Microbial influences on host cells depend upon the identities of the microbes, their spatial localization, and the responses they invoke on specific host cell populations. Multimodal analyses of both microbes and host cells in a spatially resolved fashion would enable studies into these complex interactions in native tissue environments, potentially in clinical specimens. While techniques to preserve each of the microbial and host cell compartments have been used to examine tissues and microbes separately, we endeavored to develop approaches to simultaneously analyze both compartments. Herein, we established an original method for mucus preservation using Poloxamer 407 (also known as Pluronic F-127), a thermoreversible polymer with mucus-adhesive characteristics. We demonstrate that this approach can preserve spatially-defined compartments of the mucus bi-layer in the colon and the bacterial communities within, compared with the marked absence when tissues were processed with traditional formalin-fixed paraffin-embedded (FFPE) pipelines. Additionally, antigens for antibody staining of host cells were preserved and signal intensity for 16S rRNA fluorescence in situ hybridization (FISH) was enhanced in poloxamer-fixed samples. This in turn enabled us to integrate multimodal analysis using a modified multiplex immunofluorescence (MxiF) protocol. Importantly, we have formulated Poloxamer 407 to polymerize and cross-link at room temperature for use in clinical workflows. These results suggest that the fixative formulation of Poloxamer 407 can be integrated into biospecimen collection pipelines for simultaneous analysis of microbes and host cells. Biofilms have been shown to promote colonic tumor progression in genetically susceptible mice.

While many studies have characterized the microbiome through sequencing and/or mass spectrometry, host–microbe spatial relationships can only be studied with sufficient resolution through microscopy-based techniques, such as fluorescence in situ hybridization (FISH) or electron microscopy. Moreover, while sophisticated multimodal analyses are being conducted on the microbiome, concurrent analyses of host cells have lagged. Studies on the host response to microbiome alterations to date have typically relied on macroscopic outputs, such as tumor formation, or molecular assays performed on separate, spatially disconnected specimens. We propose that concurrent, multimodal analysis of both host and microbial cells within the same tissue specimen would significantly advance knowledge into host–microbe interactions in CRC, as well as other forms of pathogenesis.

Recently developed imaging techniques enable multiplexed in situ analysis of 50 or more analytes on the same tissue section. However, one challenge has been identifying a tissue processing method that enables simultaneous spatial localization and quantifiable detection of both host components, such as proteins, and bacterial components via immunofluorescence (IF) and FISH, respectively. The colonic mucus layer acts as both a resident niche and a barrier for the luminal microbiome, playing well-documented roles in colonic pathologies such as ulcerative colitis. The hydrophilic colonic mucus is easily disrupted
by hydration and dehydration of formalin-fixed tissue with standard histological preparations. Carnoy’s solution is a rapidly dehydrating fixative that preserves mucus, but it reduces the sensitivity of IF and FISH. Our goal for this work is to devise a tissue processing strategy that preserves mucus architecture and is amenable to coincident antibody and nucleic acid detection. Herein, we describe how Poloxamer 407 fixation permits the simultaneous characterization of host cell protein expression and identification of microbial communities within the colonic mucus. Furthermore, we deployed this approach into a pilot clinical pipeline to enable multiplex in situ profiling of human tissues and associated microbes.

**RESULTS**

Carnoy’s and formalin fixation are incompatible with simultaneous mucus preservation and host cell assays

The alcohol-based Carnoy’s solution (Carnoy’s fixative/Methacarn) is a rapid dehydrating fixative that preserves the hydrophilic mucus layer for colonic microbial FISH analysis. Cross-linking fixative, such as neutral buffered formalin (NBF), is used for standard formalin-fixed paraffin-embedded (FFPE) tissue processing and has optimal antigen preservation for IF or immunohistochemistry. For this study, we aimed to identify the best tissue fixation method for simultaneous microbial FISH and host cell IF. We initially used mouse colon tissue as a model because of its abundance and availability. Mice do not naturally form mucosa-associated biofilms, but their mucus layers are arranged in bi-layer structures with different microbial compositions that can easily be evaluated.

