Protocol

Fluorescent Visualization of Germline Apoptosis in Living *Caenorhabditis elegans*

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Visualization of apoptosis using fluorescent tools is quite straightforward in living *Caenorhabditis elegans*. Several transgenic lines are available that mark dying cells with fluorescent proteins, making it possible to quantify kinetics at various stages of the apoptotic process. Proteins required for the engulfment of cell corpses are particularly useful for detecting early apoptotic stages using this approach. For example, expression of the engulfment protein CED-1 fused to green fluorescent protein (CED-1::GFP) creates a halo around cells during early apoptosis, before their refractile morphology can be detected by differential interference contrast (DIC) optics. In addition, vital dyes such as acridine orange (AO) and SYTO-12 are selectively retained in apoptotic cells and can be used to visualize apoptosis in the germlines of living animals. It is also possible to use vital dyes in combination with transgenic strains expressing fluorescent markers of cell corpses to examine, in detail, multiple stages of apoptosis in vivo. Because of the high optical contrast of fluorescent reagents, apoptosis can be visualized even at low magnification, facilitating the use of screening platforms to identify apoptosis regulators. This protocol describes multiple uses of fluorescent reagents for visualization of germline apoptosis in living *C. elegans*, including AO staining, time-course studies using fluorescent proteins, and low-throughput screening of cell death genes using RNA interference (RNAi).

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous material used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Acridine orange (AO) (10-mg/mL stock solution) (Molecular Probes, Inc.)
Immediately before use, dilute 7.5 µL of AO stock solution into 1 mL of M9 buffer for worms <R>.

C. elegans strain of interest, in which apoptosis has been stimulated.
We recommend the following fluorescent reporter strains because of their broad applications for quantifying apoptosis and utility for screening; see Discussion.

- *MD701 (CED-1::GFP fusion protein)*
  *Genotype: bcIs39 (*Plim-7*::CED-1::GFP* + lin-15*[+])V*

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- *WS2170 (YFP::ACT-5 fusion protein)*
  *Genotype: opIs110 (*Plim-7*::YFP::ACT-5* + unc-119*[+])IV*

Both are available from the Caenorhabditis *Genetics Center (CGC) and can be used as germline apoptosis indicators in conjunction with RNAi treatment, application of stressors, or after crossing into mutant strains. Alternatively, there are other fluorescent reporter strains available from the CGC that mark various stages of the engulfment process.*

Young adult worms are used for AO staining (see Step 1), while both embryos and adults can be used for time-course studies (see Step 5). The RNAi procedure begins with worms at the L1 larval stage (see Step 9). Activate apoptosis in the worms of interest by perturbing gene function (e.g., with RNAi) or by exposing the germline to various stressors (e.g., DNA-damaging agents). (See Protocol: ***Induction of Germline Apoptosis in* Caenorhabditis elegans** *[Lant and Derry 2014a].)*

Plates (35-mm or 24-well) containing solid nematode growth medium (NGM) <R>
These plates may be seeded with bacteria (e.g., HT115 or OP50) as appropriate.

RNAi clones from the *C. elegans* RNAi feeding library (Source BioScience LifeSciences)
Tetramisole (20 mM)

Equipment

Compound microscope with differential interference contrast (DIC) and fluorescence optics (e.g., Leica DMRA)
Fluorescence dissecting microscope (with optional camera attachment)
Immersion oil
Slide-mounting setup, as described in Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** (Lant and Derry 2014b)

METHOD

Staining Apoptotic Corpses in the Adult Germline with AO

AO is used for staining of the young adult germline. Vital dyes such as AO are not effective for detecting apoptosis in developing embryos because they cannot penetrate the egg shell. In addition, vital dyes rely on engulfment signals; thus, engulfment-defective mutants cannot be labeled with AO (Gartner et al. 2008; Neukomm et al. 2011).

1. Pipette 200 µL of freshly diluted AO solution (75 µg/mL) onto a plate containing at least 25 target young adult worms on a bacterial lawn. Distribute the solution evenly by carefully rotating the plate. Allow the plate to dry with its lid slightly ajar for ~20 min in the dark.

