In situ visualization of bacterial populations in coral tissues: pitfalls and solutions

Naohisa Wada [1, 2, 3] , **Frederic J Pollock** [2, 3, 4, 5, 6] , **Bette L Willis** [3, 5, 6] , **Tracy Ainsworth** [5] , **Nobuhiro Mano** [1] , **David G Bourne** Corresp. [2, 3, 6]

1 Department of Marine Science and Resources, College of Bioresource Science, Nihon University, Fujisawa, Kanagawa, Japan

2 Australian Institute of Marine Science, Townsville, QLD, Australia

3 AIMS@JCU, Townsville, Queensland, Australia

4 Eberly College of Science, Department of Biology, Pennsylvania State University, University Park, Pennsylvania, United States

5 ARC Centre of Excellence for Coral Reef Studies, James Cook University of North Queensland, Townsville, Queensland, Australia

6 College of Science and Engineering, James Cook University of North Queensland, Townsville, Queensland, Australia

Corresponding Author: David G Bourne
Email address: d.bourne@aims.gov.au

In situ visualization of microbial communities within their natural habitats provides a powerful approach to explore complex interactions between microorganisms and their macroscopic hosts. Specifically, the application of fluorescence in situ hybridization (FISH) to simultaneously identify and visualize diverse microbial taxa associated with coral hosts, including symbiotic algae (Symbiodinium), Bacteria, Archaea, Fungi and protists, could help untangle the structure and function of these diverse taxa within the coral holobiont. However, the application of FISH approaches to coral samples is constrained by non-specific binding of targeted rRNA probes to cellular structures within the coral animal tissues (including nematocysts, spirocysts, granular gland cells within the gastrodermis and cnidoglandular bands of mesenterial filaments). This issue, combined with high auto-fluorescence of both host tissues and endosymbiotic dinoflagellates (Symbiodinium), make FISH approaches for analyses of coral tissues challenging. Here we outline the major pitfalls associated with applying FISH to coral samples and describe approaches to overcome these challenges.

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3   **Authors:** Naohisa Wada[1,2,5], F. Joseph Pollock [2,3,4,5,6], Bette L. Willis [3,4,5], Tracy Ainsworth[4],

4   Nobuhiro Mano[1] and David G. Bourne [2,3,5]

6   [1] Department of Marine Science and Resources, College of Bioresource Science, Nihon

7   University, Fujisawa, Kanagawa 252-0880 Japan

8   [2] Australian Institute of Marine Science, Townsville, QLD 4810, Australia

9   [3] College of Science and Engineering, James Cook University, Townsville, QLD 4811, Australia

10   [4]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, QLD

11   4811, Australia

12   [5] AIMS@JCU, Townsville, QLD 4811, Australia

13   [6] Pennsylvania State University, Eberly College of Science, Department of Biology, University

14   Park, PA 16802, USA

17   Corresponding Author:

18   David Bourne

19   Australian Institute of Marine Science, PMB 3, Townsville, QLD 4810, Australia

20   D.Bourne@aims.gov.au

22 **Abstract**

23 *In situ* visualization of microbial communities within their natural habitats provides a powerful

24 approach to explore complex interactions between microorganisms and their macroscopic hosts.

25 Specifically, the application of fluorescence *in situ* hybridization (FISH) to simultaneously

26 identify and visualize diverse microbial taxa associated with coral hosts, including symbiotic

27 algae (*Symbiodinium*), Bacteria, Archaea, Fungi and protists, could help untangle the structure

28 and function of these diverse taxa within the coral holobiont. However, the application of FISH

29 approaches to coral samples is constrained by non-specific binding of targeted rRNA probes to

30 cellular structures within the coral animal tissues (including nematocysts, spirocysts, granular

31 gland cells within the gastrodermis and cnidoglandular bands of mesenterial filaments). This

32 issue, combined with high auto-fluorescence of both host tissues and endosymbiotic

33 dinoflagellates (*Symbiodinium*), make FISH approaches for analyses of coral tissues challenging.

34 Here we outline the major pitfalls associated with applying FISH to coral samples and describe

35 approaches to overcome these challenges.

Introduction

38   Corals form close symbiotic associations with a range of microorganisms, including

39 dinoflagellate microalgae, Bacteria, Archaea and viruses, a consortium collectively termed the

40 coral holobiont (Rohwer et al., 2002; Rosenberg et al., 2007). Coral-associated bacterial

41 communities are known to contribute to coral holobiont fitness (Rosenberg et al., 2007) and

42 disruptions in coral-associated bacterial community structure have been implicated in the onset

43 of coral disease (Bourne et al., 2009; Bourne, Muirhead & Sato, 2011). However, the

44 identification of specific bacterial pathogens directly responsible for disease causation has been

45 problematic and, in many cases, causative agents have not been definitively linked with disease

46 lesions at the cellular level (Work & Aeby, 2006; Work et al., 2008). While standardized

47 histological approaches allow investigations of disease lesions at the cellular level, *in situ*

48 hybridization (ISH) approaches allow targeted localization of specific DNA and RNA sequences

49 at the molecular scale (Levsky & Singer, 2003). Specifically, fluorescence *in situ* hybridization

50 (FISH) provides a powerful tool for simultaneous identification and visualization of bacteria

51 within host tissues (Moter & Gobel, 2000).