Initially, we compared Carnoy’s fixation and NBF fixation, followed by standard histological processing. Standard IF stains such as proliferating cell nuclear antigen (PCNA) and the epithelial marker pan-cadherin (PCAD) had lower fluorescent signals in Carnoy’s- and Methacarn-fixed mouse colons compared to NBF fixation (Fig. 1a, b and Supplementary Fig. 1). In contrast, NBF fixation results in loss of the mucopolysaccharide-containing mucus layer (Fig. 1c), because it cannot withstand hydration and shear forces during processing. NBF contains mostly water and solubilizes the hydrophilic mucus layer during the fixation process. Consistent with known knowledge, these conventional fixatives are inadequate for in situ analysis of mucus-residing bacteria and host cells within the same tissue section.

**Poloxamer 407 formulated as a fixative solution polymerizes at room temperature**

Poloxamer 407 is a bio-adhesive polymer with an A-B-A block configuration, with a hydrophobic polypropylene oxide “B” block flanked by hydrophilic polyethylene oxide “A” blocks (Fig. 2a, b). Poloxamer 407 exhibits temperature-dependent and reversible polymerization, essentially transforming from a viscous liquid at 4 °C to a solid gel at higher temperatures capable of scaffolding biological surfaces. As temperature increases, hydrophobic B blocks align at the core while hydrophilic A blocks are oriented outward in a radial pattern to form micelles that can been used for substrate alignment and hydrophilic A blocks are oriented outward in a radial pattern to form micelles that can been used for host cell analysis (Fig. 2c, d). With the outwardly arranged hydrogen bonds (Fig. 2c), with the outwardly arranged hydrogen bonds capable of forming non-covalent bonds with the highly glycosylated mucus matrix (Fig. 2d). Because of this...
transition into a gel state capable of providing scaffold support, we surmised that it would support the hydrophilic mucus architecture in histological applications.

For its adaptation into the clinical workflow, we formulated the Poloxamer 407 solution such that it had (1) fixation properties in addition to structural support, and (2) a lower ceiling temperature such that it can be used at room temperature in clinical suites. These properties enable scaffolded fixation of a specimen in a single step without additional equipment or processing steps. We formulated Poloxamer 407 to 20% (w/w) in NBF solution to enhance mucus structure integrity during fixation and to initiate polymerization at room temperature (Fig. 2e). In a clinical workflow, Poloxamer fixative is stored at 4 ºC prior to adding tissue at room temperature (Fig. 2f). The procedure listed was optimized to limit the introduction of extraneous variables and enhance ease of use in the clinic, and we have begun using Poloxamer fixative for limited human colonic biopsy specimen collection at Vanderbilt University Medical Center.

Poloxamer fixative enables in situ analysis of colonic microbes and host cells

To directly compare Carnoy’s solution and Poloxamer fixative for histological applications, a portion of intact colonic tissue from each mouse was divided and fixed separately in each solution. We first tested IF antibodies that performed poorly in Carnoy’s solution and Methacarn, and robust staining was observed with the Poloxamer fixative for PCNA and PCAD (Supplementary Fig. 1). To determine which fixation was best for visualizing both the mucus and epithelia, we performed serial multiplex FISH and IF on the same tissue sections using antibodies previously validated in Carnoy’s solution. Five feature multiplex images (GOB5, Eub—eubacterial probe, PCK—pan-cytokeratin, WGA—wheat germ agglutinin, nuclei) are shown for the distal colon, where the highest density of microbes is found (Fig. 3). IF for PCK was used to delineate the epithelial layer (Fig. 3a, b, c, g, i, j). GOB5, also known as calcium-activated chloride channel regulator 1 (CLCA1), is a mucin-processing protein that is secreted with mucus. An intact mucus layer highlighted by antibody staining against GOB5 and WGA was observed with Carnoy’s solution, Methacarn, and Poloxamer fixative (Fig. 3a, c, e, g, i, k). Notably, the Eub probe showed greater signal intensity with the Poloxamer fixative compared to Carnoy’s solution.
and Methacarn (Fig. 3a, d, h, i, l and Supplementary Fig. 2a). Bacteria were only observed with the Carnoy’s solution after increased gain (Fig. 3a—inset), which exemplifies the challenges with biofilm detection reported with Carnoy’s solution 3,7,43. Consistent with previous studies6,8,11, the Eub signal is absent in the inner mucus layer closest to the epithelium (Fig. 3i, l, white arrow and Supplementary Fig. 2b). The thickness of the inner mucus layer, as denoted by the distance spanning this bacteria-free space, was not affected by different fixatives (Supplementary Fig. 2c). To evaluate mucus layer composition further, we stained Poloxamer and Methacarn-fixed colonic tissues with MUC2, UEA1, and WGA, the latter are lectins known to bind glycans on mucin. Mucus staining was consistent between the two fixatives, with strong, patterned staining of the inner mucus layer, and weaker, more heterogeneous, staining of the outer mucus layer (Supplementary Fig. 3). However, nonspecific, non-mucus cellular staining in the colonic lumen was observed in Methacarn, but not in Poloxamer fixative (Supplementary Fig. 3b, white arrow). These results demonstrate the improved sensitivity for concurrent FISH and IF imaging in colonic tissue with Poloxamer fixative.