   We recommend using at least 25 target worms to ensure that germlines can be visualized in a minimum of 15 animals per condition.

2. Replace the lids so that plates are completely covered and allow the worms to continue feeding on the AO-soaked bacterial lawn in the dark for 1 h.

   The ingested AO will dissipate throughout the worm's body and concentrate in apoptotic nuclei.

3. Transfer the worms to a clean NGM plate containing the appropriate bacterial lawn. Incubate the plates in the dark for 3 h to clear excess AO from the intestines. Observe the worms under a dissecting microscope before slide mounting to be sure background has been reduced appropriately (Fig. 1).

   For RNAi experiments, plates used for this step can be seeded with the target RNAi bacteria or the HT115 strain, as the RNAi effect will last for several hours.

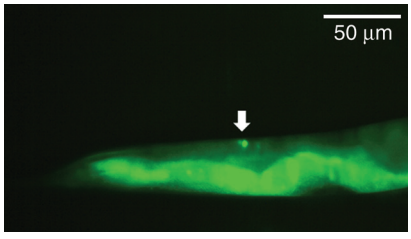

FIGURE 1. AO-stained germline viewed at 5× magnification (in the GFP channel) using a fluorescence dissecting microscope. The apoptotic corpse, indicated by the arrow, is clearly identifiable by the strong AO signal. The intensity of AO staining in the corpse relative to the surrounding cells is a good indicator (or prescreen) of how effective the staining/destaining process has been.

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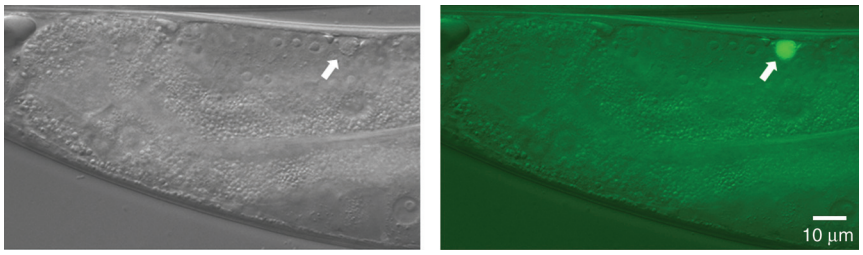

FIGURE 2. AO staining of a germline corpse. The vital dye enters the corpse as membrane permeability increases (mid- to late-stage apoptosis) and intercalates DNA. The *left* panel shows a DIC image of a germ cell corpse (arrow) among healthy germ cells in the pachytene bend region of the posterior germline. Shown in the *right* panel is the same corpse stained with AO (arrow). Images taken at 630× magnification.

The time required for destaining may require some optimization, based on the eating habits of your worms. You will rarely obtain a signal that is too low, but too much background is common. A general ratio of 1:3 staining:destaining time should be appropriate.

See Troubleshooting.

4. Slide-mount the worms on agar pads as described in Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** (Lant and Derry 2014b). Observe on a compound microscope with fluorescence optics.

   When viewing AO-stained corpses, be careful not to mistake germ cell corpses with spermatocytes, which will be closer to the uterus, past the bend region of the gonad, and will also take up AO (and SYTO-12) dyes. Stained corpses are found in the pachytene region of the distal germline (Fig. 2).

   See Troubleshooting.

Time-Course Studies Using Fluorescent Proteins

CED-1::GFP can be used to visualize apoptotic corpses in both developing embryos and the germline (Fig. 3) (Kinchen et al. 2008;Yu et al. 2008); however, we find ACT-5::YFP to be a superior marker for the fluorescent detection of apoptotic cells in the germline (Fig. 4) (Kinchen et al. 2008). (See Discussion.)

When using either of the recommended strains in conjunction with AO staining (as shown in Fig. 5), destain more rigorously to optimize detection of both fluorescent signals; we recommend a 1:4 ratio of staining:destaining time.

5. Slide-mount embryos or worms of a fluorescent strain as described in Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** (Lant and Derry 2014b).

