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RESEARCH ARTICLE

Failure of a proprietary fluorescent in situ hybridization assay to detect M. avium subspecies paratuberculosis in archived frozen brain from patients with Multiple Sclerosis

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ABSTRACT

Objectives: Multiple Sclerosis is a chronic, enigmatic, progressive central nervous system "inflammatory" disease, with no know etiology. As with other "inflammatory" diseases there is the possibility that a cryptic infectious trigger may play a role in initiating Multiple Sclerosis. *M. avium* subspecies *paratuberculosis* causes Johne's disease in ruminants and may be an infectious trigger in Crohn's disease.

In this study, frozen archived brains from patients with Multiple Sclerosis, pure culture of multiple bacteria and circulating WBCs were assayed with proprietary (Affymetrix™ RNA view®) Tissue and Cell fluorescent *in situ* hybridization assay for MAP RNA.

Results: Repetitively, false positive signal was observed in the "No-Probe" negative control. Despite advice from the technical staff at Affymetrix, multiple experimental modifications could not prevent positive signal in the "No-Probe" negative control.

When studying human white blood cells under specific storage conditions, we observe positive signal with human house-keeping genes, when no signal is seen in the No-Probe controls.

Conclusions: We conclude, that when performed according to manufactures instructions and with multiple variations on the manufactures recommended suggestions to correct for false positive signal, that the Affymetrix™ RNA view® TISSUE assay cannot be used to detect *M. avium* subspecies *paratuberculosis* in pre-frozen brains of humans with Multiple Sclerosis. In contrast, using the Affymetrix™ RNA view Cell fluorescent *in situ* hybridization system, evaluating human white blood cells, we reliably identify human house-keeping genes. This indicates that the Cell fluorescent *in situ* hybridization assay may be useful when evaluating circulating cells for specific pathogens.

Keywords: *In situ* hybridization; multiple sclerosis; *Mycobacterium avium* subspecies *paratuberculosis*; Mycobacteria; Crohn disease; Johne disease:

Abbreviations Used:

MAP= *M. avium* subspecies *paratuberculosis*
FISH =Fluorescent *in situ* hybridization
HCL= hydrochloric acid
MS = Multiple Sclerosis
CD = Crohn's disease
IBD = Inflammatory Bowel Disease.

Introduction

Multiple Sclerosis (MS) was first definitively described by Charcot in the French medical literature in 1868 [1] and subsequently in the English medical literature in 1873. [2] MS remains a chronic, enigmatic, progressive neurological disease of the central nervous system, that has no known etiology (see [3] for review.) The possibility that the etiology of MS may be consequent to an infectious cause is the cause of innumerable studies and speculations (see [4-6] for reviews.) None of which have been proven or are accepted.

Intriguingly, the relevance of vitamin D in the prevalence, incidence and clinical course of MS has revealed noteworthy correlates. [7,8]. Circumstantial data show the relevance of vitamin D include migration, its time of life and destination influence the incidence of MS.[9-12] Observational studies [13-15] show a positive clinical course when vitamin D levels are elevated and an exacerbation when vitamin D levels are low. Randomized control trials of vitamin D supplementation in MS did NOT meet their primary endpoints (which was no evidence of disease activity) but did show improvements in secondary endpoints (annualized relapse rates and new or hypointesnse T1 Lesions on MRI [16] Habitually, the role of vitamin D is assigned to its well documented effect on the immune response of the afflicted individual.[17-22] We have shown that vitamin D directly inhibits mycobacteria in culture [23,24], suggesting a possible correlate between an infectious etiology of MS and the role of vitamin D.

Johne's disease [25] is a chronic wasting intestinal infection in animals, universally acknowledged to be caused by *M. avium* subspecies *paratuberculosis* (MAP.) There is increasing concern that MAP may be zoonotic. [26] Crohn's disease is an affliction evocative of Johne's disease [27-30]. MAP is frequently implicated as a possible infectious trigger in the "inflammatory" bowel condition best known as Crohn's disease (See [24,31] for review.)

Evidence of polymicrobial (fungal as well as bacterial) have been documented in MS. [32] In 1986, an unexplained association of MS with inflammatory bowel disease was observed. [33] Subsequently [34], in a series based primarily on immunological studies [35-40], a possible MAP/MS etiological connection has been investigated. It must be emphasized, that these studies did not identify MAP in MS lesions. Accordingly, it would be of noteworthy interest if viable MAP, could be identified in MS CNS lesions. The presence of MAP RNA [30], or culture of MAP [41] would be an acceptable indication of viability.

A proprietary (Affymetrix™ RNA view®) fluorescent *in situ* hybridization (FISH) assay identifies RNA. We have shown that this FISH assay cannot be used to reliably identify MAP RNA in frozen archived intestine from ruminants with Johne disease. [42] Likewise we show that this FISH assay cannot reliably identify MAP RNA in frozen archived resected intestine from patients with Crohn's disease.[43]

The purpose of this present study was to evaluate whether the MAP Affymetrix™ RNA view® FISH assay could reliably detect MAP RNA from frozen brain autopsy specimens of humans who had demonstrable multiple sclerosis as well as normal brain controls.

Methods.

This study was approved by the Research & Development Committee at the VAMC Bronx NY (0720-06-038.) The methods and results with bovine ileal intestinal tissue, with and without Johne's disease [42], and with human tissue in Crohn disease have been published.[43] Non-identifiable brain tissue from individuals with and without multiple sclerosis were obtained from Wallace W. Tourtellotte, M.D., Ph.D. Human Brain and Spinal Fluid Resource Center. Neurology Research brainbnk@ucla.edu. Specimens were shipped on dry ice and stored at -80°C. until processed as below.

The tissue and assay were handled in an identical manner to that of the published bovine study [42], with one exception. Previously, at our request Affymetrix had generated probes that were species specific from published gene sequences. In this (and the parallel Crohn disease study [43], the housekeeping gene was Human Specific β-actin (Affymetrix Catalog # VA6-10506-1 Probe type 6) As in the previous study for MAP, an Affymetrix generated probe designed using the published sequence. [44] (Affymetrix name: M. tuberculosis Is900: Cat # VF1 19496: Lot # 195634523: Probe type 1.) Previously, the house keeping gene for ruminants was bovine β-actin (Bos Taurus actb: NCBI Reference Sequence: NM_173979.3 (Affymetrix name: Bos Taurus Actb: Cat# VF6 20062: Lot # 200642784: Probe Type 6.) All these probes are proprietary to Affymetrix. We also use 16S Bacterial, Probe type 6, Cat # VF-6-16576-01: 16S *E. Coli*, Probe type 1 Cat # VF1-19200-01: 16S *Mycobacterium tuberculosis* Probe type 1, Cat # VF1-16224-01: Human β-Actin (ACTB Human) Probe type 1, Cat # VA1-10351-01: Bovine β-Actin (ACTB BOB TAURUS) Probe type 1 Cat# VF1-20959-01: IS 900 (*M. avium* subspecies *paratuberculosis*) Probe type 6, Cat # VF6-20958-01: IS6110 (*Mycobacterium Tuberculosis*) Probe type 1, Cat # VF1-6000090-01: and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (As an additional house-keeping gene) Probe type 6 Cat # VA6-100337-01. All these probes are proprietary to Affymetrix.

Excepting for using a Human Specific β-actin probe and non-identifiable human brain tissue (instead of bovine [42] and or human intestine [43],) the assay was carried out identically as published .[42,43]

Experimental Design

Table 1: Brain Tissue, Bacteria, Cultured Cells and Human White Blood Cells studied

Figures #'s	Tissue/Cell	With Probes Figure #'s	No Probe Control Figure #'s	Indicate reliable FISH data
1-8	Human Brain MS	1,3,6	2,7,8	No
9-12	Human Brain Normal	9	10,11,12	No
13-15	BCG	13	14,15	No
16-18	MAP Dominic	16	17,18	No
19-21	E. Coli	19	20,21	No
22-24	M. tb.	22	23,24	No
25-28	M. avium avium	25,26	27,28	No
25-31	MAP Dominic	29	30,31	No
32-33	Murine Cells	32	33	No
34-43	Human WBC's	34,36,38,40,42	35,37,39,41,43	Possibly

Abbreviations used in Table 1: FISH = Fluorescent *In Situ* Hybridization assay. MAP = *M. avium* subspecies *paratuberculosis*. M. avium = *M. avium* subspecies *avium*. BCG = Bacillus Calmette–Guérin Karlson and Lessel ATCC 19015. MS = Multiple Sclerosis. Murine Cells = Murine RAW 264 NIH93 Cells. WBC's White Blood Cells isolated by Ficoll gradient.

Results

In Table 2 we show the overview of all the experiments and their results. Note that it is only in Figures 35-43 that we obtain relable results, specifically with WBCs.

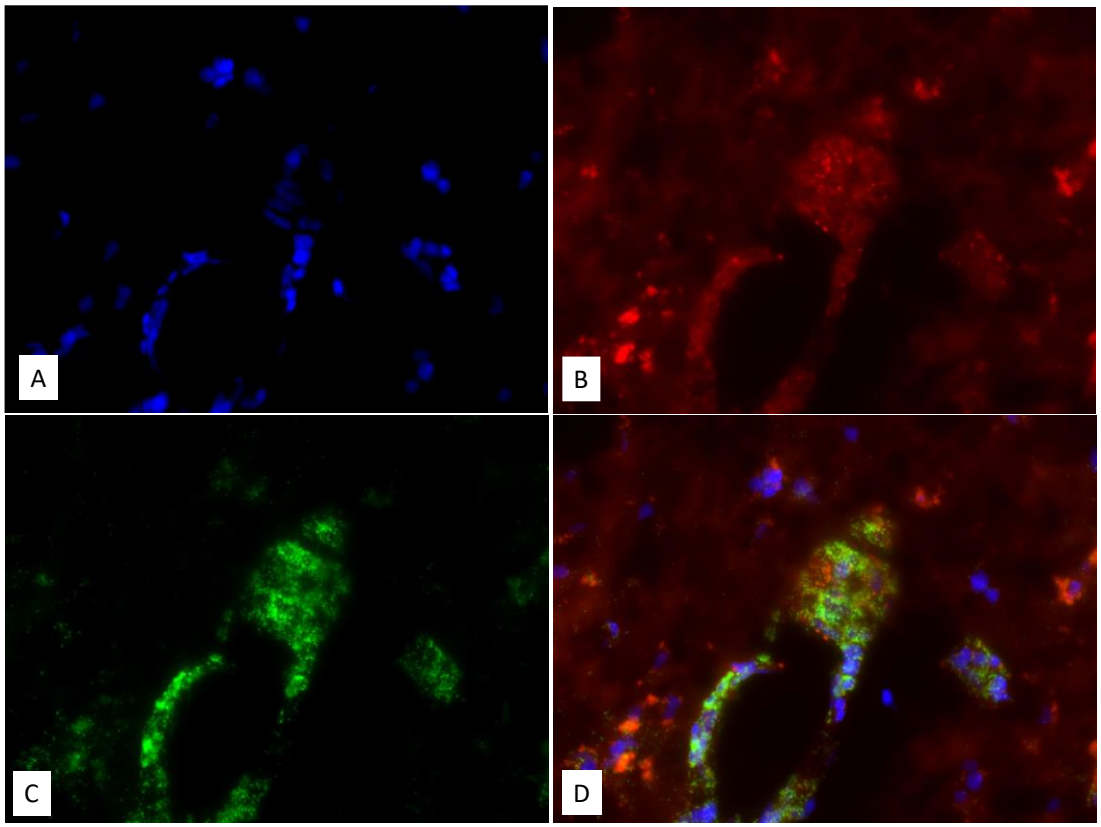
Table 2

Figure #	Probes / Stains used	Technique Modification	Filters used	FISH	"No-Probe" Control	Positive/Negative Signal
1. (Brain)	(IS900) Human B-Actin	None	Texas Red Cy-5	Yes		"Positive"
2. (Brain)	None	None	Texas Red Cy-5		Yes (for #1)	"Positive"
3. (Brain)	None	None	Texas Red Cy-5		Yes (for #1)	"Positive"
4. (Brain)	H&E					NA
5. (Brain)	Luxol Blue					NA
6. (Brain)	IS900/ B-Actin	None	Texas Red Cy-5	Yes		"Positive"
7. (Brain)	None	None	Texas Red Cy-5		Yes (for #6)	"Positive"
8. (Brain)	None	None	Texas Red Cy-5		Yes (for #6)	"Positive"
9. (Brain)	IS900/ B-Actin	True Black®	Texas Red Cy-5	Yes		"Positive"
10. (Brain)	None	True Black®	Texas Red Cy-5		Yes (for #9)	"Positive"
11. (Brain)	None	None	Texas Red Cy-5		Yes (for #9)	"Positive"
12. (Brain)	None	True Black®	Texas Red Cy-5		Yes (for #9)	"Positive"
13. (BCG)	16S (Red) 16S(Green)	RNA View® Tissue	Texas Red Cy-5	Yes		"Positive"
14. (BCG)	None	RNA View® Tissue	Texas Red Cy-5		Yes (for #13)	"Positive"
15. (BCG)	None	RNA View® Tissue	Texas Red Cy-5		Yes (for #13)	"Positive"
16. (Dominic)	16S (Red) 16S(Green)	RNA View® Tissue.	Trit C Hope	Yes		"Positive"
17. (Dominic)	None	RNA View® Tissue.	Trit C Hope		Yes (for #16)	"Positive"
18. (Dominic)	None	RNA View® Cell	Texas Red Cy-5		(Yes (for #16)	"Positive"
19. (E. Coli)	16S (Red) 16S(Green)	RNA View® Tissue.	Texas Red Cy-5	Yes		"Positive"
20. (E. Coli)	None	RNA View® Tissue.	Texas Red Cy-5		Yes (for #19)	"Positive"
21. (E. Coli)	None	RNA View® Cell	Texas Red Cy-5		(Yes (for #19)	"Positive"
22. (M. tb)	IS 6110 (Red)	RNA View®	Texas Red	Yes		"Positive"

Figure #	Probes / Stains used	Technique Modification	Filters used	FISH	"No-Probe" Control	Positive/Negative Signal
	IS 900 (Green)	Tissue.	Cy-5			
23. (M. tb)	None	RNA View® Tissue	Texas Red Cy-5		Yes (for #22)	"Positive"
24. (M. tb)	None	RNA View® Cell	Texas Red Cy-5		Yes (for #22)	"Positive"
25. (M. avium.avium)	16 S (Red) 16S (Green)	RNA View® Tissue	Texas Red Cy-5	Yes		"Positive"
26. (M. avium.avium)	IS 900 (Red) 16S (Green)	RNA View® Tissue	Texas Red Cy-5	Yes		"Positive"
27. (M. avium.avium)	None	RNA View® Tissue	Texas Red Cy-5		Yes (for # 25)	"Positive"
28. (M. avium.avium)	None	RNA View® Cell	Texas Red Cy-5		Yes (for # 26)	"Positive"
29. (Dominic)	IS 900 (Red) 16S (Green)	RNA View® Tissue	Texas Red Cy-5	Yes		"Positive"
30. (Dominic)	None	RNA View® Tissue	Texas Red Cy-5		Yes (for # 29)	"Positive"
31. (Dominic)	None	RNA View® Tissue	Trit C Hope		Yes (for #29)	"Positive"
32. (Murine RAW264 NIH 93 Cells)	IS 900 (Red) 16S (Green)	RNA View® Tissue	Texas Red Cy-5	Yes		"Positive"
33. (Murine RAW264 NIH 93 Cells)	None	RNA View® Tissue	Texas Red Cy-5		(Yes (for #32)	"Positive"
34. (Human Ficoll WBC's)	Human B-Actin (Red) Human GAPD (Green)	RNA View® Cell RT x 1 Hr	Texas Red Cy-5	Yes		Positive
35. (Human Ficoll WBC's)	None	RNA View® Cell	Texas Red Cy-5		Yes (For # 34)	Negative
36. (Human Ficoll WBC's)	Human B-Actin (Red) Human GAPD (Green)	RNA View® Cell 4°C x 24 Hr	Texas Red Cy-5	Yes		Positive
37. (Human Ficoll WBC's)	None	RNA View® Cell 4°C x 24 Hr	Texas Red Cy-5		(Yes (for #36)	Negative
38. (Human Ficoll WBC's)	Human B-Actin (Red) Human GAPD (Green)	RNA View® Cell RT x 24 Hr	Texas Red Cy-5	Yes		"Positive"
39. (Human Ficoll WBC's)	None	RNA View® Cell RT x 24 Hr	Texas Red Cy-5		Yes (For #38)	"Positive"
40. (Human Ficoll WBC's)	Human B-Actin (Red) Human GAPD (Green)	RNA View® Cell 4°C x 72Hr	Texas Red Cy-5	Yes		"Positive"
41. (Human Ficoll WBC's)	None	RNA View® Cell 4°C x 72Hr	Texas Red Cy-5		Yes (for # 40)	"Positive"
42. (Human Ficoll WBC's)	Human B-Actin (Red) Human GAPD (Green)	RNA View® Cell RT x 72Hr	Texas Red Cy-5	Yes		Positive
43. (Human Ficoll WBC's)	None	RNA View® Cell RT x 72Hr	Texas Red Cy-5		Yes (for #42)	Negative

Shown in Figure 1 are the results with MAP and Human specific β-actin.