Individual microbes are detected in Poloxamer-fixed tissue

To determine the specificity of the Eub probe with Poloxamer fixative, we tested distal colonic samples in parallel with a nonsense probe (Non-Eub). Low intensity staining was observed again with Carnoy’s solution (Fig. 4a), which was further reduced to background level with the non-Eub probe (Fig. 4b). Poloxamer-fixed tissue again had increased Eub staining sensitivity, and at higher magnification, individual, distinct rod-shaped bacteria were observed (Fig. 4c and inset). The non-Eub FISH probe also showed background level of signal in the Poloxamer-fixed tissue (Fig. 4d). These results demonstrate the enhanced sensitivity and specificity of FISH for detection of colonic microbiota with Poloxamer fixation.

To assess whether Poloxamer-fixed tissue can inform biologically relevant changes, we evaluated the mucus layer of dextran sulfate sodium (DSS)-treated animals. DSS induces localized damage to the colonic epithelium, resulting in a regenerative repair response. Regenerating epithelia initially lack glandular structure and are skewed towards proliferation instead of differentiation, resulting in a reduction of goblet cells44. Using the colonic architecture and goblet cell numbers as markers, we concurrently observed damaged and normal-appearing regions within the same mouse colon (Supplementary Fig. 4A, B). The inner mucus layer overlying the damaged regions appeared to be thinner compared to that of normal regions, with the epithelium in closer proximity to the microbiome as marked by the Eub probe (Supplementary Fig. 4). Furthermore, Eub probe signal appeared in the crypts of damaged areas (Supplementary Fig. 4A, B), a
A* and C* are higher magnified views of red insets in a, c. Scale bars (a, c) = 20 μm, (A*, b, C*, d) = 10 μm.

phenomenon not observed in normal regions or colons of healthy mice. The reduction in mucus is consistent with the decrease in goblet cells induced by DSS damage, and microbial encroachment into crypts may form the basis of inflammatory interactions with the mucosa.

Mucosa-associated biofilms are observed in colonic tissue using Poloxamer fixation

To further validate Poloxamer fixative for biofilm detection with direct implications for the human microbiome, we colonized germ-free (GF) mice with a biofilm-positive microbiota slurry isolated from a human colorectal cancer (Fig. 5a). With Poloxamer fixation, we were able to observe bacteria forming dense colonies in direct contact with the colonic epithelium, without an inner mucus layer that typically separates luminal bacteria from the epithelium (Fig. 5b). For our negative control, we observed that no bacteria were detected in sham-inoculated mice, a mucus layer was maintained (Fig. 5c). Multiplex FISH observed bacterial encroachment into crypts may form the basis of inflammatory interactions with the mucosa.

Poloxamer fixation permits detection of mucosa-associated biofilms in human colonic adenomas

To demonstrate the clinical utility of our method, portions of human colonic adenomas collected during colonoscopy were immediately placed into Poloxamer fixation for subsequent FISH-based biofilm analysis (Fig. 2f and Supplementary Fig. 6). Using a scoring scheme defined previously, a biofilm detection (>2.5 score) was consistent in Poloxamer fixative compared to prior methods (Table 1). Mucosa-associated biofilms were detected on adenomas arising from the ascending colon, compared with ascending normal colons from the same patient which lack biofilm (Fig. 6). Bacterial invasion into the adenoma was observed in one polyp (Fig. 6e, white arrows). The advantages of existing repertoires of antibodies and effective bacterial FISH staining enabled robust screening for biofilms in human specimens fixed in the Poloxamer fixative, suggesting that this approach can be a useful clinical tool.