52   The application of FISH to coral samples has been complicated by the need for time-

53 consuming and labor-intensive processing, combined with specialized, and often expensive,

54 microscopes and image processing software to separate probe fluorescence from high

55 background tissue auto-fluorescence (Ainsworth et al., 2007; Pollock et al., 2011). Optimized

56 FISH protocols have been developed to overcome some of these limitations, allowing

57 researchers to target specific nucleic acid sequences in coral histological studies (Ainsworth et al.,

58   2006a; Ainsworth, Hoegh-Guldberg & Leggat, 2008). Consequently, FISH approaches have

59   become a valuable tool to elucidate how microbial communities are spatially located within both

60   healthy and diseased coral tissues (Bythell et al., 2002; Webster et al., 2004; Lesser et al., 2004;

61   Ainsworth et al., 2006a; Ainsworth, Hoegh-Guldberg & Leggat, 2008; Ainsworth & Hoegh-

62   Guldberg, 2009; Apprill et al., 2009). For example, Bayer et al. (2013) recently employed FISH-

63   based techniques to identify abundant communities of *Endozoicomonas*-related bacteria residing

64   within healthy coral tissues, and Neave et al. (2016) showed that *Endozoicomonas* species, which

65   can comprise as much as 90% of the microbiome, form cyst-like aggregations at the interface of

66   the epidermal and gastrodermal cell layers of *Stylophora pistillata*. Ainsworth et al. (2015)

67   identified a core coral microbiome using sequencing-based approaches and applied FISH

68   techniques to localize some members of this core microbiome within microhabitats of the coral

69   host. FISH also allows detection, identification and visualization of potential coral pathogens

70   (Ainsworth et al., 2007), which is important for studies of disease etiology and the development

71   of diagnostic tools for coral diseases (Pollock et al., 2011).

72   Autofluorescence associated with corals is the direct result of high densities of

73   chlorophyll-containing dinoflagellates within the corals' gastrodermal layers and an abundance

74   of fluorescent pigments, including green fluorescent protein-like molecules within coral cell

75   layers (Salih et al 2000; Dove et al., 2001; Yokouchi et al., 2003; Ainsworth et al., 2006a).

76   Despite the demonstrated utility of direct localization and visualization of specific DNA and

77   RNA targets within coral tissues and recent advances to overcome high levels of coral auto-

78   fluorescence, non-specific probe binding (i.e. erroneous hybridization of FISH probes to non-

79   target structures leading to the detection of false positives) impedes the application of ISH and

80   FISH techniques to coral samples (Bythell et al., 2002; Ainsworth et al., 2006b; Apprill et al.,

81   2009). Clear and consistent guidelines and methodological criteria are therefore needed to

82   differentiate specific and non-specific probe binding. In this manuscript, we outline current

83   barriers to the application of FISH techniques to coral samples and provide clear guidelines to

84   help researchers and practitioners overcome these challenges.

85

86   **Materials and Methods**

87   *Sample collection*

88   Samples of healthy and white syndrome (WS) infected colonies of *Acropora hyacinthus* (i.e.

89   colonies displaying diffuse, acute to sub-acute areas of tissue loss revealing white, intact

90   skeleton; see Fig. 1) were collected from reefs near Lizard Island (14º40'S, 145º27'E) in the

91   Northern sector of the Great Barrier Reef (GBR) on SCUBA (1–5 m depth), in September 2010,

92   July 2011 and February 2012. At each sampling time point, ~ 3 cm coral fragments were

93   collected from each healthy colony (n=4) and from each WS-infected colony (i.e., from the

94   lesion-healthy tissue interface [n=7] and from apparently healthy tissue [n=3] approximately 10

95   cm away from the lesion). Coral fragments were placed in individual sterile bags underwater.

96   Sampling was undertaken on Permit G11/34003.1 issued by the Great Barrier Marine Park

97   Authority.

98

99   *Fixation, decalcification and sectioning*

100 Within 15 minutes of collection, coral samples were placed in freshly prepared 4%

101 paraformaldehyde (Electron Microscopy Sciences, USA), 10 mM phosphate buffered saline

102 (PBS) solution at 4°C. After 8-10 hrs, the paraformaldehyde solution was exchanged for a 1:1

103 solution of 10 mM PBS and ethanol. Samples were rinsed with 10 mM PBS and then embedded

104 in 1.5% agarose (~55 to 60 °C) to maintain tissue conformation during the decalcification process.

105 Once the agarose was set, excess agarose was removed and a small hole was punched through to

106 the coral fragment to allow liquids to reach the sample. Agarose-embedded samples were placed

107 in histological cassettes and decalcified in a 20% EDTA solution (pH 8.0), with the solution

108 maintained at 4°C and exchanged approximately every 2 days for 2-3 weeks. Following

109 decalcification, the agarose-embedded samples were rinsed in PBS, dehydrated sequentially in a

110 70%, 80%, 90%, 95%, 100% and 100% ethanol series for 60 min each, then processed through

111 three, 30 min xylene rinses and embedded in Paraplast paraffin wax. Paraffin-embedded samples

112 were serially sectioned at 4 μm and collected on Superfrost Plus slides (Menzel, Germany).

113 Sections were dewaxed at 60 °C prior to histological processing.

114

115 *Histology*

116 Prior to staining, one serial section from each sample was dewaxed in xylene (2 x 3 min) and

117 rehydrated through 3 x 5 min 100% ethanol, 1 x 5 min 70% ethanol, and 1 x 2 min water washes.

118 Hydrated sections were counterstained in Mayer's Hematoxylin for 8 min, rinsed in tap water (1

119 x 20 dips), differentiated in Scott's tap water substitute for 30 sec for bluing, rinsed in water (1 x

120 2 min) and stained in Eosin for 3 min. Stained section were dehydrated through an ethanol series

121 (1 x 2 min 70% ethanol and 2 x 5min 100% ethanol) and washed in xylene (2 x 5 min). Sections

122 were mounted in DPX mounting medium, observations were recorded using a Leica DMI 6000B

123 light microscope (Leica, Germany) and microphotograph images were processed using the LAS

124 imaging software (Leica, Germany).