6. Using the 10× lens on a compound microscope, identify the samples on the slide. Move to the 63× oil immersion lens and focus on a point of the embryo or germline with readily visible corpses (the more, the better).

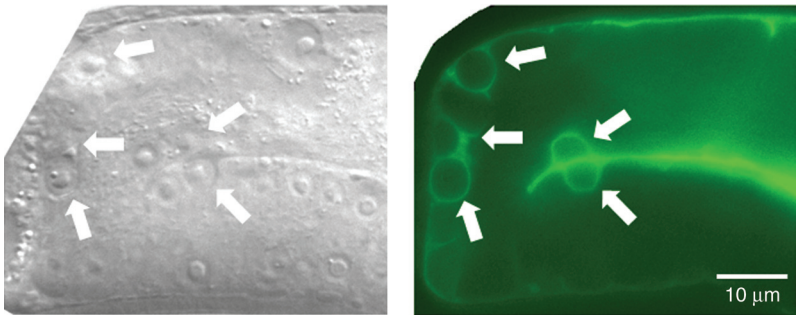

FIGURE 3. Germline corpses in a worm expressing the CED-1 protein fused to GFP in the gonadal sheath cells using the *lim-7* promoter following irradiation (60 Gy). The CED-1::GFP marker indicates early-stage corpses, as gonadal sheath cells surround the apoptotic bodies. The *left* panel shows the corpses under DIC optics, while the *right* panel shows the corpses in the GFP channel. Images taken at 630× magnification.

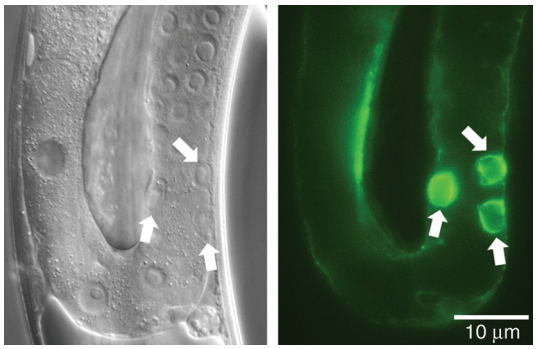

FIGURE 4. Germline corpses in worms expressing the actin protein ACT-5 fused to the yellow fluorescent protein (YFP) following irradiation (60 Gy). As gonadal sheath cells surround the apoptotic bodies, actin cables are remodeled, pushing the corpse up (giving the "button"-like appearance). The *left* panel shows the germ cell corpses under DIC optics (bright field), and the *right* panel shows the same germline in the YFP channel. ACT-5::YFP, like CED-1::GFP, forms halos around early stage corpses. White arrows indicate corpses. Images taken at 630× magnification.

Corpses detected by CED-1::GFP and ACT-5::YFP mark early apoptotic stages, so you may not always observe refractile bodies coinciding with strong fluorescence signals. We and others have also noticed CED-1::GFP signal surrounding some cells that do not undergo apoptosis (Craig et al. 2012), so exercise caution when quantifying corpses with this marker.

7. Capture a sequence of images over the span of ~1 h; one image in several *Z* planes every 3–5 min is ideal. Shutter the fluorescent light source in between images to minimize photobleaching. If possible, note the depth (in the *Z* plane) of the starting image because the agar pad will dry over time and offset the image stacks. Compensate for shrinkage of the agar pad and/or stage drift by adjusting the stage periodically to maintain the bottom plane of the worm or embryo where the first image was taken.

   Alongside the fluorescent image, an image in the corresponding plane in white light should also be taken to allow for comparison of changes in corpse morphology in both spectra—specifically the formation of button-like corpses, which follows loss of fluorescent signals. The difference between fluorescence and white light imaging is shown in Figure 5.

   See Troubleshooting.