DISCUSSION

Microbiota–host interactions are implicated in a range of disorders, including CRC. Specifically, human CRC-associated stool microbiota transplanted into GF or carcinogen-treated mice exacerbates colon epithelial cell proliferation and the tumor-associated immune response. Colonic mucosa-associated biofilms are defined by the direct interaction between bacteria and epithelial cells. They are present in ~89% and 12% of right-sided and left-sided colon tumors, respectively. CRC in human FAP appears to be uniformly biofilm positive. Some human colonic biofilms are carcinogenic in GF ApcMin mice. With a reported range of 13–35% colonic biofilm positive in healthy subjects, these findings indicate clinical relevance for using biofilm to stratify high-risk precancer lesions.

The established tissue fixation for detecting colonic biofilms is Carnoy’s solution. A major limitation of Carnoy’s solution is that IF
sensitivity is reduced and inadequate for detecting epithelial antigens (Fig. 1). Generally, dehydration fixation approaches are able to retain mucus in samples, but they also reduce the efficacy of FISH staining. Conventional FFPE tissue is limited by the poor preservation of the mucus layer (Fig. 1). Our goal was to develop a broadly applicable reagent that preserves mucus and biofilms without compromising IF detection of host factors. With the Poloxamer fixative, we overcame the need to apply heat for polymerization and developed a clinically compatible, room temperature reagent (Fig. 2).

We demonstrated how Poloxamer-fixed samples were superior to Carnoy’s-fixed samples for visualizing both mucosa-associated bacteria and host cells in the same tissue section (Fig. 3). Whereas agarose has been shown to also provide physical scaffold, the physical and chemical properties of Poloxamer make it the ideal substrate for preservation of mucus. The Poloxamer fixative polymerizes at room temperature, enabling its incorporation into existing clinical protocols for human studies. Methods that enable multimodal analysis will become very valuable in future endeavors for understanding complex tissue ecosystems. Our efforts represent an advance in the field of multiplex imaging, and one of the first to simultaneously evaluate composition and organization of both microbes and host cells.

### Table 1. Biofilm characteristics of human specimens.

| Sample ID   | Mucus present | Bacteria abundance per 200 μm CEC | Percentage of biofilm coverage | Is there DAPI staining without Cy3? | Notes                                                                 |
|-------------|---------------|-----------------------------------|---------------------------------|-------------------------------------|----------------------------------------------------------------------|
| MPP00048A1  | 0.5           | 3                                 | 50%                             | No                                  | Mostly rods, some cocci in BF; not great mucus though; some autofluorescent objects (larger than bacteria) to watch out for in some regions |
| HTA11_1429  |               |                                   |                                 |                                     |                                                                      |
| MPP00049A1  | 1             | 3                                 | 5%                              | No                                  | Lots of nice rods everywhere, BF region located within crypts of CEC in one area |
| HTA11_1391  |               |                                   |                                 |                                     |                                                                      |

*0 = none, 1 = some, 2 = excellent.

*1 = ≤5 bacteria, 2 = ≤20 bacteria or < 200 μm long.

*0–100% of CEC.
Previous assessments of biofilms relied on consensus of experts to score samples for determining biofilm positivity. The increased FISH signal observed with Poloxamer fixative potentially enables the identification of biofilms with improved technical proficiency and machine learning. Unlike sequencing approaches to characterize the microbiome, FISH requires preselection of candidates, but it provides spatial information important for determining microbial behaviors.

To our knowledge, this is the first use of Poloxamer 407 for tissue fixation. Our application of Poloxamer fixation is directed towards room temperature, standard histological applications. Substitution of Poloxamer for OCT as a scaffold for frozen tissue application has not been successful at this time due to the gelation point of Poloxamer at about 19 °C. Nevertheless, our successful implementation of this fixative to conventional histological pipelines will enable its wider deployment in clinical pipelines for human microbiome and biofilm research.