125

126 *Fluorescent* in situ *hybridisation* (FISH)

127 Prior to staining, three serial tissue sections were dewaxed in xylene (2 x 3 min), dehydrated

128 through 100% ethanol washes (3 x 5 min) and air-dried. Dried sections were washed in a 0.2 M

129 HCl solution for 12 min and a 20 mM Tris HCl solution (pH 8.0) for 10 min at room temperature.

130 To digest bacterial cellular membranes and allow easier probe penetration into tissues, sections

131 were mounted in a proteinase K (50 μg/ml), 20 mM Tris HCl solution (pH 8.0) for 5 min at 37

132 °C and rinsed in 20 mM Tris HCl (pH 8.0) prior to probe hybridisation. Oligonucleotide probes,

133 including a probe targeting the 16S rRNA gene (EUB338 mix: 5'-GCT GCC TCC CGT AGG

134 AGT-3', 5'-GCA GCC ACC CGT AGG TGT-3', 5'–GCT GCC ACC CGT AGG TGT-3') and a

135 nonsense, negative control probe (NonEUB338: 5'–ACA TCC TAC GGG AGG C-3'), were

136 labeled with the Cy3 flurochrome (Thermo Fisher Scientific, Germany) (Wallner, Amann &

137 Beisker, 1993; Daims et al., 1999). Tissue sections were covered with hybridization buffer (30%

138 v/v formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.01% SDS), oligonucleotide probes

139 were added to a final concentration of 25 ng μl^{-1}, and samples were incubated at 46°C for 1.5

140 hours. Sections were washed in 50 ml Falcon tubes containing preheated wash buffer (0.112M

141 NaCl, 20 mM Tris-HCl (pH 8.0), 0.01% SDS, 5mM EDTA) in a water bath at 48°C for 10 min.

142 Following washing, sections were immediately soaked in cold, filtered water for 10 seconds to

143 remove excess salts, air dried and mounted in Citifluor AF1 (ProScitech, Australia). As a

144 negative control, one serial tissue section was processed as described above, but no

145 oligonucleotide probe was added. To detect true and false binding simultaneously, one serial

146 section was processed with the Cy3-labelled EUB338 mix probes as described above, and a Cy5-

147 labelled NonEUB338 probe applied to the same section. An LSM710 confocal laser scanning

148 microscope (Carl Zeiss, Germany) combined with spectral emissions profiling was used to

149 visualize tissue-associated, FISH-labeled bacterial communities, as described by Ainsworth et al.

150 (2006). Detection of the Cy3 fluorochrome label was in the emission range 519 – 580 nm and the

151 target signal was recorded at 561 nm. Auto-fluorescence detection and spectral removal included

152 emission ranges of 407 – 486 nm for removal of coral tissue autofluorescence and 627 – 704 nm

153 for removal of dinoflagellates (*Symbiodinium*) autofluorescence. Micrographs of bacterial

154 communities associated with coral tissue sections were processed using Zen 2009 software

155 (Zeiss, Germany). The linear unmixing function of the Zen 2009 software (Zeiss, Germany) was

156 utilized when multiple probes were visualized simultaneously.

157

158 *Trouble shooting approaches*

159 To determine if the sequence of the oligonucleotide probe contributes to non-specific binding,

160 three probes were assessed: EUB338 mix probes, NonEUB338 and a Vib-GV (5'–AGG CCA

161 CAA CCT CCA AGT AG-3'; Giuliano et al., 1999). To determine if the fluorochrome attached

162 to the oligonucleotide probe affects non-specific binding, three flurochromes were assessed: Atto

163   647 (excitation λ = 645 , emission λ = 669) Cy3 (excitation λ = 548 , emission λ = 561) and

164   Cy5 (excitation λ = 647 , emission λ = 665) (i.e. Cy3-labeled EUB338 mix, Cy3-labeled

165   NonEUB338, Cy5-labeled NonEUB338 and Atto 647-labelled Vib-GV). To assess the utility of

166   incorporating hybridising agents in the FISH workflow to avoid non-specific binding, "Blocking

167   Reagent" (Roche, Germany) was added to the hybridisation buffer (30% formamide, 0.9M

168   NaCl, 20mM Tris-HCl with adjusted pH8.0, 0.01% SDS, 10% "Blocking Reagent" with maleic

169   acid buffer), alongside Cy3-labelled NonEUB338 probe.

170

171   **Results and Discussion**

172   *The challenges of applying FISH approaches to corals*

173   The challenge of non-specific FISH probe binding within coral tissues is clearly

174   highlighted in FISH-labeled sections of healthy samples of *Acropora hyacinthus* from Lizard

175   Island in the northern Great Barrier Reef (GBR) (Fig. 2). Under fluorescence excitation light,

176   coral tissues targeted with probes for Bacteria (EUB338) show multiple strongly fluorescent

177   cellular structures within anatomical features of coral polyps (e.g., mesenterial filaments, Fig.

178   2A), suggesting the presence of targeted bacteria within gastrodermal and epidermal cells.

179   However, direct comparison with serial sections targeted with nonsense negative control probes

180   (NonEUB338) (i.e. probes specifically designed to detect non-target binding) (Fig. 2B)

181   highlights strong non-specific binding of both probes to granular gland cells within the

182   cnidoglandular band of mesenterial filaments. Granular gland cells are secretory cells commonly

183   distributed throughout the gastrodermis, including in regions of coenosarc, stomodeum and

184   mesenterial filaments, where secretions are released into the corals' coelenteron (gastrovascular

185   cavity) to aid extracellular digestion of prey (Galloway et al., 2007). The lack of granular cell

186   fluorescence in probe-free, negative control sections (Fig. 2C) indicate that these false positives

187   are caused by non-specific binding of the oligonucleotide probes rather than auto-fluorescence.