Low-Throughput RNAi Screening Using Fluorescent Reagents

The following method describes low-throughput screening of RNAi-induced apoptosis using low magnification (i.e., a dissecting microscope). This is appropriate for screening <100 genes. When screening very low numbers, you may instead wish to use Protocol: ***Induction of Germline Apoptosis in*** **Caenorhabditis elegans** *(Lant and Derry 2014a).*

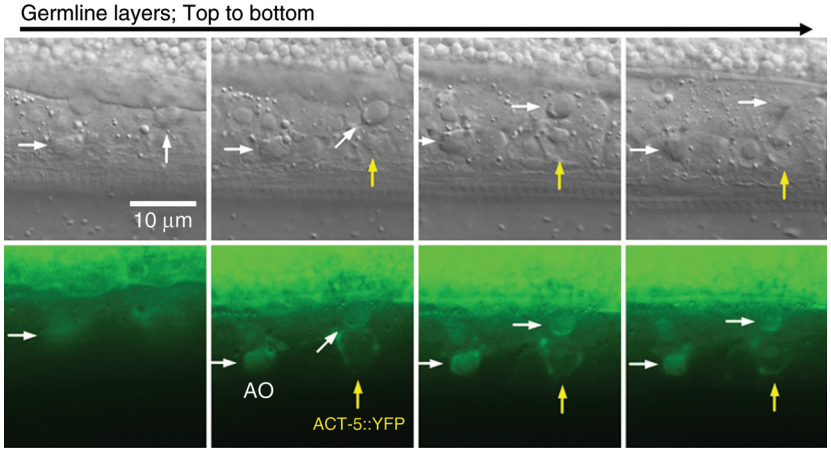

FIGURE 5. Concurrent imaging of early- and late-stage apoptotic corpses in the *C. elegans* germline. The images from *left* to *right* represent layers (from *top* to *bottom*) of the germline. White arrows indicate AO-stained late-stage corpses, while the yellow arrows indicate an ACT-5::GFP-labeled early-stage corpse. Images taken at 630× magnification. (Scale is indicated in the first panel.)

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To screen 100 or more genes, see Protocol: ***High-Throughput RNAi Screening for Germline Apoptosis Genes in*** **Caenorhabditis elegans** *(Lant and Derry 2014c). Low-magnification prescreening of apoptosis induced by various stressors can also be performed on single plates by following Steps 11–13.*

8. Add 30 µL of RNAi liquid culture to each well of a 24-well plate containing solid NGM.

   Plates should be thoroughly labeled, or a template created for reference, to avoid confusing RNAi samples between wells.

9. Using the hypochlorite method to synchronize populations of worms at the L1 (Stiernagle 2006), add 20–30 L1 worms to each well and grow to the young adult stage.

10. (Optional) Stain the worms with AO.

    i. Transfer the worms to a fresh 24-well plate containing solid NGM.

    ii. Pipette 20 µL of freshly diluted AO solution (75 µg/mL) into each well. Allow the worms to stain in the dark for 1 h.

    iii. Transfer the worms to a fresh 24-well plate containing solid NGM seeded with OP50 bacteria. Destain for 3 h.

    As mentioned above, a 1:3 stain:destain ratio is a starting point, but may require optimization.

11. Observe the worms under the lowest magnification lens of a dissecting microscope. Move to the desired well and select the appropriate fluorescence channel.

12. Focus on worms that show fluorescence at a low magnification. Apply a drop of tetramisole to the desired well. Wait ~30 sec for the tetramisole to immobilize the worms.

    This process—maintaining your eye on the moving worm through the eyepiece, while attempting to drop tetramisole onto the plate in the correct location—can be tricky at first.

    Note that CED-1::GFP and ACT-5::YFP should both produce visible signals under low magnification with a dissecting microscope, but the resolution of single cells (i.e., with visible individual halos) is clear only at higher magnifications. Thus, these markers are largely inappropriate for quantification of apoptosis during low-magnification screening; see Discussion.

13. Increase the magnification to visualize germlines in more detail. For high resolution imaging, carefully pick and transfer the worms to slides for mounting as described in Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** (Lant and Derry 2014b).

    As with DIC optics, the intestine can sometimes obscure the signal and make it difficult to resolve individual corpses.

    If your dissecting microscope has a camera attachment, it may be possible to quantify apoptosis using a snapshot of the image; see Discussion.

