcein red-orange biofilm stain may cause cancer by inhalation.
- Avoid prolonged or repeated exposure. Wear appropriate gloves, protective clothing and eyewear and follow safe laboratory practices. Wash thoroughly after handling.
- If eye or skin contact occurs, wash affected area with water for 15 minutes and seek medical advice. If inhaled, move individual to fresh air and seek medical advice. If swallowed, seek immediate medical advice.
- Use appropriate protective equipment and methods to clean up spilled substances promptly. Absorb spill onto an appropriate material. Collect and dispose of all waste in accordance with applicable laws.

Experimental Protocols

Guidelines for Staining

- If you need to stain and image multiple samples, do not stain more than two samples at a time. Evidence suggests that, in many cases, stain might be drawn from cells over time as they sit in water. Stagger staining, so that samples are stained, rinsed, and imaged following the same schedule. Image immediately following rinsing.
- We recommend performing staining in water as the phosphates in buffers may interfere with fluorescent staining.
- For imaging biofilm on CDC reactor coupons, use glass coupons only. In particular, avoid polycarbonate coupons for imaging purposes as polycarbonate is autofluorescent, and the rough surface interferes with imaging.
- If you follow the protocol below, you do not need to use fixatives on the biofilm.

Staining Protocol

- 1.1. Prepare staining solutions by diluting 20 μL of the stock solution into 980 μL filter-sterilized water.
- 1.2. Add 200 μL (or appropriate volume) of staining solution onto the biofilm sample. Add the stain very gently so as not to disturb the biofilm. It is important to immediately add the stain before the biofilm dries.
- 1.3. Incubate the sample for up to one hour at room temperature, **protected from light**.
- 1.4. Rinse the sample gently with filter-sterilized water. Remove all excess stain and rinse water from the base of the support material.
- 1.5. For best results with reactor coupons, place coupon in a 60-mm dish, fill the dish with filter-sterilized water to cover the coupon surface by 1–3 mm, and observe on the microscope using a 40X 0.7NA 3.3 mm WD water objective or a 63X 0.9NA 2.2 mm WD water immersion objective.

References

1. J Bacteriol 189, (2007);
2. Microscopy Today 16, 18 (2008);
3. J Biomater Sci Polym Ed 19, 1035 (2008).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
F10319	FilmTracer™ calcein red-orange biofilm stain	20 × 50 µg
F10320	FilmTracer™ calcein violet biofilm stain	20 × 25 µg
F10322	FilmTracer™ calcein green biofilm stain	20 × 50 µg
Related Products		
F10317	FilmTracer™ FM® 1-43 green biofilm cell stain	1 mg
F10318	FilmTracer™ SYPRO® Ruby biofilm matrix stain	200 mL
L10316	FilmTracer™ LIVE/DEAD™ Biofilm Viability Kit	1 kit
S34854	SYTO® 9 green fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL

Contact Information

Molecular Probes, Inc.

29851 Willow Creek Road
Eugene, OR 97402
Phone: (541) 465-8300
Fax: (541) 335-0504

Customer Service:

6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338
Fax: (541) 335-0305
probesorder@invitrogen.com

Toll-Free Ordering for USA:

Order Phone: (800) 438-2209
Order Fax: (800) 438-0228

Technical Service:

8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 335-0353
Toll-Free (800) 438-2209
Fax: (541) 335-0238
probestech@invitrogen.com

Invitrogen European Headquarters

Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100
Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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