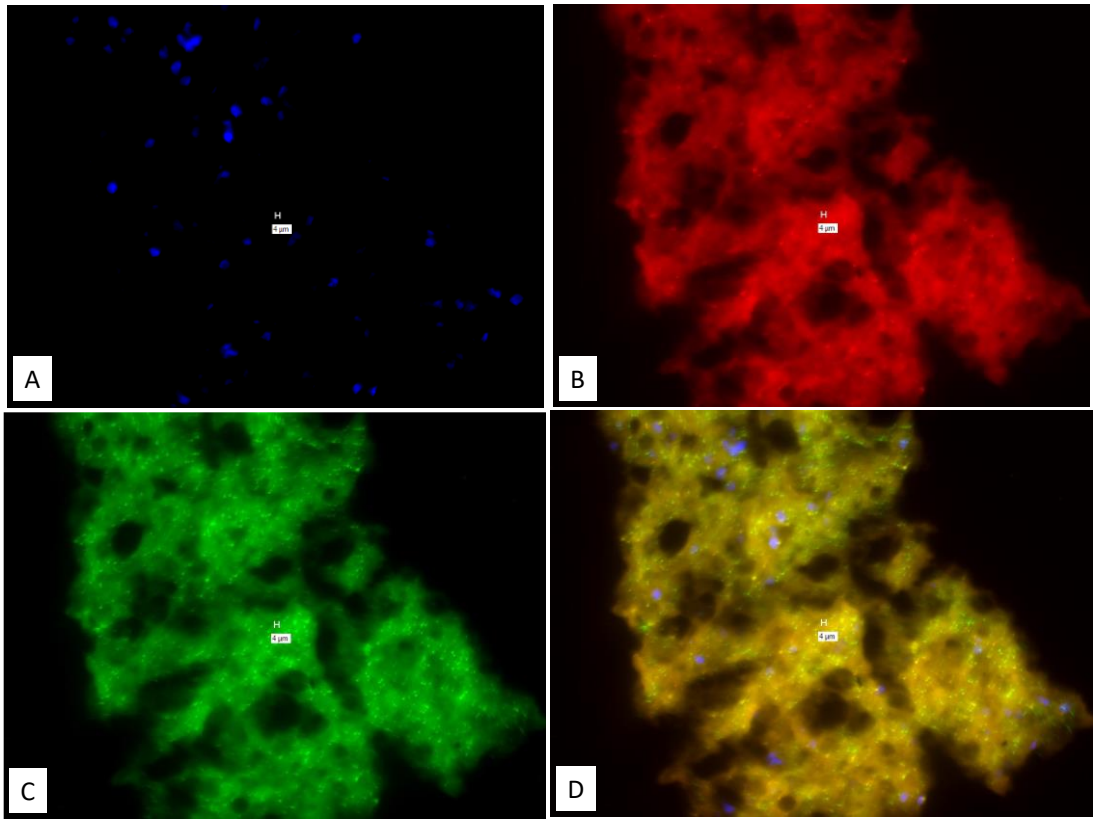
Figure 1



Legend to Figure 1. A composite of four images of the same section of human Multiple Sclerosis brain tissue. A= DAPI; B=Texas Red (IS900); C=Cy-5 (Human β-actin) D= composite of A, B & C: Note positive signal in Figures 1B, C and D. Section #021 from Z-stack image. Magnification = x60.

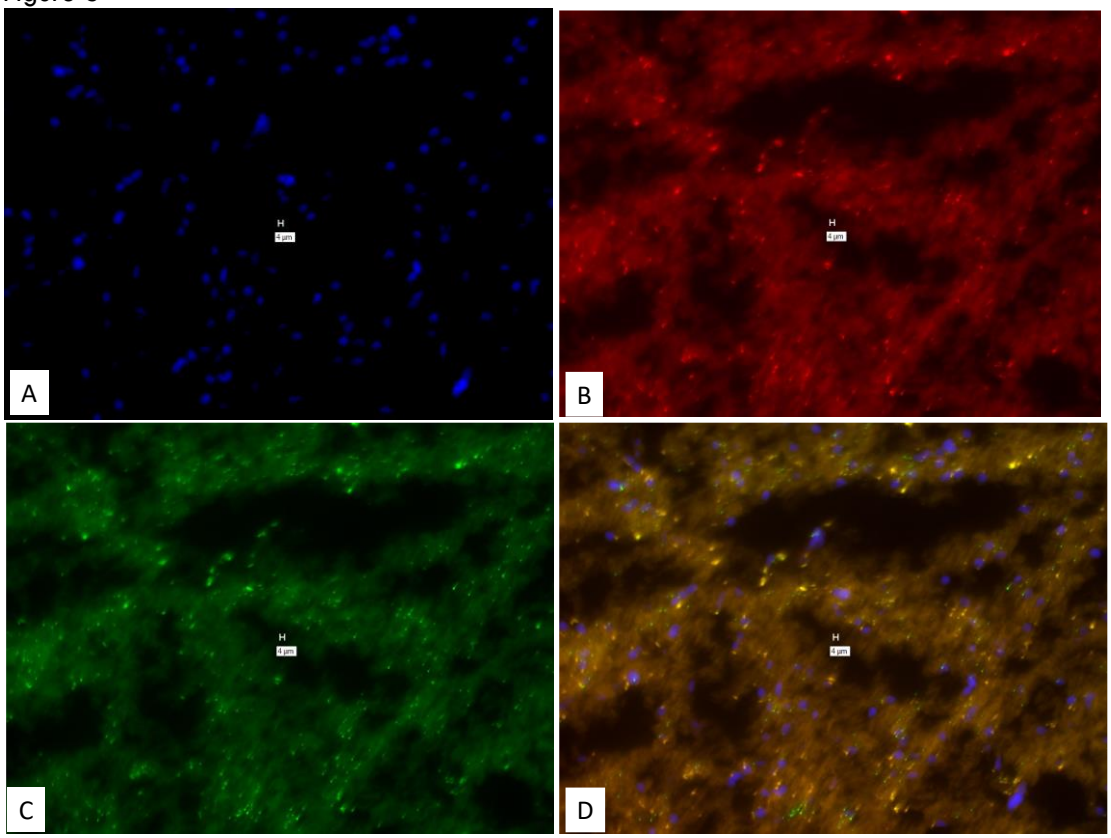
Repetitively a clear background could not be observed in the control slides, from which probes had been excluded during the Probe Set Hybridization steps. (Figure 2 & 3).

Figure 2



Legend to Figure 2. No-Probe control for Figure 1. Processed identically as in Figure 1, during the same experiment, but No probes were added during the hybridization step. A= DAPI; B=Texas Red; C=Cy-5; D = composite of A, B & C. On this No-Probe control, note "positive" signal in Figures 2 B, C and D. Marker bars in µm indicates magnification of x 40.

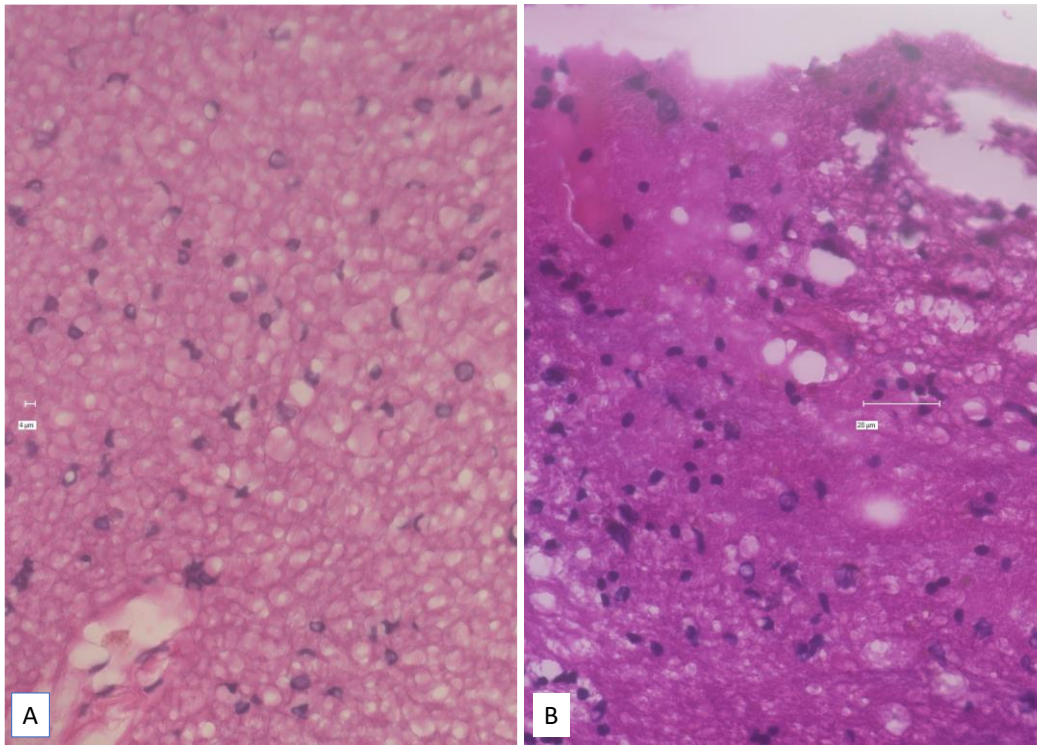
Figure 3



Legend to Figure 3. "No-Probe" negative from a different patient (Pt # 3860; Multiple Sclerosis) but processed identically in the same experiment as in Figures 1 & 2. Note the "positive" signal in this "No-Probe" control in Figures 3 B, C and D.

Shown, in Figure 4, are Hematoxylin and Eosin stains of multiple sclerosis (Panel A) and normal brain (Panel B).

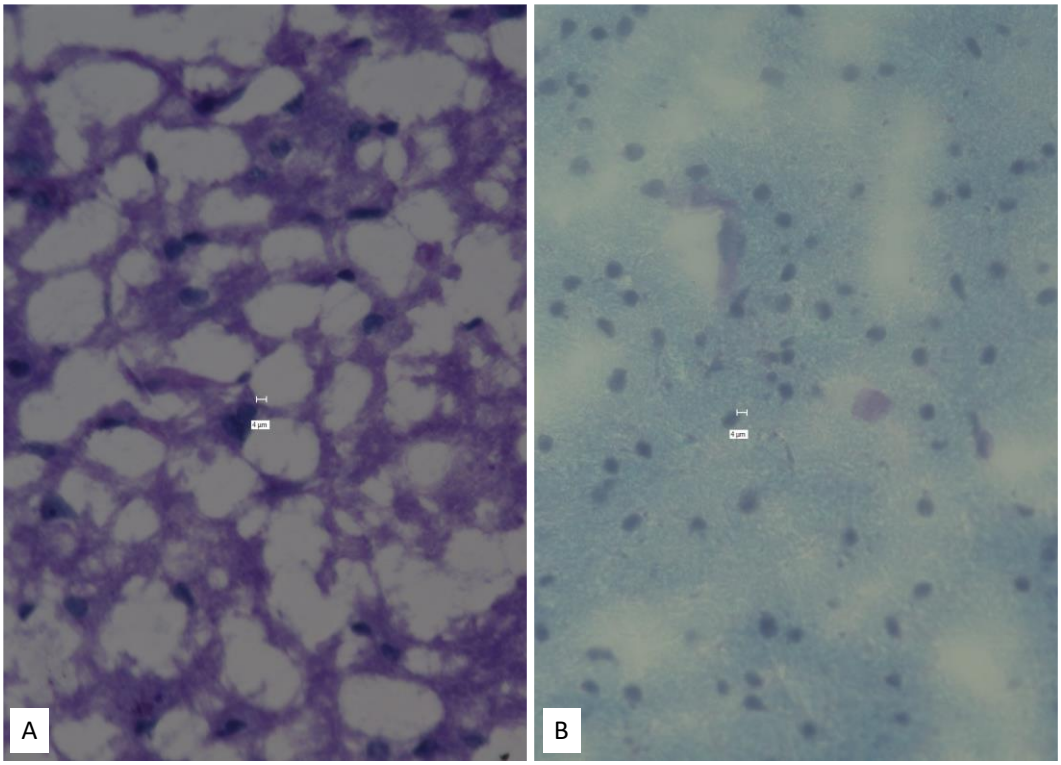
Figure 4



Legend to Figure 4. Brain with Multiple Sclerosis (Panel A. Left) and Normal Brain (Panel B. Right) Hematoxylin and Eosin. Magnification x 40.

In Figure 5 are the Luxol Fast Blue stains of MS (Panel A; Left) and Normal brain (Panel B; Right). These confirm the samples received from the MS samples from the Human Brain and Spinal Fluid Resource Center. Neurology Research brainbnk@ucla.edu, are brain.

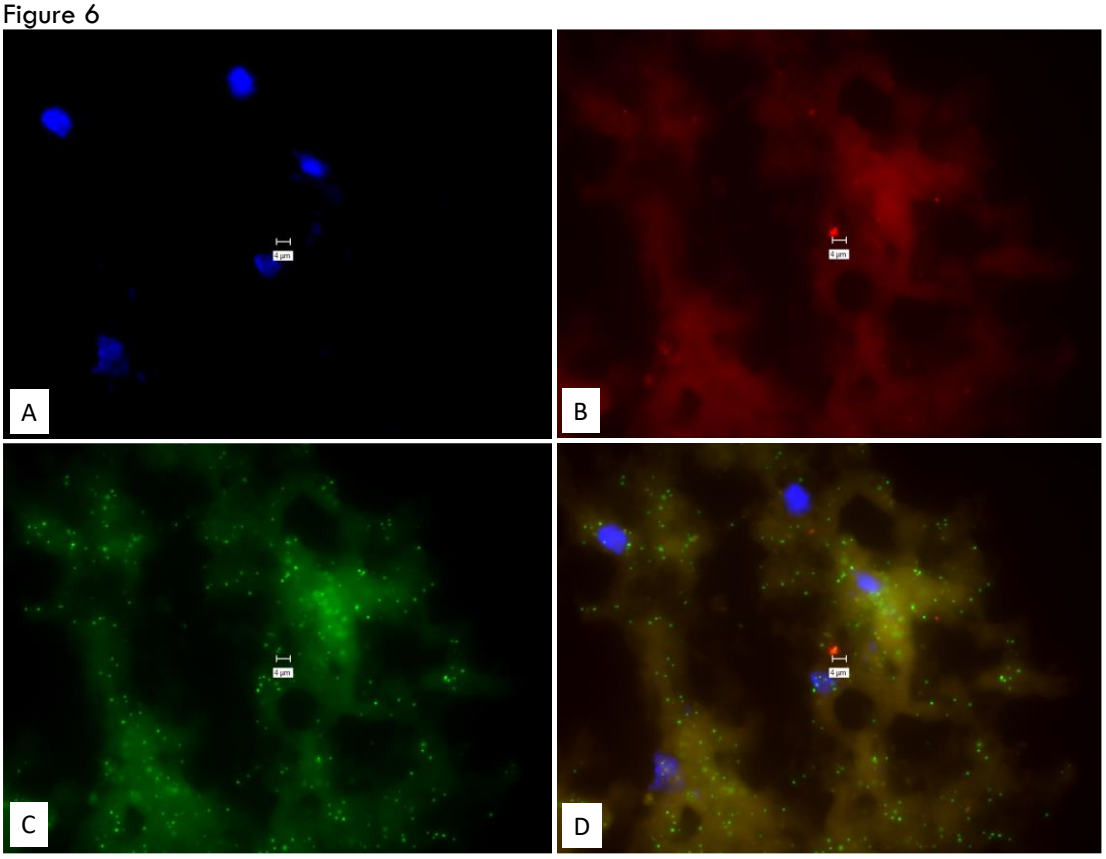
Figure 5



Legend to Figure 5. MS (Panel A; Left Panel) and Normal brain (Panel B; Right) Luxol Fast Blue stain. Magnification = x 40