TROUBLESHOOTING

Problem (Step 3): The AO background signal is too strong.

Solution: While a 1:3 staining:destaining ratio is suggested, it may be necessary to increase the destaining time, especially when using the aforementioned fluorescent strains in conjunction with AO. Check the worms in 20- to 30-min intervals until the background signal is minimal. Luckily, using a dissecting microscope with a fluorescence attachment, you can prescreen the signal ratio before slide mounting.

Problem (Step 4): The AO corpse signal appears faded.

Solution: Fading can be caused by prolonged exposure to fluorescent lighting, which eventually will cause photobleaching of the image. Alternatively, there may have been insufficient stain uptake, in which case prescreening for corpses should be used (Fig. 1). Note that ablation of genes required for the engulfment of corpses prevents vital dyes from entering apoptotic cells (Neukomm et al. 2011).

Problem (Step 7): More corpses are visible by DIC optics than with fluorescence in the CED-1::GFP strain.

Solution: This is a fairly common caveat of using the CED-1::GFP strain, which shows a dissipation of signal at high levels of apoptosis.

Problem (Step 7): Corpses labeled with CED-1::GFP or ACT-5::YFP persist for longer than expected periods of time, sometimes not completing engulfment.

Solution: Alas, this also seems to be a caveat of using these strains. Given the role of CED-1 and actin in the engulfment process, the labeling of these proteins can prevent some corpses from completing engulfment. We have also noticed mildly reduced levels of overall apoptosis in strains expressing ACT-5::YFP.

DISCUSSION

Markers for apoptosis and apoptotic corpses themselves can be detected through physically staining the *C. elegans* adult germline, because one of the hallmark features of apoptotic cells is the condensation of chromatin into pycnotic bodies. DNA intercalating dyes (such as 4′,6-diamidino-2-phenylindole [DAPI]) and covalent labeling of fragmented DNA using terminal deoxynucleotidal transferase (TdT)-mediated dUTP nick end labeling (TUNEL) have long been used for visualizing apoptosis, but these reagents are limited to use in fixed specimens.

More accurate imaging of early and late stages of apoptosis in the germline of living animals can be achieved using vital dyes (such as AO and SYTO-12; Gumienny et al. 1999; Derry et al. 2001; Lettre et al. 2004) as well as transgenic lines that express fluorescent markers. Visualization of cell corpses by fluorescence depends on the resolution and intensity of the markers used. Many transgenic strains expressing fluorescent markers specific for various stages of apoptosis are bright enough to create detectable changes using a low-resolution fluorescence dissection microscope. However, some markers are less bright and are only detectable when visualized using a higher resolution compound fluorescence microscope (see below).

Fluorescently tagged proteins such as CED-1 are regularly used to visualize apoptosis in both embryos and the germline of living animals. CED-1 is required for the engulfment of apoptotic cells, and labels corpses before and during engulfment (Zhou et al. 2001). Strains carrying mutations in the engulfment genes (i.e., *ced-1[e1754]*) are useful for determining whether subtle changes in corpse numbers are statistically significant, because subtle differences are greatly enhanced when they accumulate in the absence of engulfment. The CED-1::GFP marker is also a good tool for quantifying apoptosis, as it forms distinct rings around early-stage apoptotic corpses in the soma and germline (Fig. 3). One caveat is that it is difficult to distinguish individual corpses at high levels of apoptosis because the signal becomes distorted. Conditions that cause higher numbers of apoptotic corpses—judged by morphological criteria in the bright field or with fluorescent reagents—should be validated in apoptosis-resistant mutants, such as *cep-1(gk138)* for genotoxic stress and *ced-3(n717)* or *ced-4(n1162)* to inhibit the core apoptotic pathway. Another potential problem is that a fraction of CED-1::GFP-labeled cells do not undergo apoptosis (Craig et al. 2012), which will skew quantification if not carefully addressed.