188   Granular gland cells within the gastrodermis appear as pink aggregations in hematoxylin and

189   eosin (H&E) stained histological sections (Harrison 1980; Fig. 2D). Due to their strong

190   fluorescence signal in FISH images (Fig. 2A,B,E), combined with their spherical shape and

191   similar size to bacteria (approximate diameter = 0.5μm), non-specific binding of granular gland

192   cells can easily be confused with true binding to small cocci bacterial cells.

193   Spirocysts, which are a type of cnidae comprised of a single-walled capsule containing a

194   tightly coiled tubule bearing microtubules (Galloway et al., 2007), were another common site for

195   non-specific FISH probe binding. Spirocysts were frequently detected in the epidermal layer and

196   were particularly prevalent in the coenosarc and tentacles (Fig. 2F,G). Unlike granular gland

197   cells, spirocysts can be easily differentiated from bacterial aggregations due to their coiled

198   tubules (Fig. 2F,G). However, this distinction may not be obvious to the untrained practitioner.

199   Non-specific binding of FISH probes to common cellular structures (granular gland cells and

200   spirocysts, in particular) within coral tissues highlights the importance of including non-target

201   (i.e. NonEUB338) probe controls and extensive familiarization with coral cellular structure to

202   differentiate true positive signals from false positives.

203   Common approaches designed to avoid non-specific binding failed to reduce non-

204 specific fluorescent signals generated by false probe binding to spirocysts and granular gland

205 cells. The incorporation of alternative oligonucleotide probes, which targeted different 16S

206 rRNA sequences with different hybridization kinetics and efficiencies (competitor probes), also

207 yielded non-specific hybridization to granular gland cells (Fig. 2C) and spirocysts (Fig. 3).

208 Increased probe hybridization stringencies through higher formamide concentrations in the

209 hybridization buffers, plus higher stringency post-hybridization washing as recommended by

210 Wallner, Amann & Beisker (1993), also failed to reduce the strong fluorescent signal associated

211 with these cellular structures. Furthermore, the application of commercially available blocking

212 solutions, which are specifically designed to prevent non-specific binding, also failed to prevent

213 non-specific binding of oligonucleotide probes to granular gland cell and spirocysts (Fig. 3D).

214 Difficulties discriminating true detection of bacterial cells from non-specific binding to

215 non-target cellular structures is demonstrated in tissue sections of *Acropora hyacinthus* affected

216 by a WS disease (Fig. 2H). Coral-associated microbial aggregates (CAMAs) can be observed

217 within the calicoblastic layer of healthy tissues sampled from WS diseased colonies (n=3) and

218 probed with the EUB338 probe (Fig. 2H). CAMAs have been identified in healthy tissues of

219 many coral species (Work and Aeby 2014), although their exact functional role is unknown and

220 their influence on a coral's position along a healthy-diseased continuum needs further

221 exploration. Non-specific binding to granular gland cells was also observed within gastrodermal

222 cells that were in the vicinity of a bacterial aggregate, and could be mistaken for a true positive

223 without detailed understanding of coral cellular structures (Fig. 2H). True positive detection of

224 bacterial assemblages and individual bacterial cells were observed in all regions (n=6) of

225   sections from actively progression WS lesions (Fig. 2I).

226

227   *Recommendations to avoid common pitfalls*

228   The inability to prevent non-specific binding of oligonucleotide probes to cellular

229   structures within coral tissues represents a major challenge for accurate identification of specific

230   bacterial targets. However, by taking into account the issues highlighted above and the

231   recommendations laid out below, these challenges can be overcome.

232   • Combining appropriate microscope hardware with advanced image acquisition software

233   significantly improves detection of bacterial cells within coral tissues. Laser confocal

234   scanning microscopes (LSCM) provide the ideal platform for optimized image resolution,

235   fluorescence signal acquisition, and removal of non-specific background auto-fluorescence.

236   While standard fluorescence microscopes can be used to visualize coral samples for some

237   applications (e.g. coral mucus-associated microorganisms), high sample auto-fluorescence

238   often confounds coral FISH studies. Unfortunately, LSCMs are relatively expensive, which

239   limits access for some researchers, but many of the suggestions outlined below should prove

240   helpful, regardless of the microscope/software available.

241   • Selecting an appropriate fluorochrome with an emission spectrum distinct from that of coral

242   tissues (e.g. background green fluorescent proteins) and *Symbiodinium* cells (i.e.

243   background fluorescence in red and far red due to the presence of chlorophyll) is an

244   important consideration (Ainsworth et al., 2006a). Certain fluorochromes (e.g. FITC) are not

245 ideal for visualizing targets within coral tissues, while others (i.e. Cy3, Cy5 and Atto647)

246 are commonly used for coral samples. However, it is important to note that few, if any,

247 fluorochromes provide emission spectra completely distinct from that of coral tissue and

248 *Symbiodinium* auto-fluorescence.

249 • When using microscopes that allow spectral profiling at the pixel scale, comparison of

250 background emission spectra and intensity with that of potential positive signals (i.e. probe

251 binding) can greatly improve differentiation between background fluorescence and true

252 probe binding.

253 • Alternative approaches to FISH, such as catalyzed reporter deposition FISH (CARD-FISH)

254 that which increase probe emission signal intensity, can aid in the differentiation of target

255 versus non-target fluorescence. Such approaches have been widely used in other

256 environmental samples (Pernthaler, Pernthaler & Amann, 2002) and are now being

257 successfully used for corals (see examples in Ainsworth et al 2006a; Bayer et al., 2013;

258 Neave et al 2016).

259 • Serial sections should be visualized using both FISH and traditional staining (e.g. H&E stain

260 or Trichrome stains) to identify and localize granular gland cells within the gastrodermal

261 cell layer, particularly within the glandular band of mesenterial filaments, and spirocysts

262 within epidermal cell layers, particularly in coenosarc and stomodeum regions of the polyp.