**METHODS**

**Tissue fixation and processing**

For Methacarn fixation, tissues were immersed in Methacarn solution (6:3:1 ratio of methanol:glacial acetic acid:chloroform) and processed using standard procedure. A similar procedure was followed for Carnoy’s solution fixation with the exception of the use of ethanol (6:3:1 ratio of ethanol:glacial acetic acid:chloroform). Tissues were also fixed using standard NBF fixation. For Poloxamer fixation, the Poloxamer 407 solution was made at 4 °C, where 20% w/w Poloxamer was mixed into 10% NBF. Aliquots were kept on ice prior to the addition of the tissue specimen. Once the specimen was immersed in the solution, the mixture was brought to room temperature at 25 °C and allowed to polymerize. The mixture was visually inspected for polymerization by checking for the reduction of movement when the conical tube was inverted. From here, the tissue was fixed at room temperature for 24-h, followed by standard histological processing. Tissue blocks prepared from fixed tissues were sectioned at 5 microns onto slides.

**Rheological testing**

To test for polymer gelation properties, rheological testing was performed using Rheometer AR2000 ex machine with both the Poloxamer fixative formulation (20% w/w Poloxamer 407 into 10% NBF) and normal Poloxamer formulation (20% w/w Poloxamer 407 into water). To test sol–gel transitions, temperature was modulated from 5 °C to 40 °C with controlled stress and rate and half gelation temperature was calculated by a dose response non-linear log fit in Prism (Graphpad).

**Tissue collection**

Conventional mouse experiments were performed under protocols approved by the Vanderbilt University Animal Care and Use Committee and in accordance with NIH guidelines. Animals were euthanized and colonic tissues were collected using previously published procedures. GF mouse experiments were performed at Johns Hopkins University under protocols approved by the Johns Hopkins University Animal Care and Use Committee in accordance with NIH guidelines. Human colonic tissues were collected from participants in the Colorectal Molecular Atlas Project (COLON MAP), a study approved by Vanderbilt Institutional Review Board. All participants provided written informed consent. Participants underwent colonoscopy as part of their routine care following standard bowel cleansing preparation. Polyps were removed according to standard of care and bisected. Visually normal mucosal biopsies were obtained in the mid-ascending colon using jumbo biopsy forceps. To increase likelihood of biofilm positivity, only participants with a polyp in the cecum or ascending colon were included in this analysis. Each tissue (either normal biopsy or polyp portion) was placed in individual labeled tubes of Poloxamer fixative, and gently inverted twice to ensure full coverage of the sample.

**Multiplex fluorescence imaging**

Sequential antibody staining and dye inactivation was performed as described. FISH probes, antibodies, and lectins used are listed in Tables 2 and 3. Modifications to the protocol includes the incorporation of FISH, as described below, prior to IF. Fluorescence of FISH probes was inactivated using the same methods as antibodies. For direct comparisons, tissues were imaged with the same exposure time using an Olympus XB1 inverted microscope with a motorized stage with filter sets for DAPI, GFP, CY3, CY5, and CY7. Images were also collected on a Zeiss Axio-Imager M2 microscope.

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**Fig. 6** Biofilm-positive human colonic polyps demonstrate invasion of bacteria into the mucosa. ×63 confocal images of human colonic tissues fixed in Poloxamer showing regions of biofilm positivity using Eub (green) probes to identify bacteria. a Patient polyp sample HTA11_1429_20_2013 with (b) normal ascending colon. c Normal ascending colon with (d, e) patient polyp sample HTA11_1391_20_1011. White arrows indicate invaded bacteria. White dotted lines represent epithelial borders in normal colon. Scale bars = 50 μm.
Fluorescent in situ hybridization

FISH probes were suspended in sterile water at a concentration of 0.2 nmol/μl (200 μM) and stored at ~20 °C. Hybridization buffer (20 mM Tris-HCl [pH 8.0], 0.9 M NaCl, 0.01% sodium dodecyl sulfate) and FISH wash buffer (225 mM NaCl, 20 mM Tris, 5 mM EDTA) were pre-warmed in a 46 °C oven. Slides were then de-paraffinized through a 3 × 5 min dips in Histoclear under the fume hood. Re-hydration of slides was done using an ethanol gradient. Slides were placed for 5 min in each of the following solutions: -100% ETOH, 100% ETOH, 95% ETOH, 95% ETOH, 70% ETOH, followed by 2 h at 46 °C in a humidified chamber (1.5 h for the universal probe; ~1.5 h for specific taxa probes). Slides were washed with FISH wash buffer in an anaerobic hood to a dilution of 1:20 (weight/volume). Prior to gavage into GF mice, the inoculum was further diluted 1:10 in PBS, for a final of 1:200 w/v dilution from the original tissue.