An alternative germline apoptosis marker expresses actin (ACT-5) fused to yellow fluorescent protein (YFP) in the gonadal sheath cells (Fig. 4). Like CED-1::GFP, ACT-5::YFP forms a fluorescent halo around cells beginning engulfment, but in our experience there is less background compared with CED-1::GFP, making it easier to distinguish individual corpses. However, the ACT-5::YFP strain has a modest resistance to DNA damage-induced apoptosis in the germline (B Lant and WB Derry, unpubl.), so exercise caution if using it for this purpose. Regardless, both strains can be used to indicate which cells should undergo the stereotypical morphological changes of apoptosis. As such, time-course experiments can be performed in which both GFP/YFP and white light channels are

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photographed over a period of ~45 min (the standard time for corpse development and engulfment). It is also possible to use the combination of a vital dye on a fluorescent strain to visualize distinct steps of early, mid- and late-stage corpses in real time (Fig. 5). The main caveat with this approach is that the fluorescence of the dye will often overwhelm the signal of the integrated fluorescent marker. Thus, extensive optimization of dye signal is necessary.

QUANTIFICATION OF APOPTOSIS USING FLUORESCENT REAGENTS

If multiple genes are being assessed for apoptosis defects in fluorescent strains (or using vital dyes), we recommend prescreening samples using a fluorescence dissecting microscope. This method is rapid, relatively light on handling, and does not kill the worms during observation. Corpses stained with vital dyes or marked with fluorescently tagged proteins can be detected at 10× magnification, but are ideally viewed at higher magnifications (i.e., 50×), especially in the case of weaker reporters. Both CED-1::GFP and ACT-5::YFP can be used to differentiate high and low levels of apoptosis using a fluorescence dissecting microscope. However, the signals of both are low compared with AO staining, and are therefore not ideal for quantification at this level of magnification. For quantification it is best to visualize corpses in these strains at 630× using a high-resolution compound microscope.

From experience, only AO staining facilitates consistent quantification potential for screening at lower magnification (10×–50×; magnifications typically found on standard dissecting microscopes). The ability to quantify AO-stained corpses is also dependent on magnification capacity; quantification is possible with the 2× lens, but a 5× lens is preferable. Because worms move out of view quickly at higher magnification, it is best to anesthetize them before attempting quantification. Using AO-stained or fluorescently labeled corpses to quantify apoptosis is much like standard corpse counting (see Protocol: **Visualizing Apoptosis in Embryos and the Germline of *Caenorhabditis elegans*** [Lant and Derry 2014b]). Images taken in a fluorescent channel should be confirmed for corpse morphology in the bright field. If you are unsure about the different stages of corpse morphology, practice identifying early-stage corpses (Figs. 2–4). Again, it should be noted that while screening can give an approximation of corpse numbers (a qualitative measure more than anything), quantification in strains which have been stained or contain fluorescent markers should only be used on images taken at high magnification (>400×).

RELATED TECHNIQUES

The TUNEL assay can be used to identify apoptotic corpses in *C. elegans*, and protocols are readily available elsewhere (Wu et al. 2000; Parusel et al. 2006; Craig et al. 2012).

RECIPES

M9 Buffer for Worms

1. Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O.
2. Autoclave for 20 min.
3. Add 1 mL of 1 M $MgSO_4$.
4. Store at room temperature. After 2 wk, check for visible contamination before use.

Nematode Growth Medium (NGM)

1. For solid NGM, mix 3 g of NaCl, 2.5 g of peptone, and 20 g of agar and bring to 1 L with H_2O. (For liquid NGM, prepare without agar.)
2. Autoclave for 1 h.
3. Let cool for 1 h in a 55°C water bath.
4. Add 1 mL of cholesterol (5 mg/mL in ethanol), 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, and 25 mL of 1 M (pH 6.0) KPO_4, mixing after each addition.
5. Using an automated plate pourer (peristaltic pump), pour the medium into sterile plastic plates to set. During pouring, keep the NGM on a hot plate with stirrer to prevent the medium from solidifying.
6. Store NGM plates at 4°C until use. Seed NGM plates with bacteria the day after pouring. Warm the plates to room temperature before adding the worms. (For liquid NGM, store at room temperature for up to 1 mo. Regularly check for excess cloudiness to ensure bacterial contamination is absent.)

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