263 • A nonsense probe representing a true negative control must be applied to serial tissue

264 sections (or on the same section) to help distinguish specific probe binding to target

265 bacterial populations from non-specific probe binding to coral cellular structures (Wallner,

266   Amann & Beisker, 1993).

267   • Familiarity with coral tissue structures is imperative to distinguish morphological

268   characteristics, such as size and shape that differentiate bacteria from coral tissue structures,

269   including spirocysts and granular gland cells that are prone to non-specific probe binding

270   (Posch et al., 1997, 2009). Undertaking practical courses that focus on histological analysis

271   of cellular structures, particularly focused on coral tissues, can aid in the familiarization

272   process, along with close collaborative networks with practitioners already trained in this

273   area.

274   Non-specific probe binding constitutes a significant impediment to FISH-based detection

275 of coral-associated bacterial communities. However, the inclusion of appropriate control

276 measures combined with observational rigor can greatly increase the ability of practitioners to

277 accurately distinguish non-specific probe binding and host auto-fluorescence from true positive

278 bacterial signals. Detailed approaches for the application of FISH to both healthy and diseased

279 corals are summarized in supplementary Fig. S1 and further details of pitfalls and possible

280 solutions are summarized in Table S1. Following these recommendations will allow practitioners

281 to avoid confusion associated with non-specific binding and allow FISH techniques to be

282 employed to their full potential in studies of coral tissues.

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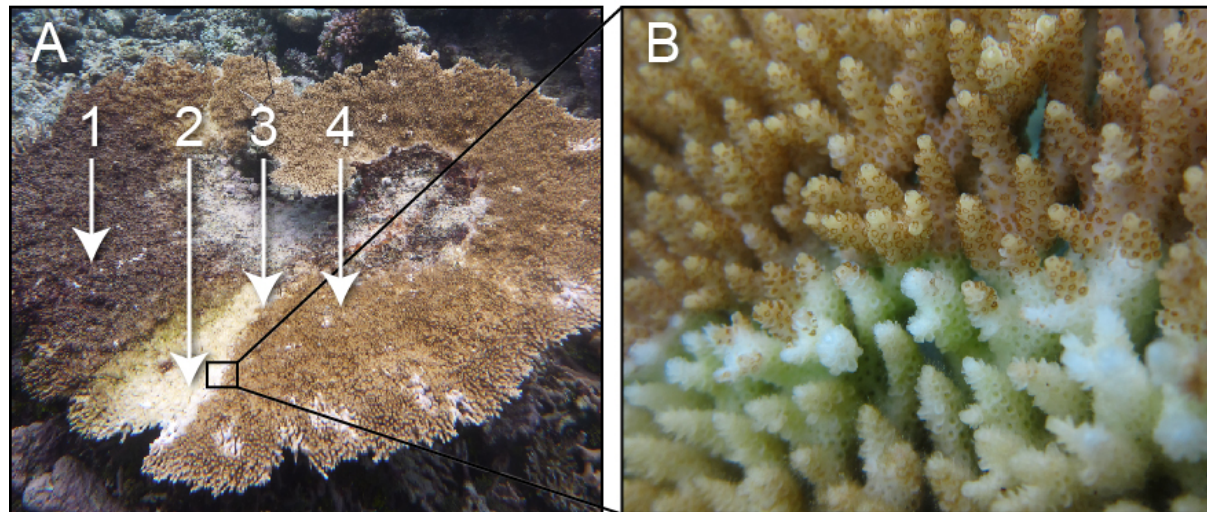
392 **Figure 1.** Characteristic field appearance of a white syndrome (WS) on a colony of the coral

393 *Acropora hyacinthus* from reefs near Lizard Island (14º40'S, 145º27'E) in the Northern sector of

394 the Great Barrier Reef (GBR): (A) WS lesion on a coral colony with numbers and arrows

395 displaying (1) dead coral skeleton overgrown with algae, (2) recently exposed coral skeleton

396 prior to algal overgrowth, (3) actively progressing lesion boundary where samples derived for

397 this study were taken, (4) healthy tissue ahead of the lesion and from which samples were also

398 derived.   (B) Close up image of lesion boundary displaying diffuse, acute to sub-acute areas of

399 tissue loss revealing white, intact skeleton.