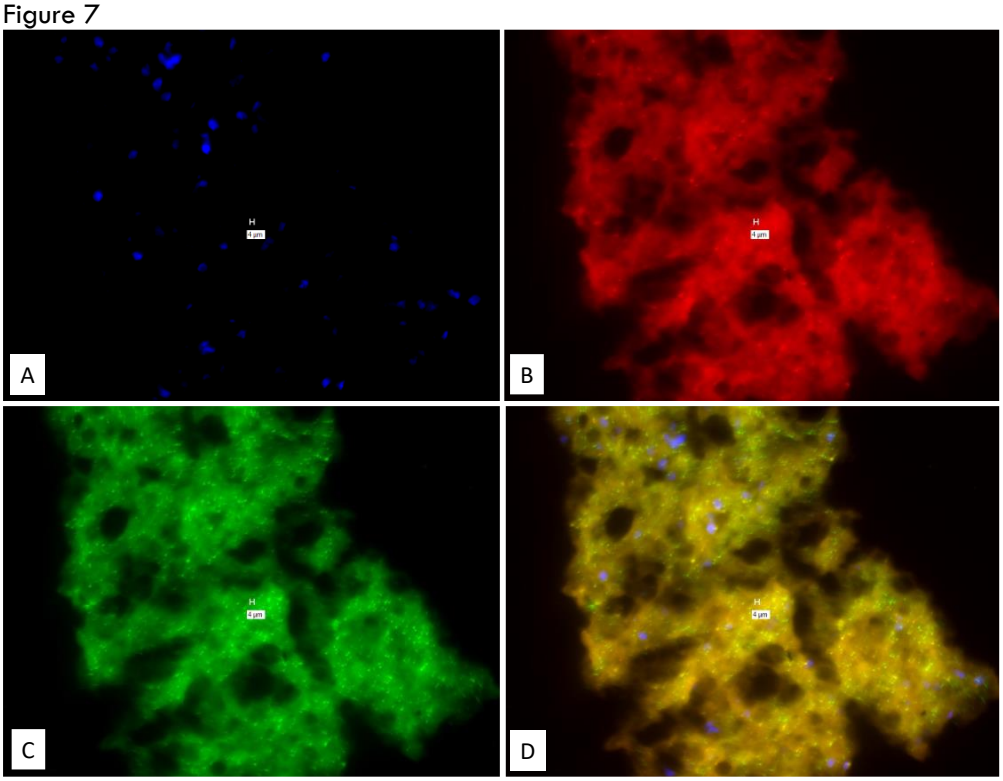
A patient with MS was studied with probes (Figure 6) and without probes (Figure 7).

Figure 6



Legend to Figure 6. Human brain with Multiple Sclerosis. Slide was processed identically and in the same experiment as Figures 1, 2 & 3. With probes: A= DAPI; B=Texas Red (Probe is IS 900 Type 1 Red); C=Cy-5 (Probe is Human β-actin; Type 6 Green) D= composite of A, B & C. Note "positive" signal in panels B, C & D) Marker bars in μm indicates magnification of x 100.

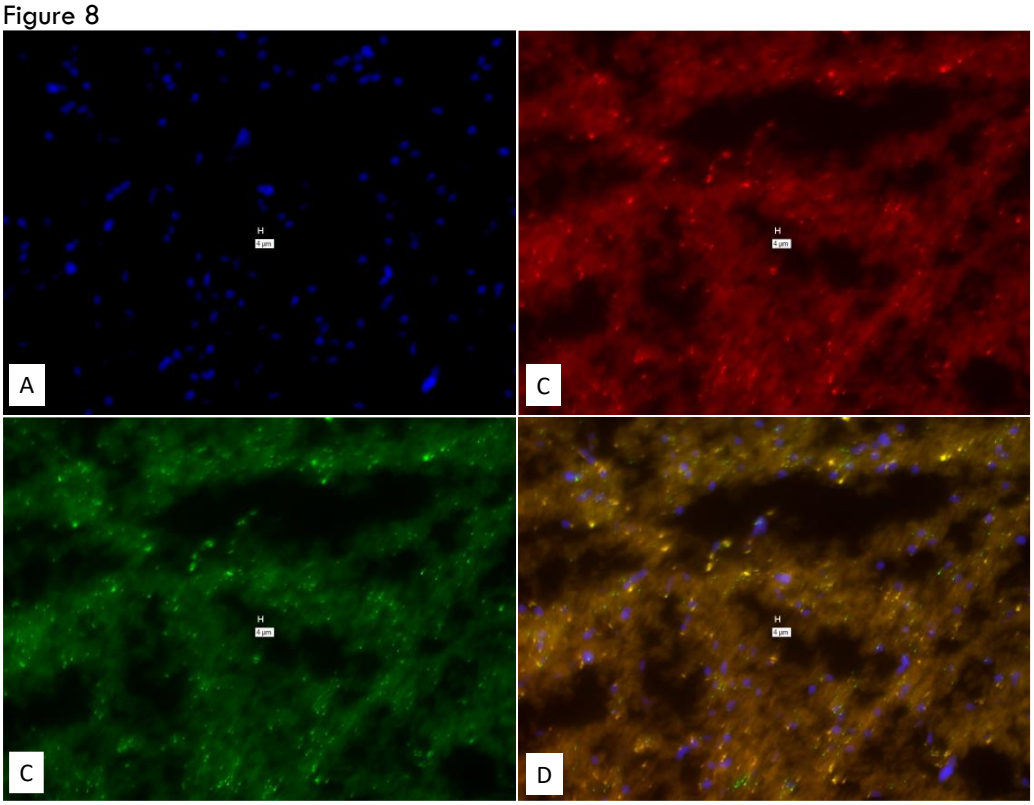
Figure 7



Legend to Figure 7. This is the No-Probe control for Figure 6. Human Multiple Sclerosis brain. Tissue is from the same patient as in Figure 6. Slide was processed identically and in the same experiment as Figures 1, 2, 3 & 6. A= DAPI; B=Texas Red; C=Cy-5, D= composite of A, B & C. Note "positive" signal in panels B, C & D. Marker bars in µm indicates magnification of x 40.

Both MS No-Probe controls (Figures 7 & 8) show positive signal. These corroborate the data presented in Figures 1, 2 & 3.

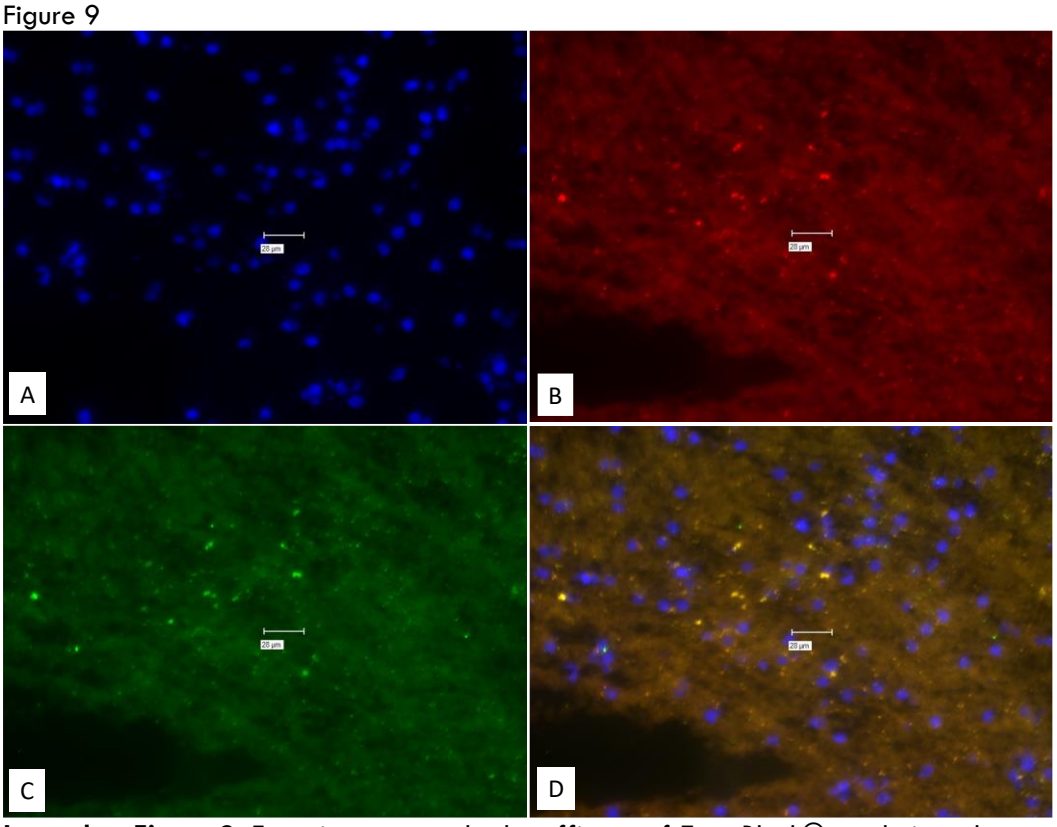
Figure 8



Legend to Figure 8. This is another **"No-Probe"** control. Additional patient with Multiple Sclerosis. Slide was processed identically and in the same experiment as Figures 1, 2, 3, 6 & 7. A= DAPI; B=Texas Red; C=Cy-5, D= composite of A, B & C. Note "positive" signal in panels B, C & D. Marker bars in µm indicates magnification of x 40.

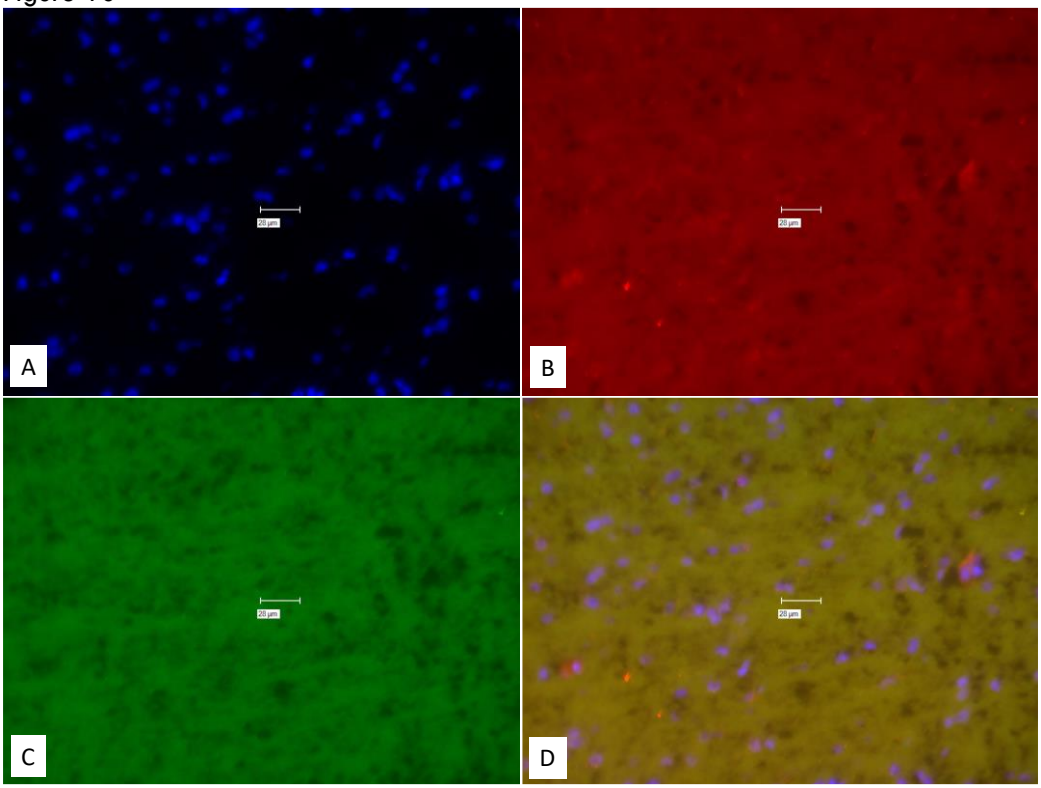
A commercially available product, True Black®, claims to prevent factitious auto-fluorescence. Shown are TrueBlack® on normal brain with probes (Figure 9) and without probes (Figure10.) Although there is diminution of false positive signal in the No-Probe control, false positive signal (Figure 10 Panels B & D) is still observed.

Figure 9



Legend to Figure 9. Experiment to study the efficacy of True Black® to obviate the auto-fluorescence. No-True-Black control with Probes. Specimen is Normal brain. WITH probes: A= DAPI; B=Texas Red (Probe is IS900 Type 1 Red); C=Cy-5 (Probe is Human β-actin; Type 6 Green) D= composite of A, B & C. Marker bars in µm indicates magnification of x 40.

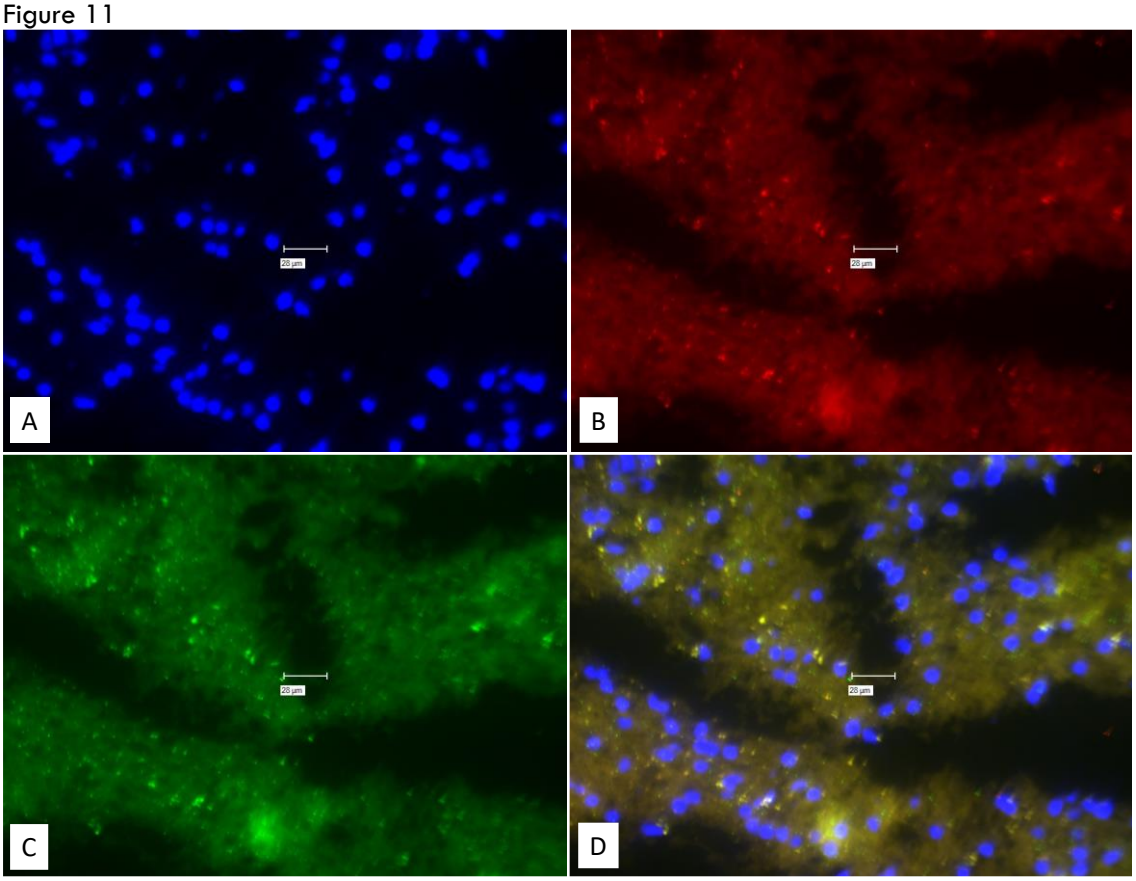
Figure 10



Legend to Figure 10. NO PROBE control for Figure 9. Experiment to study the efficacy of True Black® to obviate the auto-fluorescence. In this study there was No-True-Black added as a control and no Probes were used. Specimen is Normal brain. WITHOUT probes: Note positive signal in Panels B & D. A= DAPI; B=Texas Red (Type 1 Red); C=Cy-5: Type 6 Green) D= composite of A, B & C. Marker bars in µm indicates magnification of x 40.