Mouse colonization

GF C57BL/6 wild-type animals were transferred to gnotobiotic isolators (separate isolator for each experimental group) and gavaged with 100 μl of the human inoculum. Mice were euthanized at indicated time points. About 1 to 2 × 0.5 cm snips were taken from the proximal and distal colon, and then fixed in accordance to the protocols above. Tissues were processed, paraffin-embedded, and sectioned.

DSS administration

Conventionally housed C57BL/6 wild-type animals were administered 2.5% DSS in drinking water for the following 6 days, prior to a 9-day rest period, followed by a second round of DSS. Tissues were collected at the end of the second DSS round.

Quantitative image analysis

For the host mucosa, binary mucosal masks were generated through DAPI channel thresholding to create regions of interest limited to the mucosa and not lumen. For the colonic lumen, binary luminal masks were generated through thresholding EUB expression and the elimination of the mucosal mask. For intensity quantifications, mean fluorescent intensities were obtained for FISH or antibody staining within the respective binary masks. For calculating inner mucus layer thickness, the outer edges of the mucosal and luminal masks were identified. The minimal distances between the two edges were calculated iteratively along their lengths by sampling 100 random points along the luminal edge. All image analyses were performed using MATLAB (MathWorks).

Biofilm screening

Patient tissues were collected and screened for biofilms using the universal bacterial probe (EUB338), as described previously. Briefly, tissues were defined as biofilm positive if there were more than 10^9 bacteria/ml invading the mucus layer (within 1 μm of the epithelium) for at least 200 μm of the epithelial surface.

Human tissue inocula preparation

A sample was prepared from a 3-mm diameter tissue piece collected from a resected CRC, snap-frozen, and stored at ~80°C. The inoculum was prepared anaerobically by mincing and homogenizing tissue pieces in PBS in an anaerobic hood to a dilution of 1:20 (weight/volume). Prior to gavage into GF mice, the inoculum was further diluted 1:10 in PBS, for a final of 1:200 w/v dilution from the original tissue.

Table 2. Antibodies and lectins used in this study.

| Marker | Channel | Exposure | Concentration | Product ID | Clone/Cat # | Clonality | Host species |
|--------|---------|----------|---------------|------------|-------------|-----------|--------------|
| PK2-6  | FITC    | 100 ms   | 1:200         | ab11214    | PCK-26      | Monoclonal| Mouse        |
| EPR12254-88 | 100 ms | 1:200 | CS 85805 | P10 | Monoclonal | Mouse | Rabbit |
| RB-9036 | Cy3     | 100 ms   | 1:200         | DC13090    | sc-15334    | Polyclonal| Rabbit |
| BM8     | Cy5     | 1000 ms  | 1:200         | BL123115   | H-300       | Polyclonal| Rat         |
| JM     | Cy5     | 1000 ms  | 1:200         | sc-15334   | H-300       | Polyclonal| Rabbit |
| WGA     | Cy5     | 20 ms    | 1:200         | W7024, W32466 | NA | NA | NA |
| UEA1    | Cy5     | 10 ms    | 1:1000        | B-1065     | NA          | NA | NA |

Table 3. Nucleic acid probes used in this study.

| Probe | Probe sequence | Exposure | Concentration | Label |
|-------|----------------|----------|---------------|-------|
| Eub   | 5′-Cy3-GCTGCTCCCTCGTGAAGT-3′ | 100 ms | 1:100 | Most bacteria |
| CFB286 | 5′-Fluorescein-TCCCTCTGACACCGCTAC-3′ | 150 ms | 1:100 | Bacteroidetes |
| FUS714 | 5′-Cy7-GGCCCTCCTCCTCCGATT-3′ | 200 ms | 1:100 | Fusobacterium |
| Lac435 | 5′-Cy3-TCTTCTCCTCCTGATAG-3′ | 100 ms | 1:100 | Lachnospiraceae |
| Gam42a | 5′-Cy5-GCTTCCCTCCTACATGGT-3′ | 500 ms | 1:100 | Gammaproteobacteria |
| Non-Eub | 5′-Cy3-CGACGGAGGGCCTCCTCA-3′ | 200 ms | 1:100 | Nonsense probe |

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