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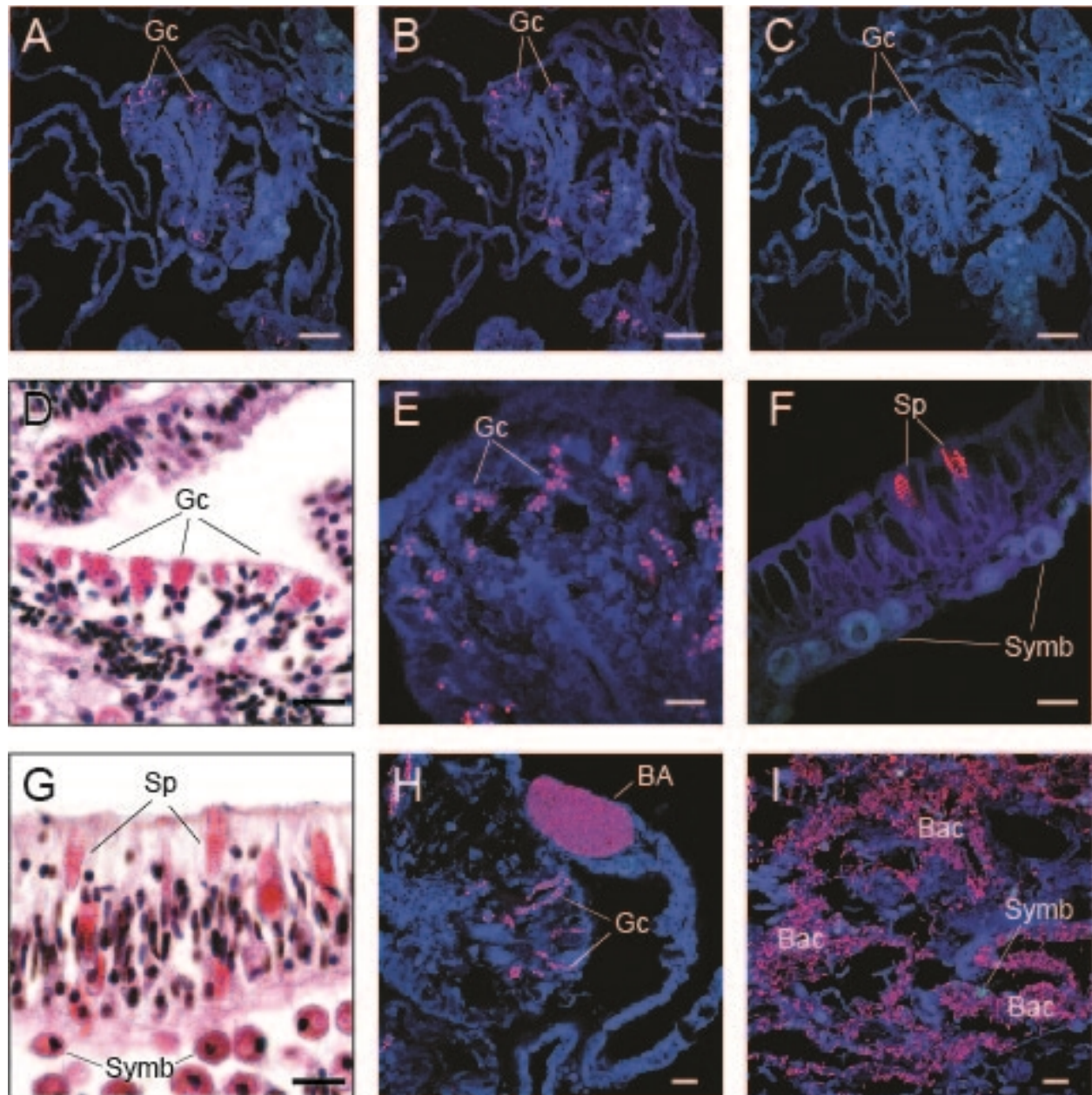
404 **Figure 2**. Detection and characterization of specific and non-specific fluorescence *in situ*

405 hybridization (FISH) probe binding to target bacteria (BA and Bac), granular gland cells (Gc),

406 and spirocysts (Sp) using Cy3-labelled FISH probes (A-C,E,F,H,I) and Hematoxylin and Eosin

407 (H&E) staining of coral tissue sections (D,G). Non-specific binding of EUB338 (A) and

408   nonEUB338 (B) FISH probes to granular gland cells and lack of auto-fluorescence of these

409   structures in probe-free treatments (C) is demonstrated through serial tissue sections. Detailed

410   granular gland cell morphology within the gastrodermis is shown in H&E stained (D) and

411   EUB338 FISH-hybridized (E) tissue sections. Non-specific binding of EUB338 FISH probes to

412   spirocysts within epidermal cells (F) was detected in tissue sections and was particularly

413   prevalent in coenosarc and tentacle regions of polyps. Detailed spirocyst morphology is shown

414   through H&E staining (G). A bacterial aggregate within the calicoblastic layer is shown near to

415   non-specific binding signals of granular gland cells (H) in healthy coral tissues hybridized with

416   EUB338 FISH probes. Bacteria assemblages were detected within necrotic tissues associated

417   with WS disease using EUB338 FISH probes (I). Scale bars represent 50 μm in A – C and 10 μm

418   in D - I. Abbreviations: Gc, granular gland cell; Sp, spirocysts; Symb, *Symbiodinium*; BA,

419   bacterial aggregation; Bac, bacterial assemblages.

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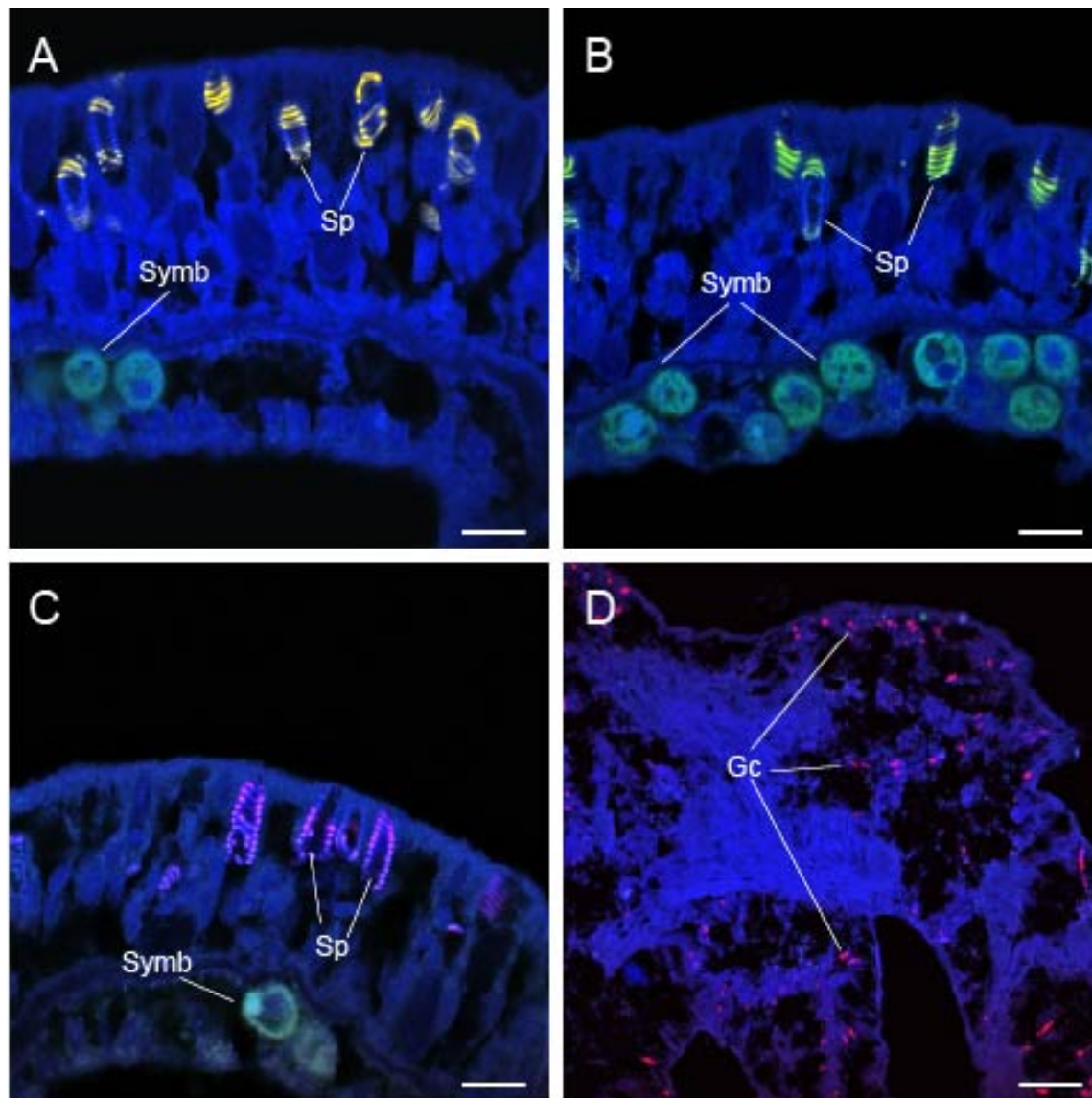
Figure 3. The application of common approaches designed to ameliorate non-specific