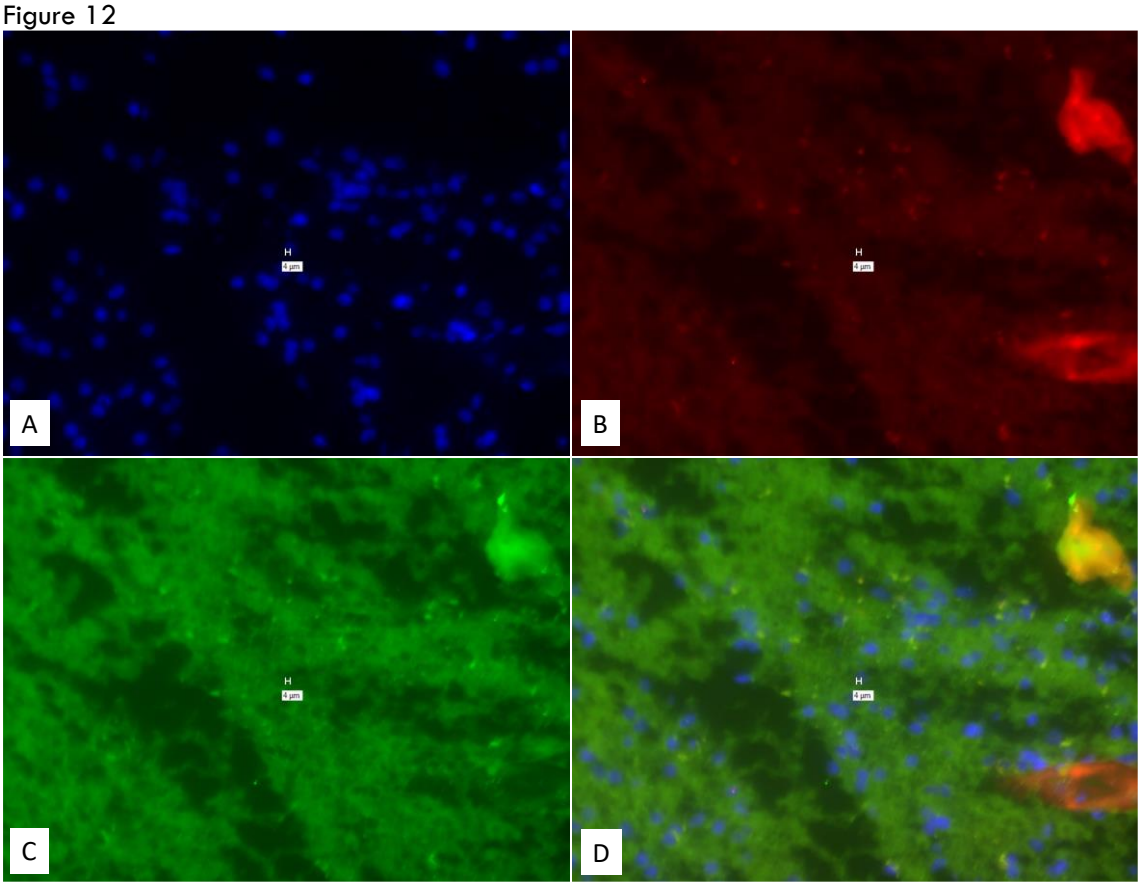
Similarly, a comparison of No TrueBlack No-Probe (Figure 11) with +TrueBlack-No-Probe shows less auto-fluorescence, but it is still present (Figure 12; Panels B & D.) We conclude that the improvement using True-Black® does not completely remove false positive signal and that True-Black® cannot be used in a binary (present or absent) study.

Figure 11



Legend to Figure 11. Experiment to study the efficacy of True Black® to obviate the auto-fluorescence. Specimen is Normal brain. This is a No-Probe, No True-Black® control. A= DAPI; B=Texas Red; C=Cy-5, D= composite of A, B & C. Note "positive" signal in panels B, C & D. Marker bars in µm indicates magnification of x 40.

Figure 12

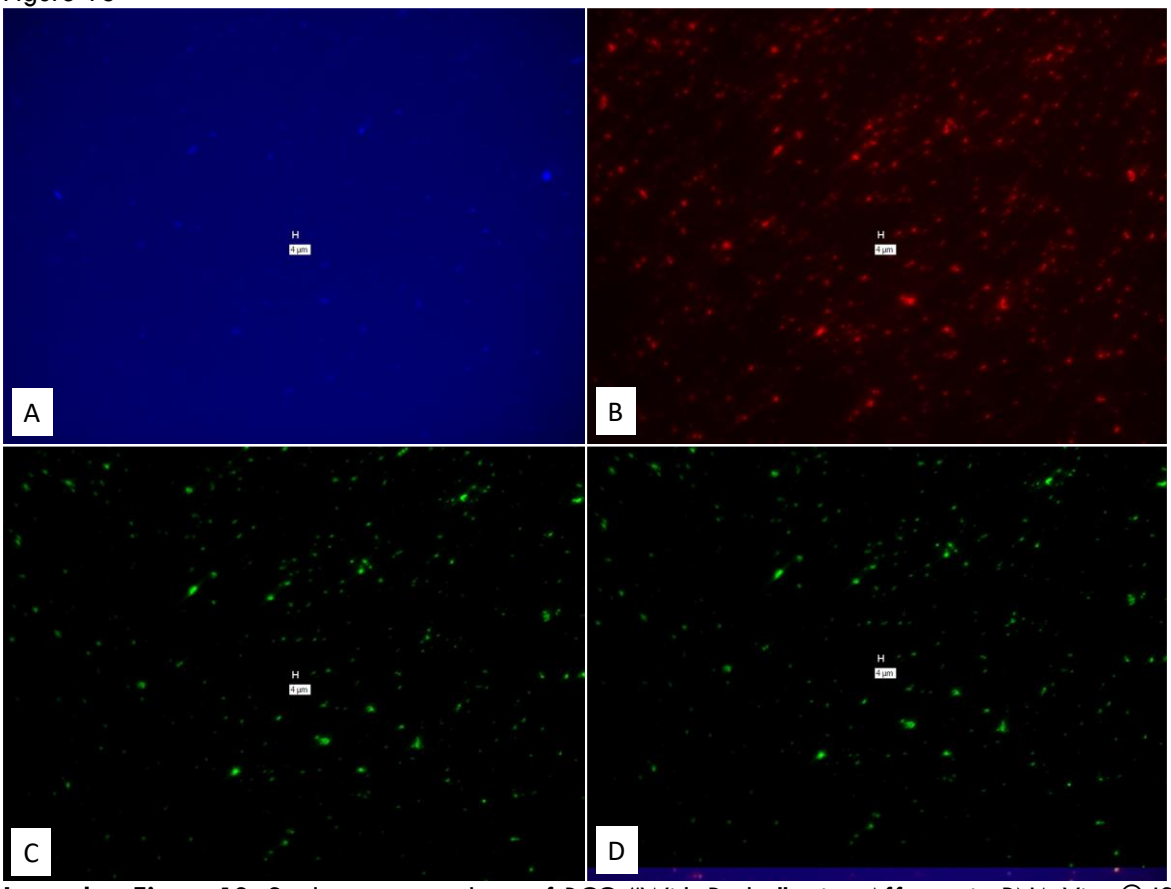


Legend to Figure 12. Experiment to study the efficacy of True Black® to obviate the auto-fluorescence. Specimen is Normal brain. This is a **plus** True-Black® **No-Probe** slide. A= DAPI; B=Texas Red; C=Cy-5, D= composite of A, B & C. In contrast to Figure 10, where the Cy-5 auto-fluorescence was diminished, here it is the Texas Red auto-fluorescence that is diminished, whereas false positive signal is seen in Panel C (Cy-5) and D. Marker bars in µm indicates magnification of x 40.

Initially a kit was used designed specifically for tissue (Affymetrix™ RNA view®. View ISH "TISSUE" Assay Kit 96 2-Plex. Thermo-Fisher Catalog Number: QVT0013.) Because it repetitively gave false positives in the No-Probe control, we subsequently evaluated a product specifically designed for isolated cell (Affymetrix™ ViewRNA ISH "CELL" Assay Kit; Invitrogen by Thermo-Fisher Scientific: Catalog Number: QVC0001.) Both the Tissue and the Cell assays were evaluated on pure culture of different species of bacteria, cultured eukaryotic cells and isolated human WBC's obtained from buffy coat using Ficoll gradient.

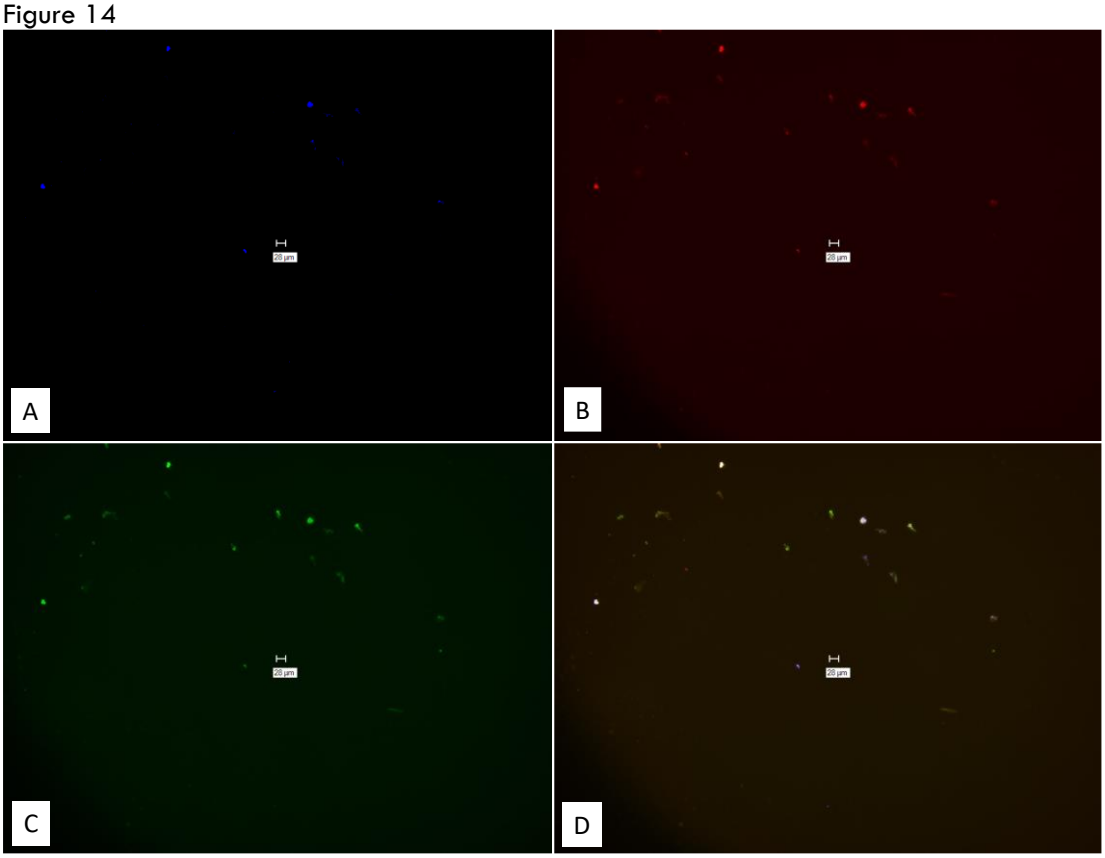
Cultured BCG "With-Probe" gives intense signal (Figure13.) Although less, positive signal is seen with the BCG "No-Probe" Affymetrix™ TISSUE control (Figure 14.) Likewise, false positive signal is seen with the Affymetrix™ CELL assay (Figure 15.)

Figure 13



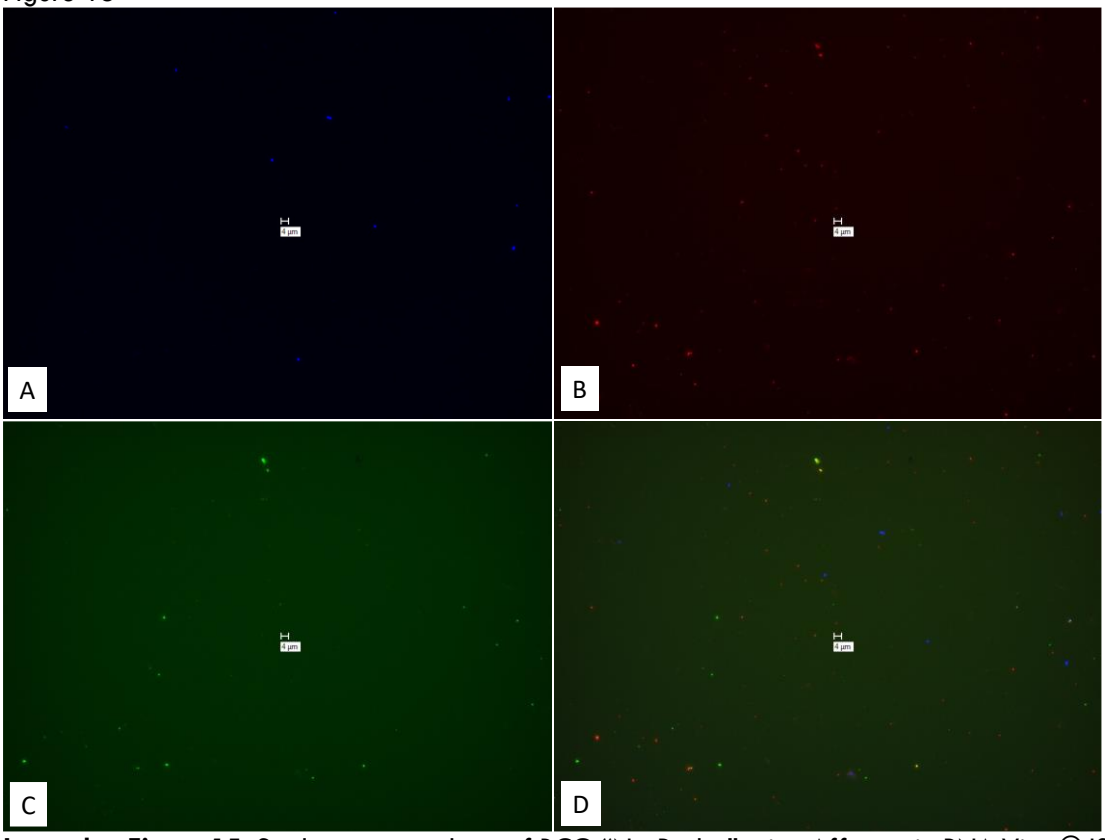
Legend to Figure 13. Study on pure culture of BCG "With-Probe" using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Mycobacteria 16S (Type 1 = Red.) Panel C = All Bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

Figure 14



Legend to Figure 14. Study on pure culture of BCG "No-Probe" using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

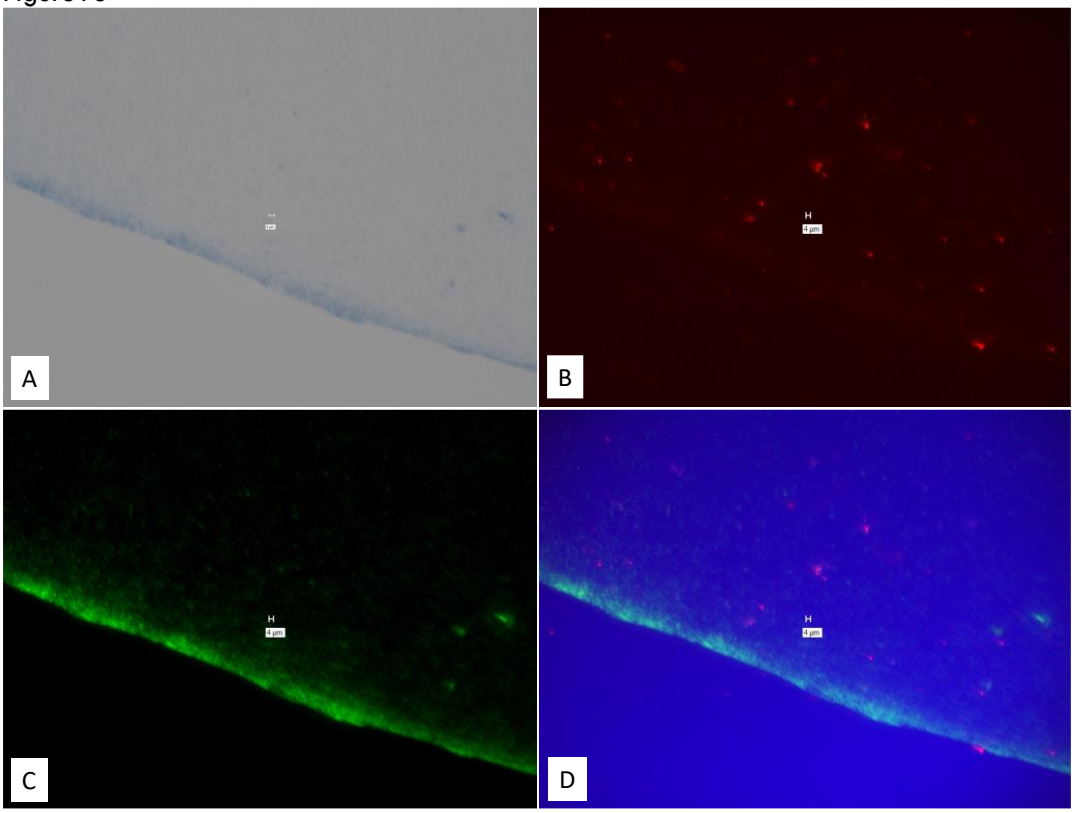
Figure 15



Legend to Figure 15. Study on pure culture of BCG "No-Probe" using Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 60.