fluorescence *in situ* hybridization (FISH) probe binding failed to inhibit hybridization to granular

gland cells (Gc) and spirocysts (Sp). Cy3 (A), Cy5-labelled NonEUB338 (B) and Atto 647-

labelled Vib-GV (C) FISH probes all hybridized to spirocysts. Blocking solution also failed to

prevent non-specific binding of oligonucleotide probes to granular gland cells (D). Scale bars

represent 100 μm in A – C and 50 μm in D. Abbreviations: Gc, granular gland cell; Sp,

spirocysts; and Symb, *Symbiodinium*

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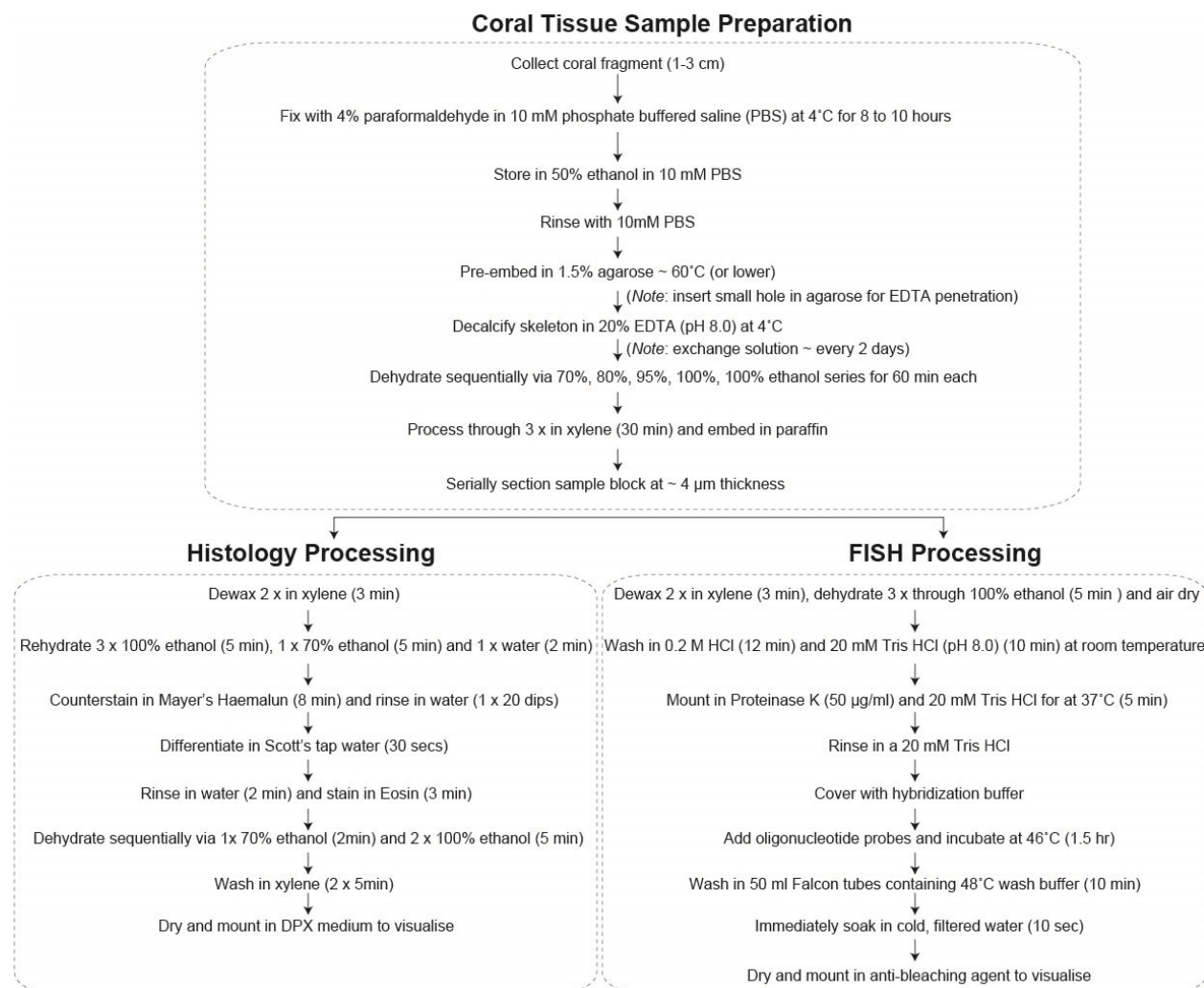
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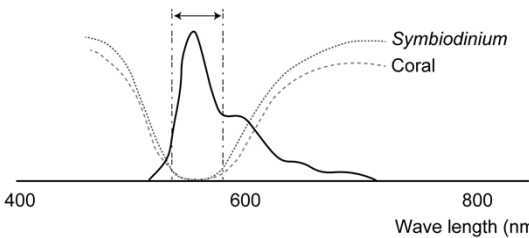
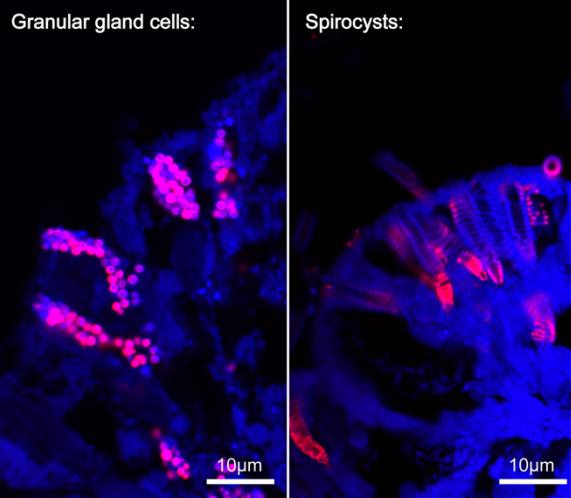
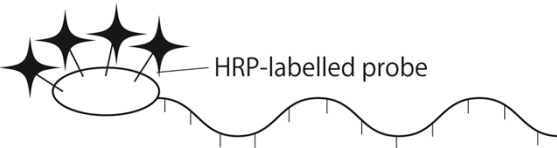
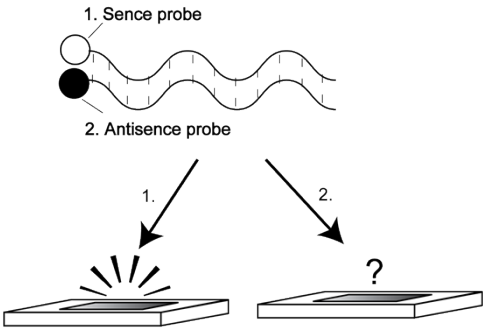
431 **Supplementary Figure S1.** Schematic flow chart detailing the protocol to process coral samples