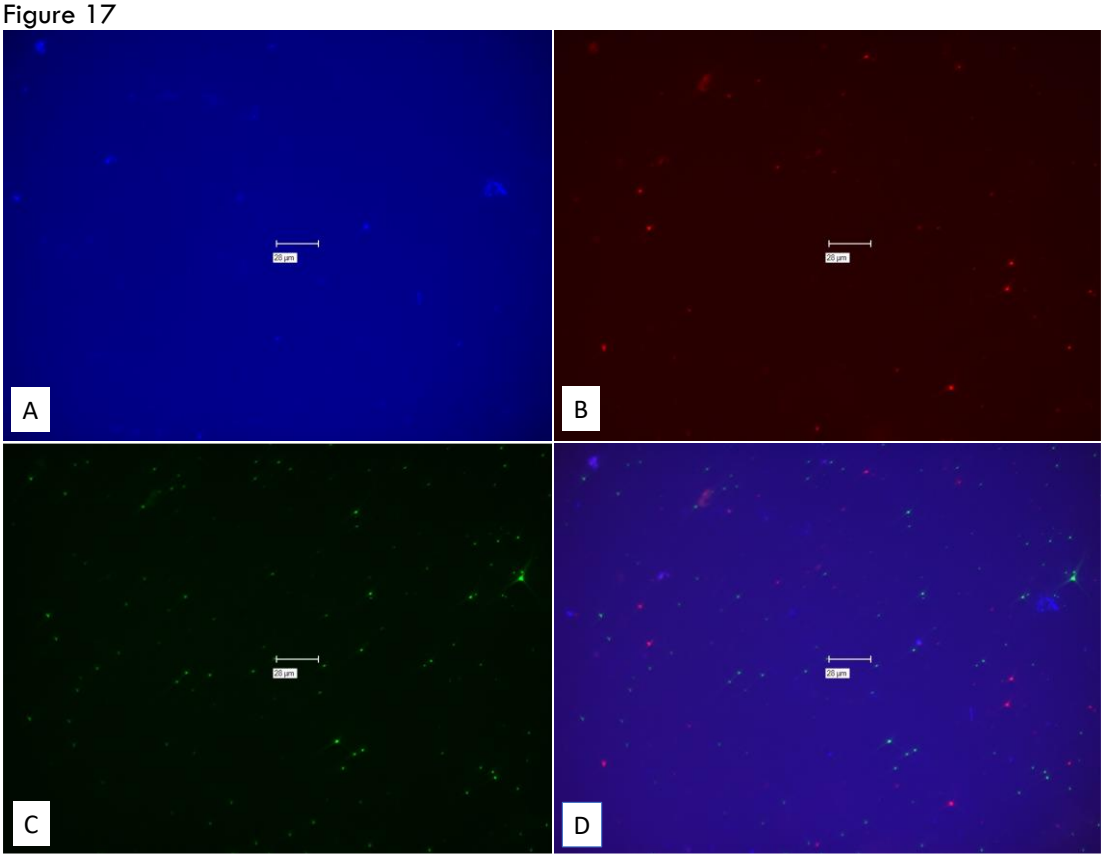
Cultured MAP Dominic has positive signal "With-Probe" (Figure 16), "No-Probe" Affymetrix™ TISSUE (Figure 17 and "No-Probe" TISSUE assay using different filters (Figure 17) False positive signal is seen with MAP Dominic "No-Probe" when using the CELL assay (Figure 18.)

Figure16



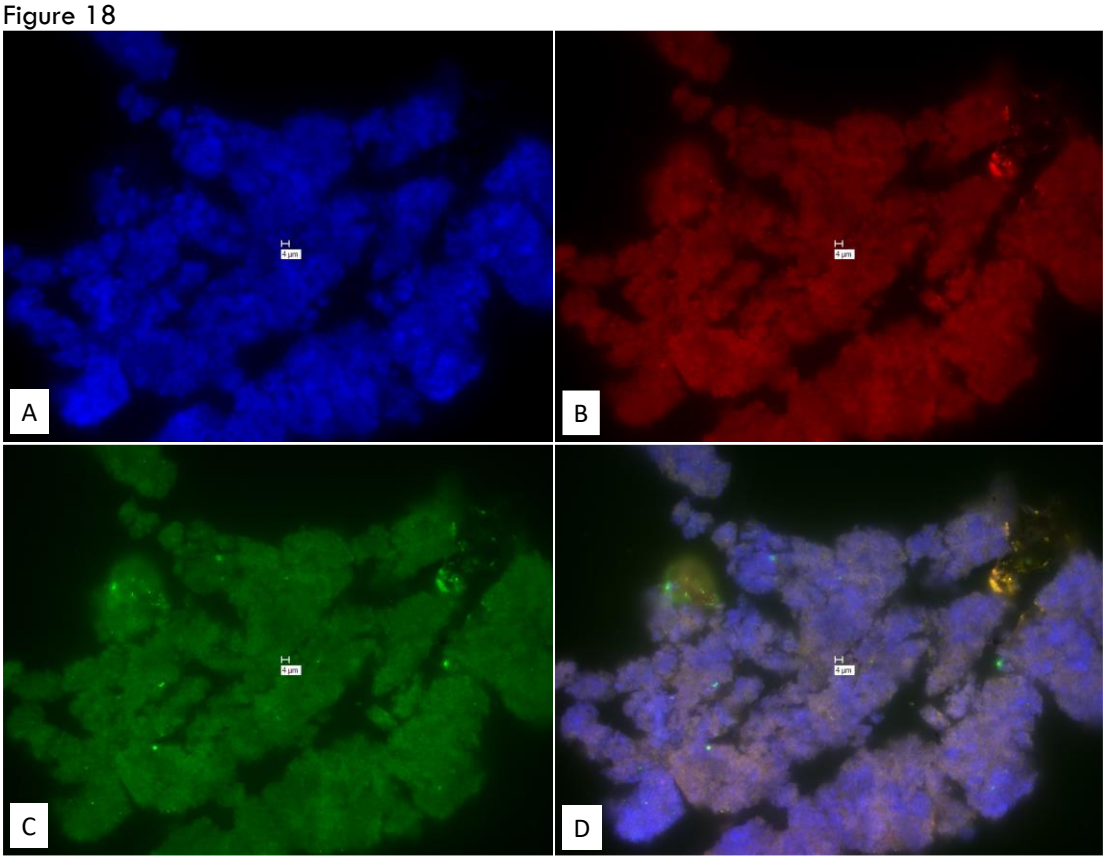
Legend to Figure 16. Study on pure culture of MAP Dominic "With-Probe" using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Different filters were employed at the suggestion of the technical staff at Affymetrix: TritC for Texas Red and a custom recommended "Hope" for Cy-5 (See main text for specifics.) Panel A= DAPI Panel B = Mycobacteria 16S (Type 1 = Red.) Panel C = All Bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

Figure 17



Legend to Figure 17. Study on pure culture of MAP Dominic **"No-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Different filters were employed at the suggestion of the technical staff at Affymetrix: TritC for Texas Red and a custom recommended "Hope" for Cy-5 (See main text for specifics.) Panel A= DAPI Panel B = TritC; Red Panel C = All "Hope" (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

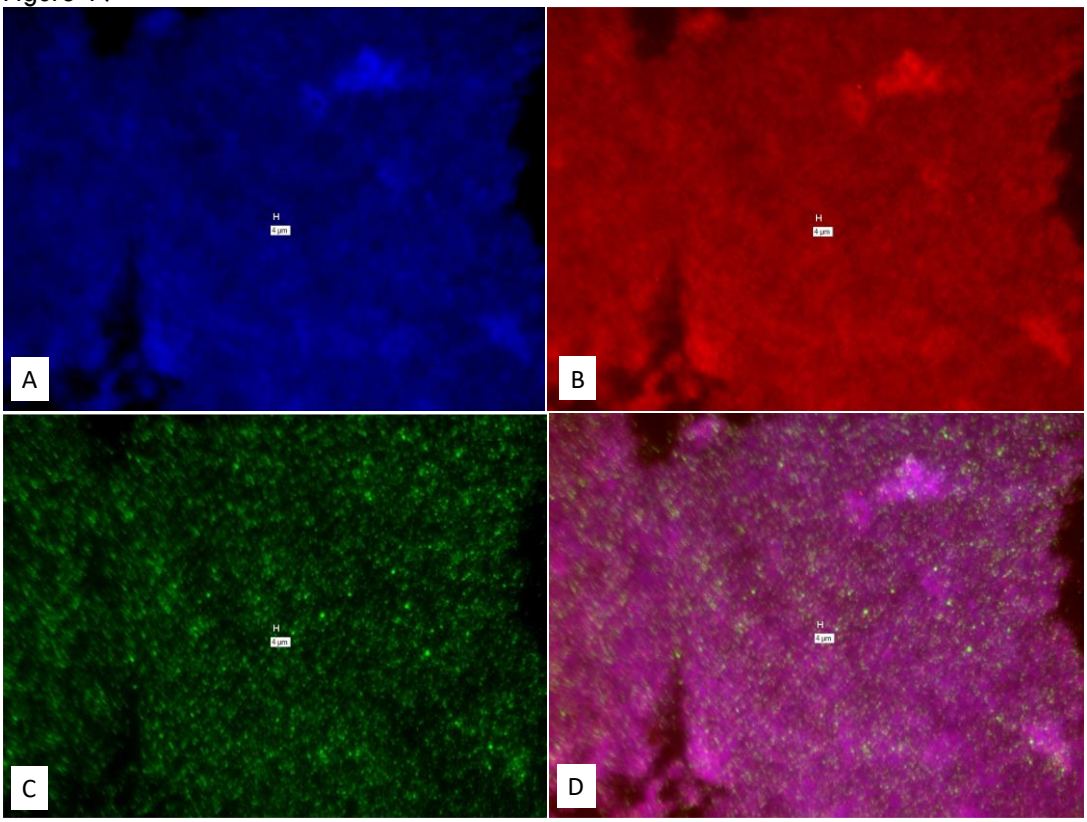
Figure 18



Legend to Figure18. Study on pure culture of MAP Dominic. **"No-Probe"** using Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 60.

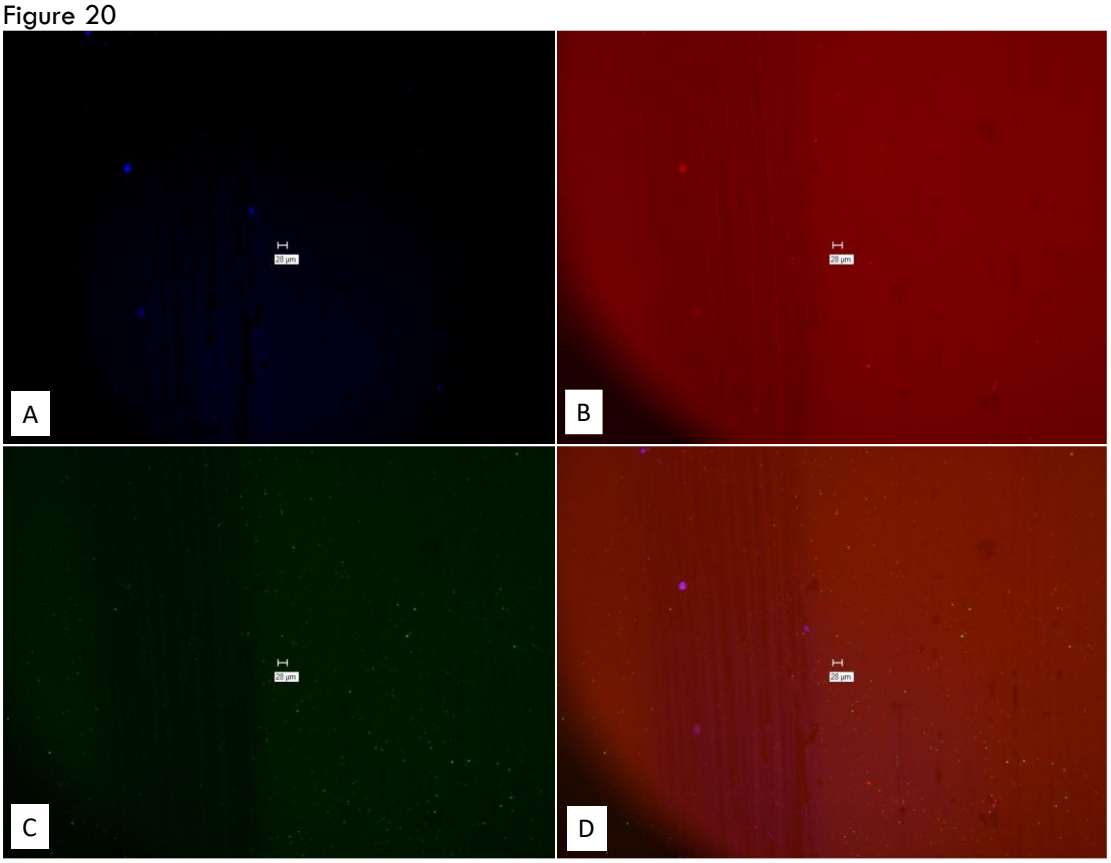
Cultured *E. Coli* "With-Probes" (Figure 19,) the "No-Probe" (Figure 20) and "No-Probe" CELL assay (Figure 21,) all show false positive signal.

Figure 19



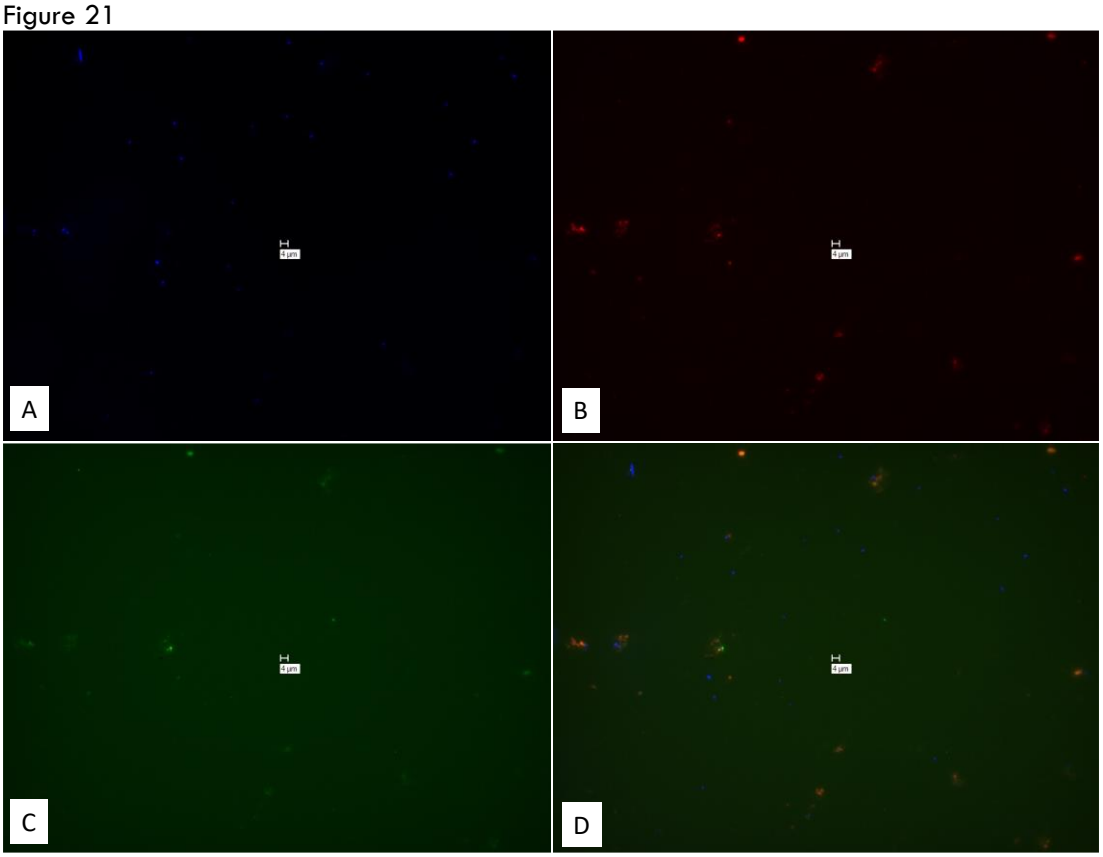
Legend to Figure 19. Study on pure culture of *E. Coli* **"With-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Mycobacteria 16S (Type 1 = Red.) Panel C = All Bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B (should be specific for Mycobacteria 16S), C & D. Marker bars in µm indicates magnification of x 40.

Figure 20



Legend to Figure 20. Study on pure culture of *E. Coli* **"No-Probe"** control for Figure 19. Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 10.

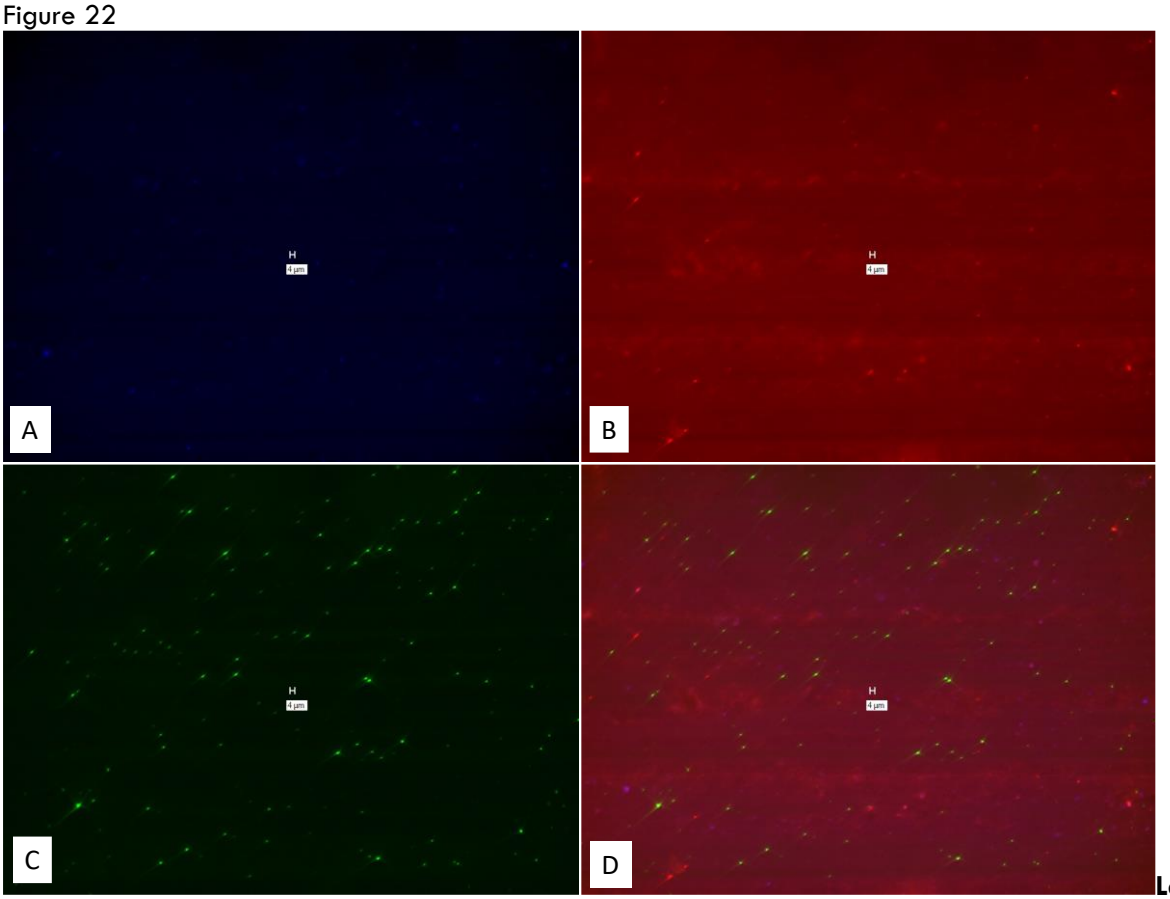
Figure 21



Legend to Figure 21. Study on pure culture of *E. Coli* **"No-Probe"** control using Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 60.

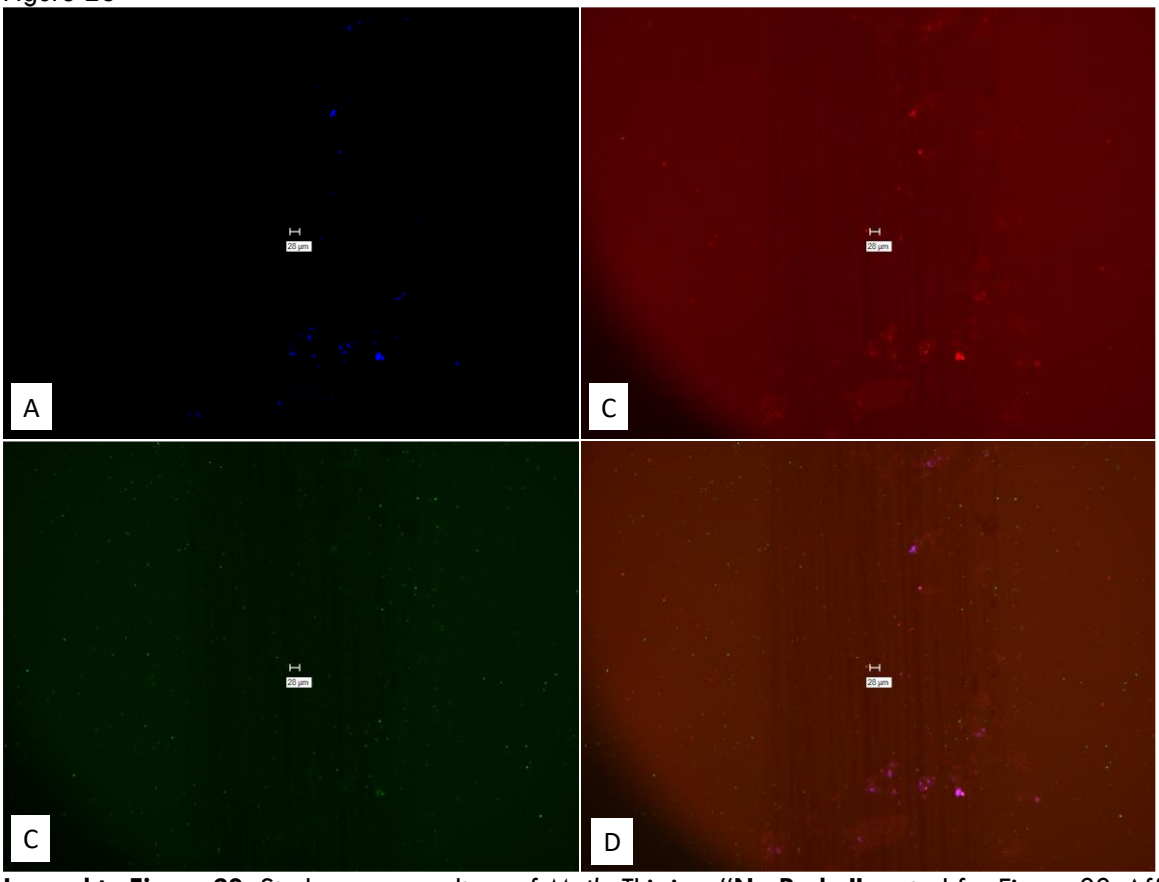
Cultured *M. tb.* Gives positive signal "With-Probe" (Figure 22.) and "No-Probe" (Figure 23) with the TISSUE assay as well as the "No-Probe" CELL assay (Figure 24.)

Figure 22



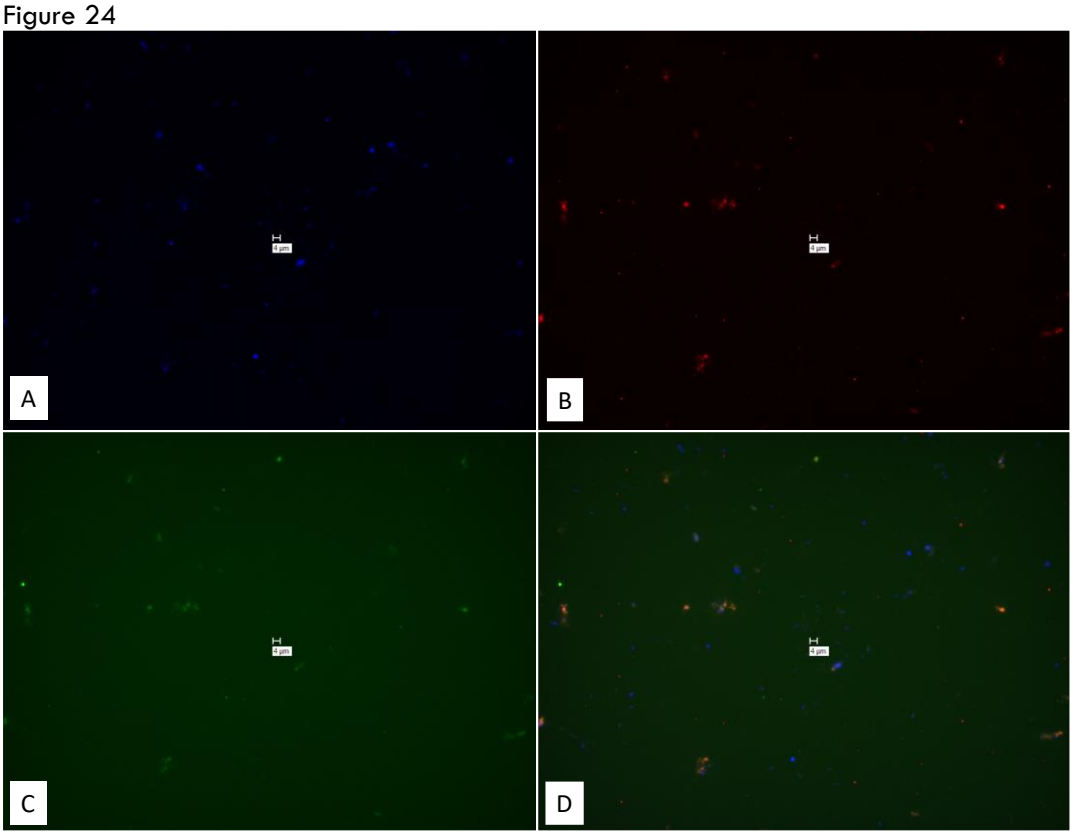
Legend to Figure 22. Study on pure culture of *M. tb.* **"With-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = IS6110 (Tb specific: Type 1 = Red.) Panel C = MAP IS900 (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Specifically the Panel C signal is spurious as IS900 should only be positive for MAP, not M. Tb. (see Main Text.) Marker bars in µm indicates magnification of x 40.

Figure 23



Legend to Figure 23. Study on pure culture of *M. tb.* This is a **"No-Probe"** control for Figure 22. Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 10.

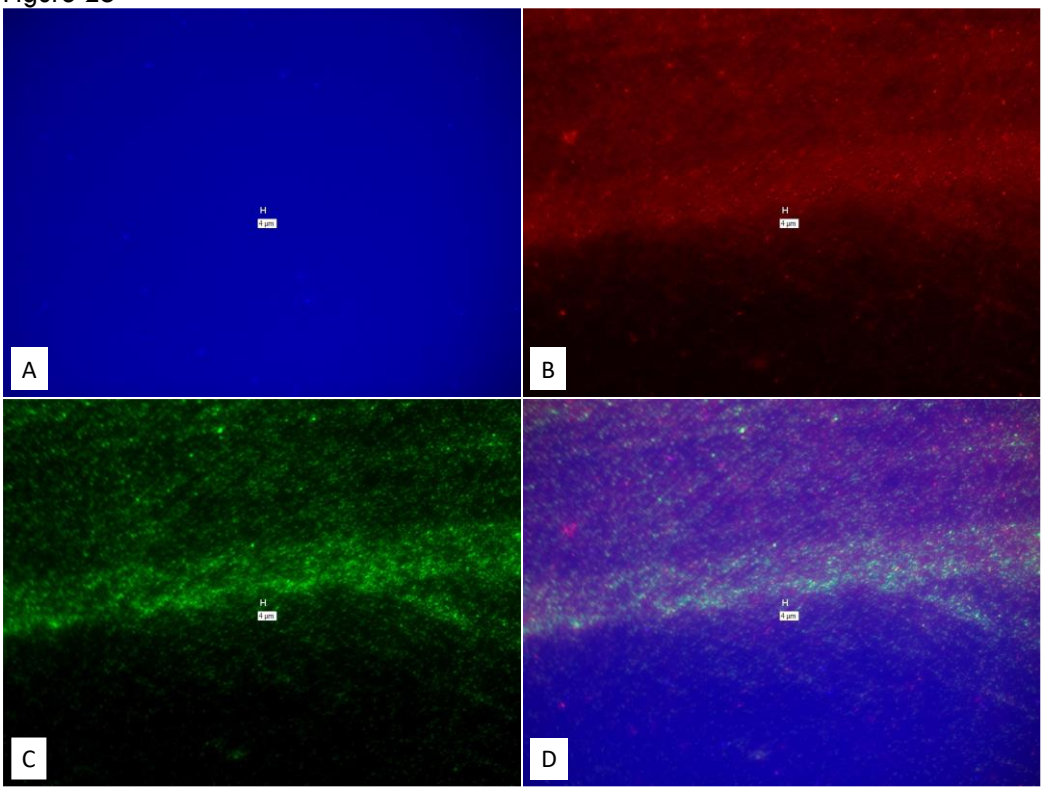
Figure 24



Legend to Figure 24. Study on pure culture of *M.tb*. This is the **"No-Probe"** control for Figures 22 & 23, using Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 60.