432 for both histological and FISH based visualization of coral tissue structure and identification of

433 bacterial communities associated with coral tissues.

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435 **Supplementary Table S1**. Identified pitfalls and potential solutions to overcome challenges of applying FISH approaches to coral tissues

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Pitfall: Coral tissue and *Symbiodinium* autofluorescence emission spectra often overlap with FISH probe fluorochrome emission spectra. This overlap confounds effective differentiation between probe signal and background autofluorescence.	**Solution:** Careful selection of FISH probe fluorochromes is essential to minimise overlap between the emission ranges of probes and coral and *Symbiodinium* autofluorescence. A strong understanding of coral and *Symbiodinium* autofluorescence emission spectra is therefore required. Use of advanced imaging hardware, such as laser confocal scanning microscopes (LSCM), can significantly improve detection of probe signal over background autoflorescence and are highly recommended when available.	
Pitfall: Coral cellular structures, including nematocysts, spirocysts and granular gland cells, bind non-specifically to FISH probes and are thus difficult to distinguish from true positive detection.	**Solution:** Familiarity with coral cellular structures is essential to distinguish non-specific fluorescent cell types from specific bacterial hybridisation based on morphological characteristics.	

Pitfall: Low fluorescent signal due to low copy number of target rRNA molecules can make it difficult to visualise bacterial cells in coral tissue sections.	**Solution:** Florescent signal amplification techniques such as CARD-FISH can increase signal intensity when target rRNA copy numbers are low. These approaches have proven successful in coral FISH studies (see Bayer et al 2013, Neave et al 2016).	
Pitfall: The use of nonsense probes to detect non-specific binding can be problematic as the nematocysts, spirocysts and granular gland cells bind the probe. During a 2-step process of hybridising with a non-sense probe labelled with one flourochrome and then binding with another specific target probe labelled with a different flourochrome, these cellular structures hybridise to both probes.	**Solution:** Nonsense probes should still always be applied to tissue sections to detect and diagnose non-specific probe binding. However, familiarity with coral cellular structures is essential to distinguish these non-specific fluorescent cell types from targeted probe signals.	

Pitfall: While the use of advanced imaging hardware (such as a LSCM) combined with florescent signal amplification techniques (such as CARD-FISH) is recommended, such approaches have the potential to confound the specific detection of bacteria in coral tissues. For example, the signal intensity of non-specific binding to spirocysts and granular gland cells is often much greater than that of specific probe binding to bacteria. This property allows a distinct colour to be assigned to these false positives so that they can be disregarded. The application of CARD-FISH can intensify true positive signals to a level comparable to those associated with non-specific binding making intensity-based differentiation difficult.	**Solution:** Care must be exercised when using florescent signal amplification techniques in combination with advanced imaging hardware that allows spectral profiling at the pixel scale. Again, familiarity with coral cellular structures is essential to ensure all detected signals are from bacteria specific targets and not background non-specific cellular structures.	

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