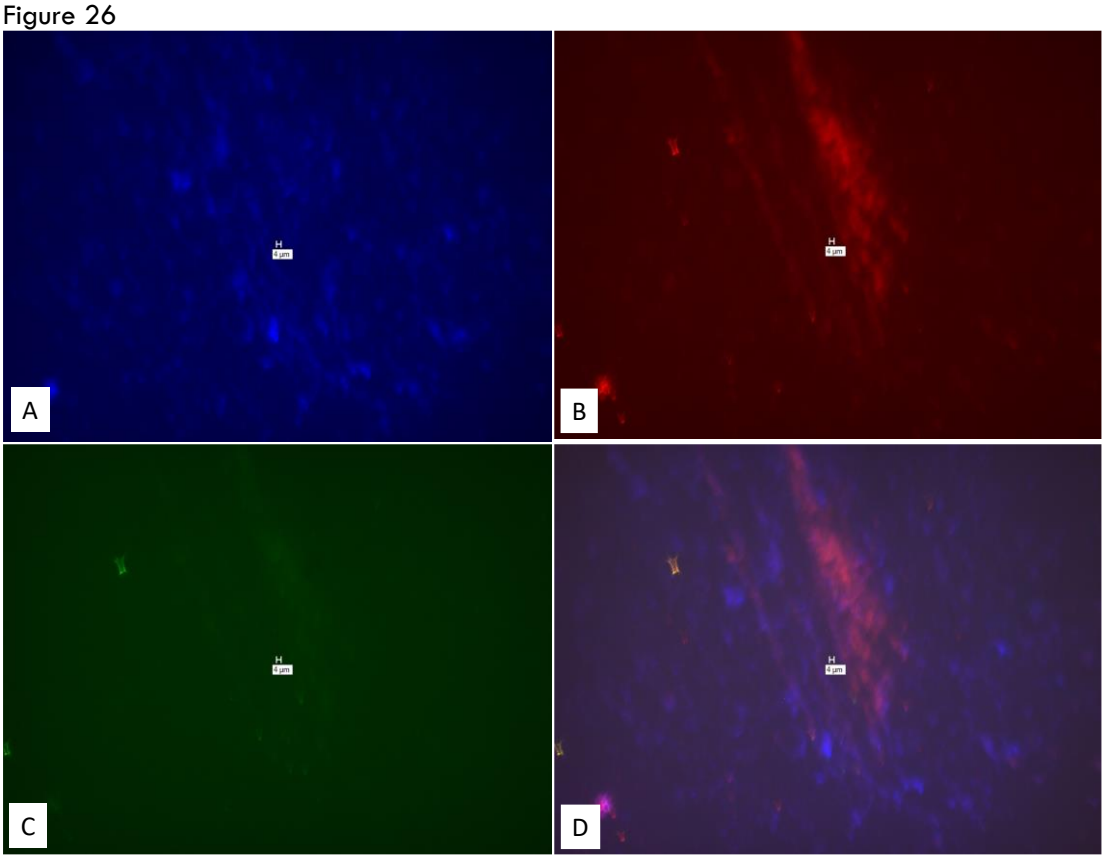
Cultured *M. avium* subspecies *avium* gives positive signal (Figures 25 & 26) with different "With -Probes." Note that spurious positivity in Figure 26 Panel B which should only be positive for MAP is here "positive" for *M. avium* subspecies *avium*. The "No-Probe" control for both the TISSUE (Figure 27) and CELL assay (Figure 28) both show false positive signal.

Figure 25



Legend to Figure 25. Study on pure culture of *M. avium* subspecies *avium*. **"With-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = (Mycobacterium 16S: Type 1 = Red.) Panel C = All bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

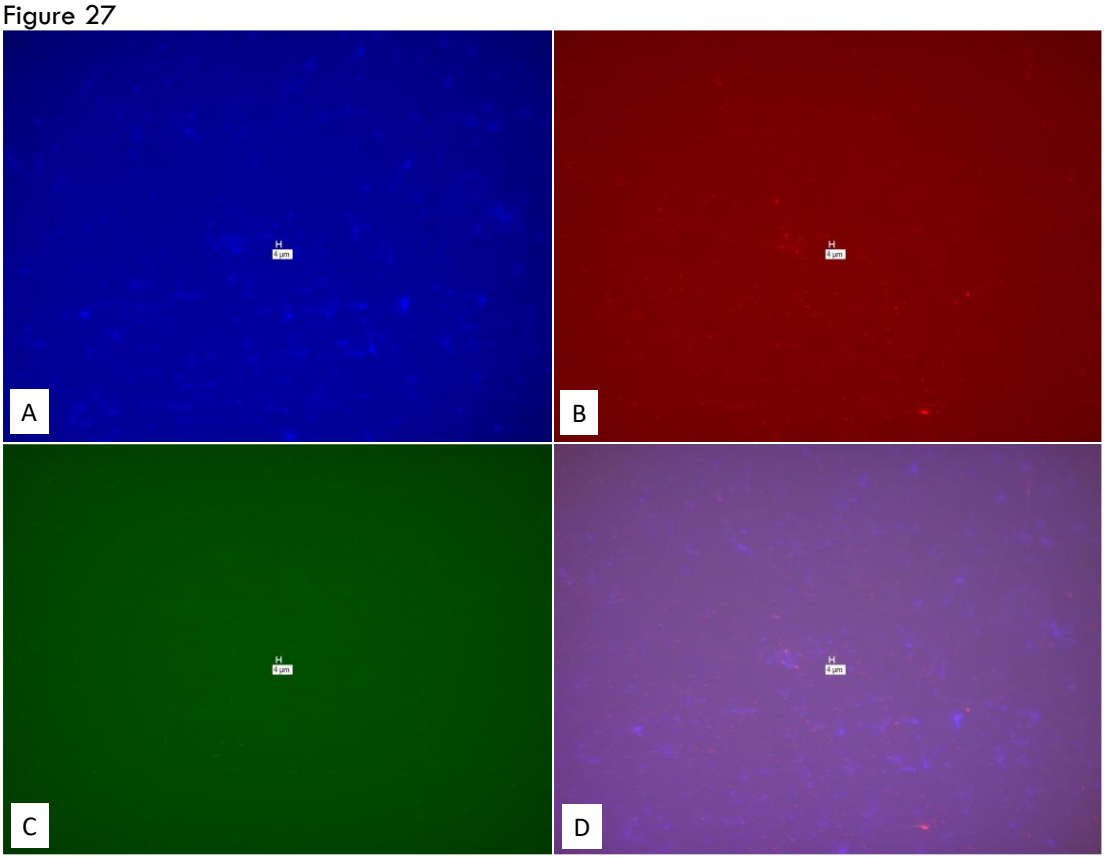
Figure 26



Legend to Figure 26. Study on pure culture of *M. avium* subspecies *avium*. Different probes than those used in Figure 25. **"With-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = (MAP IS900: Type 1 = Red.) Panel C = All-bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

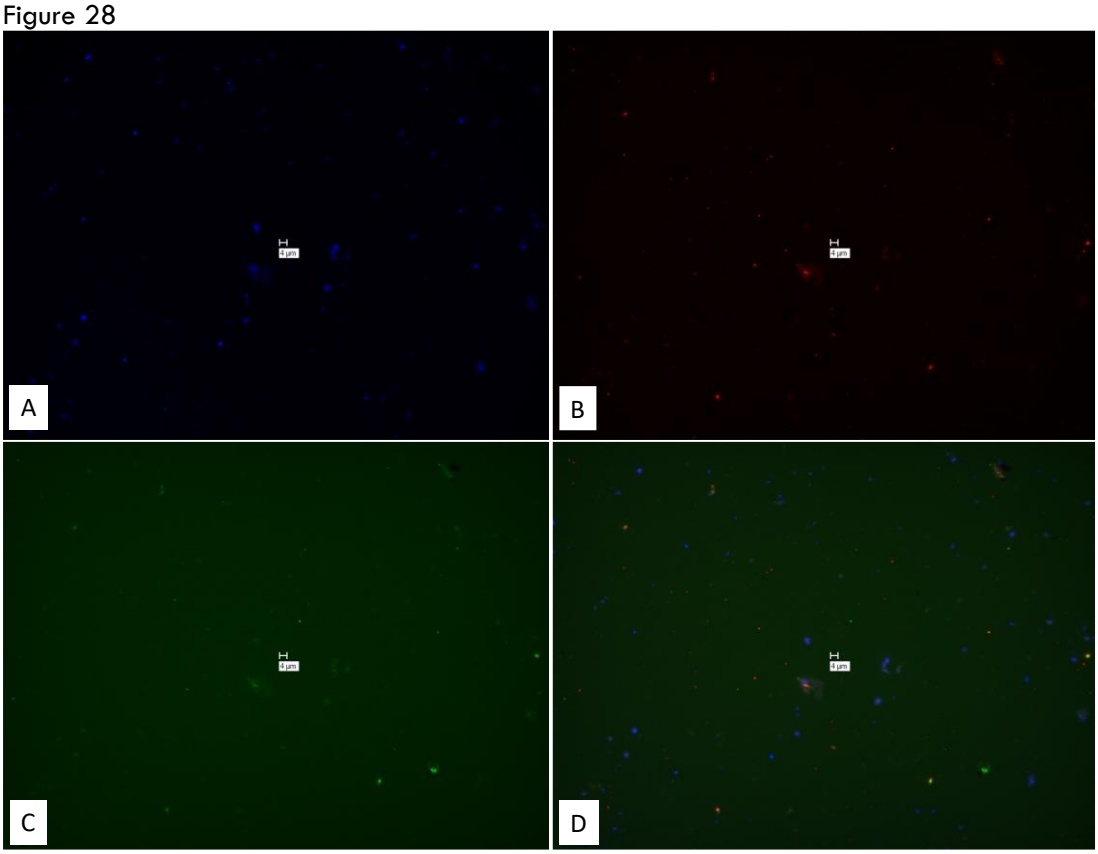
Note the "positive" signal in Panel B, which should be specific to MAP, is here positive for *M. avium* subspecies *avium*.

Figure 27



Legend to Figure 27. Study on pure culture of *M. avium* subspecies *avium*. **"No-Probe"** control for Figures 25 & 26 using Affymetrix RNA View® TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

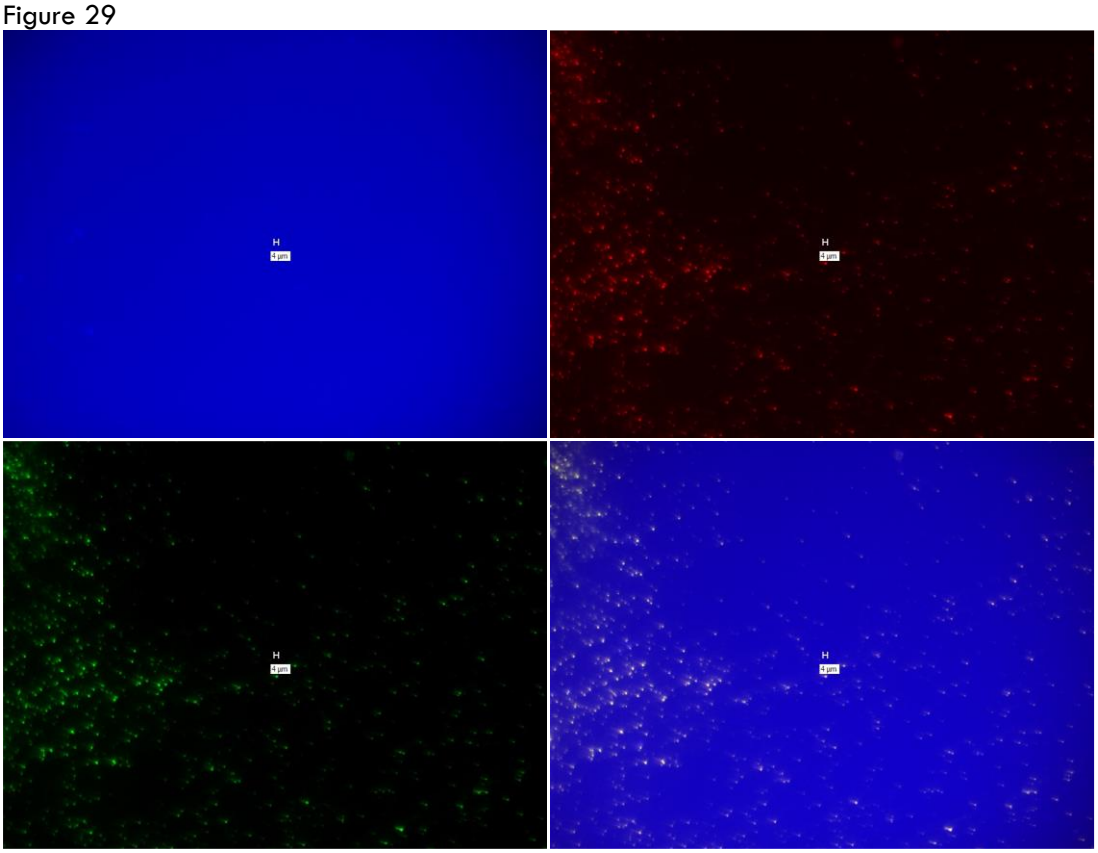
Figure 28



Legend to Figure 28. Study on pure culture of *M. avium* subspecies *avium*. **"No-Probe"** control for Figures 25 & 26, using Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 60.

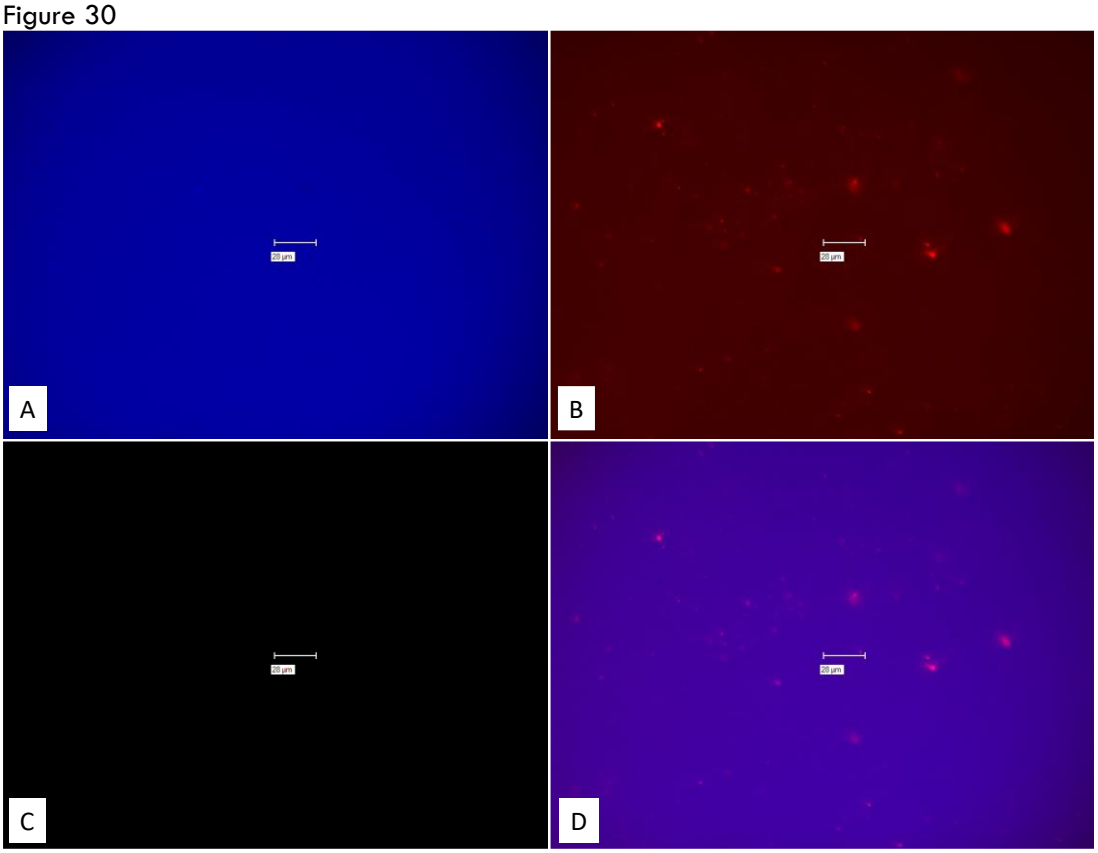
We next studied a pure culture of MAP Dominic with probes that should only have identified *M. tb* specific IS 6110.

Figure 29



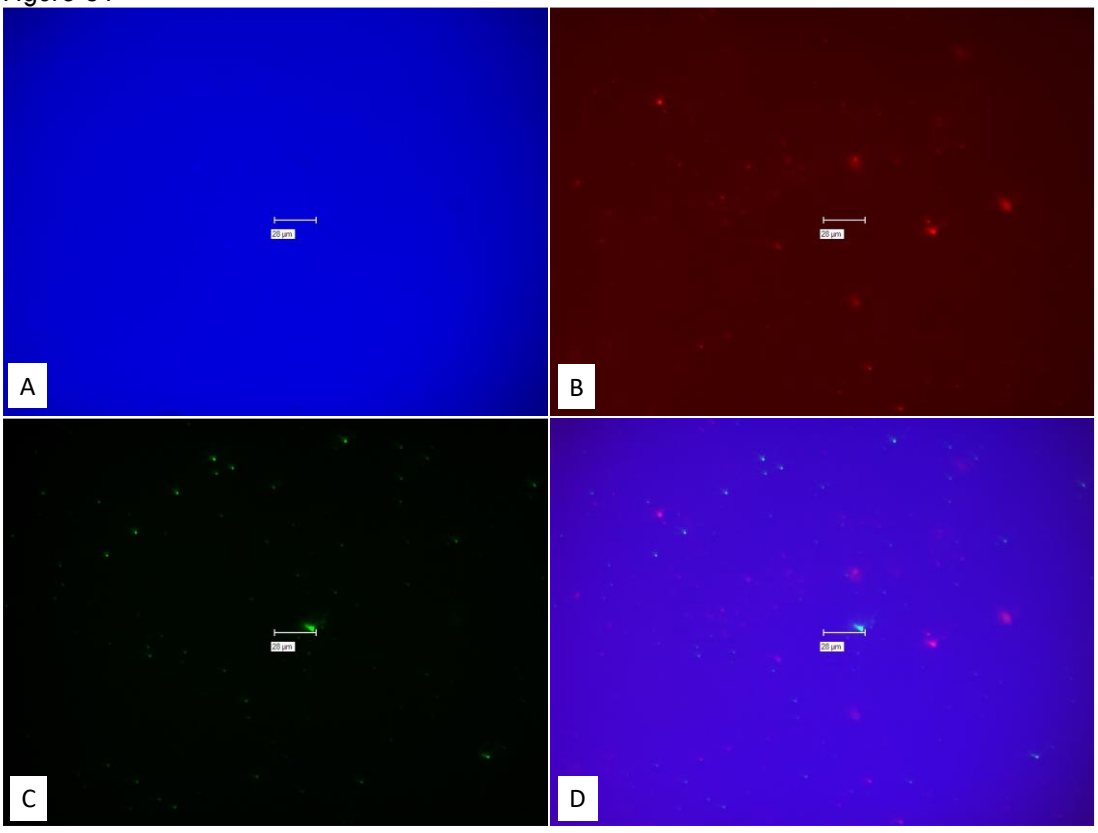
Legend to Figure 29. Study on pure culture MAP Dominic. **"With-Probe"** using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = IS6110 (Tb specific: Type 1 = Red.) Panel C = IS900 (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. In particular, Panel B should be negative as IS6110 is *M. tb* specific. Marker bars in µm indicates magnification of x 40.

Figure 30



Legend to Figure 30. Study on pure culture of MAP Dominic. **"No-Probe"** control for Figure 29. Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

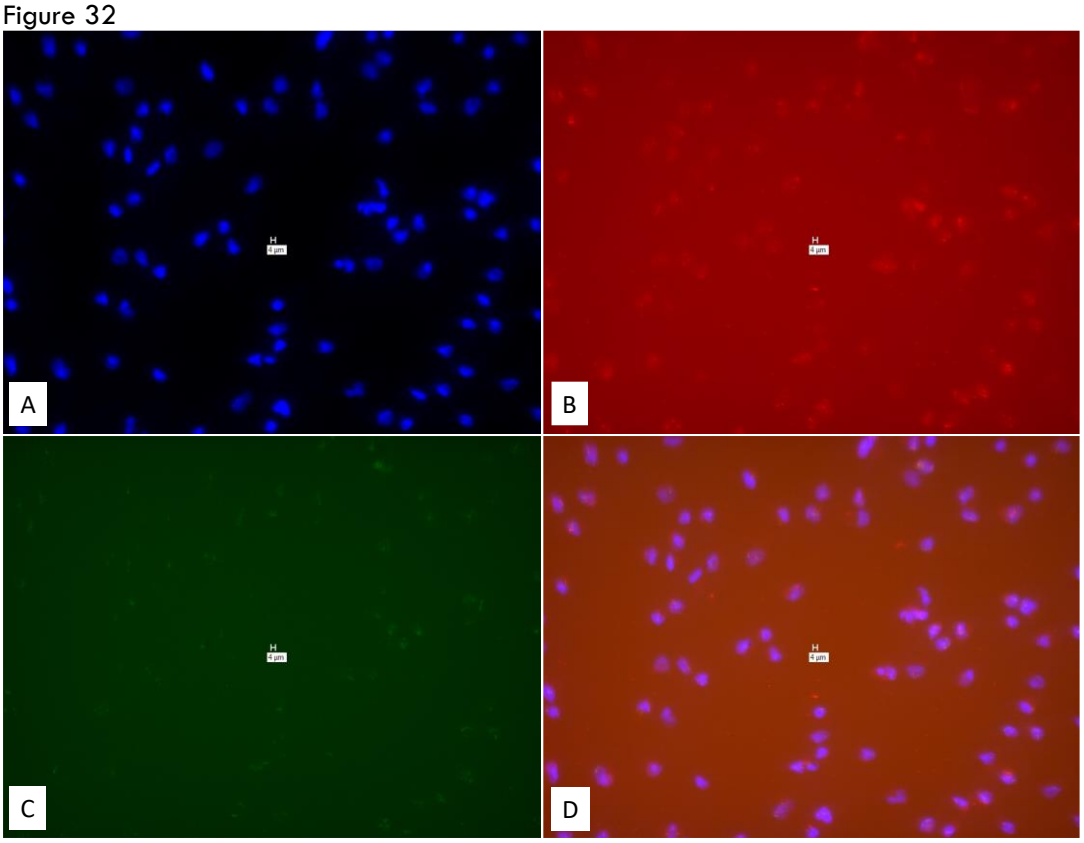
Figure 31



Legend to Figure 31. Study on pure culture of MAP Dominic. **"No-Probe"** comparison of different filters from Figure 26. Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = TritC. Panel C = "Hope" (see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40.

In contrast to the prediction of the Affymetrix Technical staff (see Main Text), Cy-5 gives less spurious signal than "Hope" in the identical view. Compare Panel C from Figure 30 with Panel C in Figure 31.

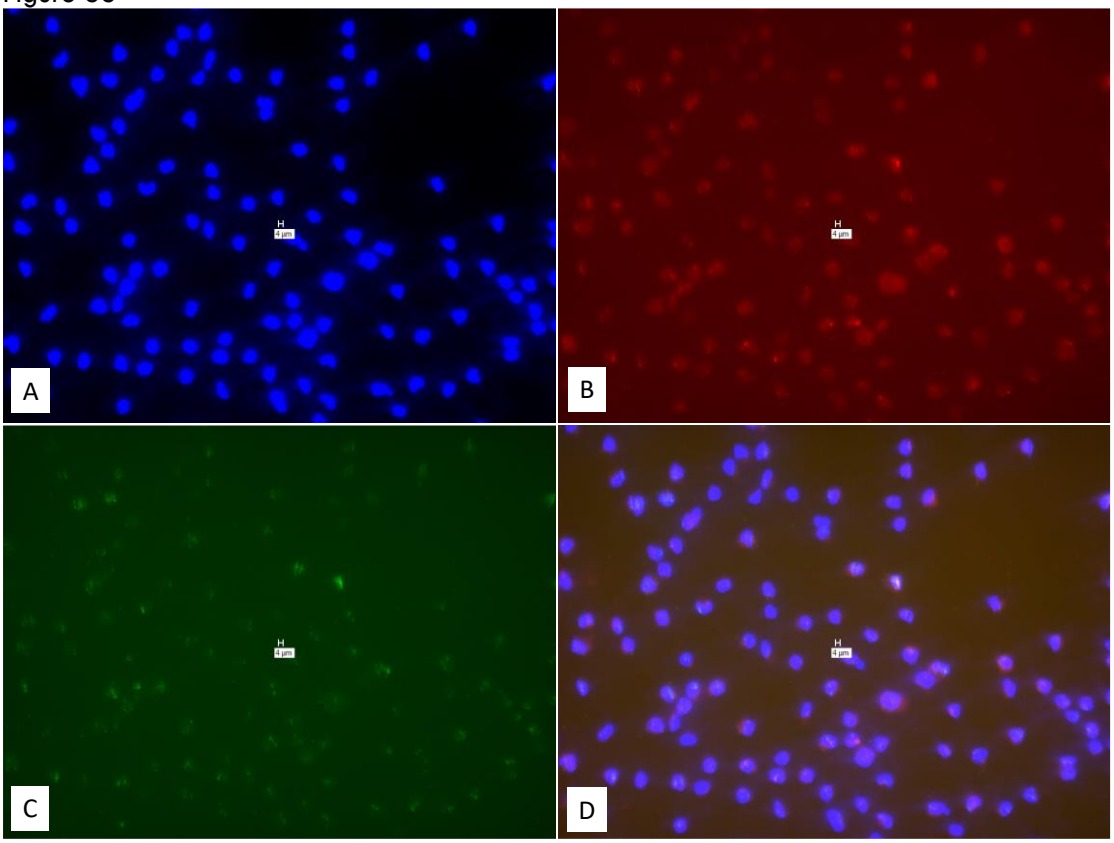
Figure 32



Legend to Figure 32. Pure culture of eukaryotic cells (Mouse RAW264 NIH 93 cells.) "With-Probe" using Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = (MAP IS900: Type 1 = Red.) Panel C = All-bacteria 16S (Type 6 = Green, see Main Text.) Panel D = composite of A, B & C. Note "positive" signal in Panels B & D. Marker bars in µm indicates magnification of x 40

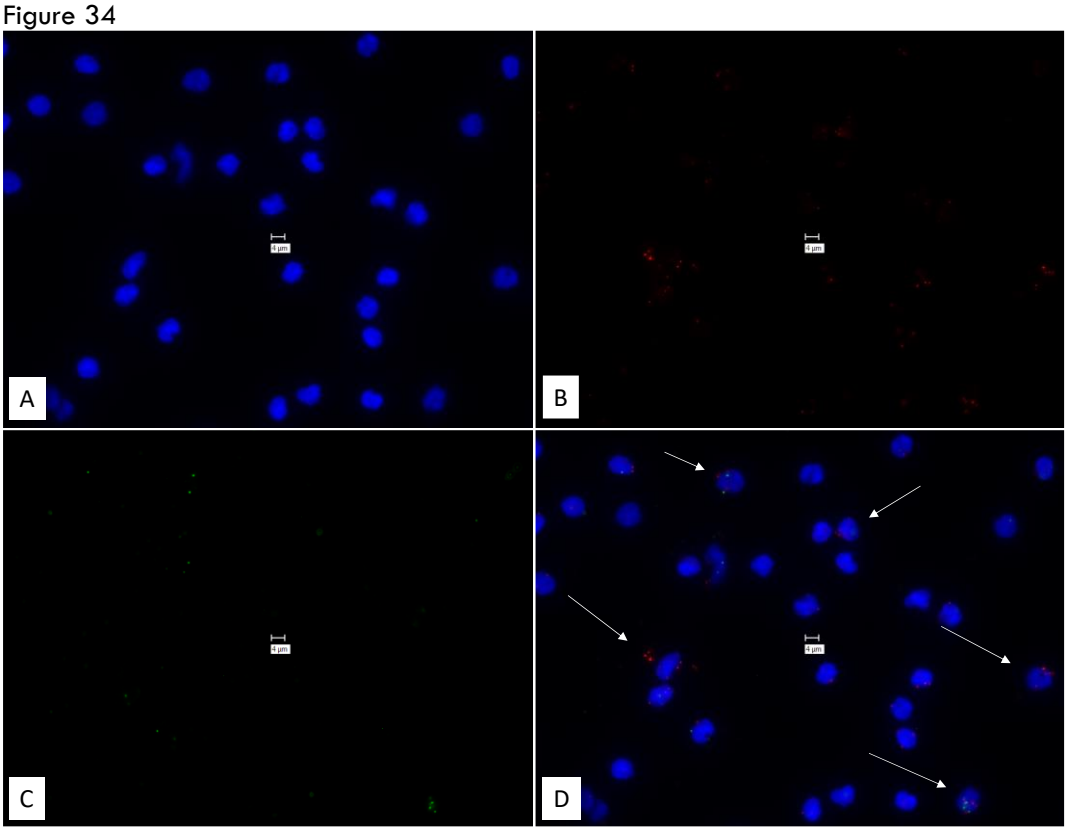
In this pure culture of an uncontaminated mouse cell line, panels B (MAP IS900) & D should be negative.

Figure 33



Legend to Figure 33. "No-Probe" control for Figure 32. Pure culture of eukaryotic cells (Mouse RAW264 NIH 93 cells.) Affymetrix RNA View® ISH TISSUE Assay Kit Catalog # QVT 0013. Panel A= DAPI Panel B = Texas Red. Panel C = Cy-5. Panel D = composite of A, B & C. Note "positive" signal in Panels B, C & D. Marker bars in µm indicates magnification of x 40

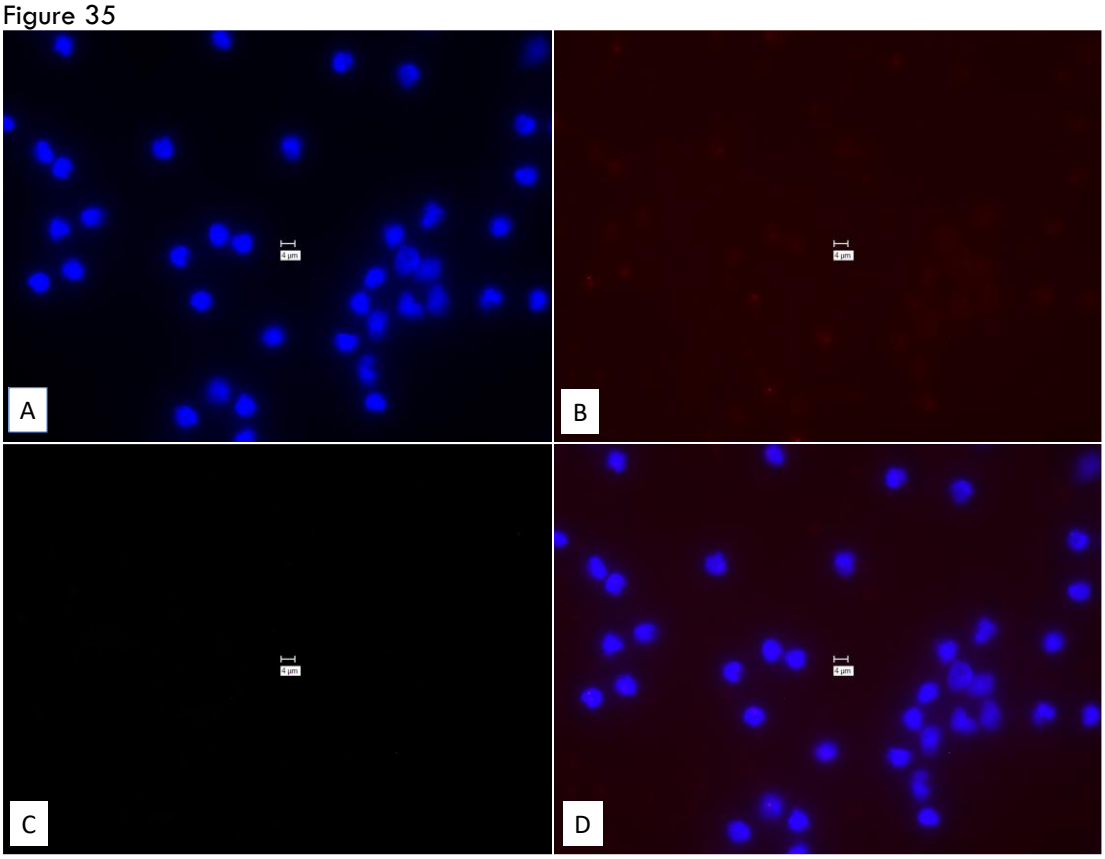
Figure 34



Legend to Figure 34. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood left at room temperature for one hour. Probes were two human house-keeping genes: Human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (Type 6 = Green.) Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note positive signal, samples indicated with white arrows, always associated with DAPI positive signal, in Panel D.

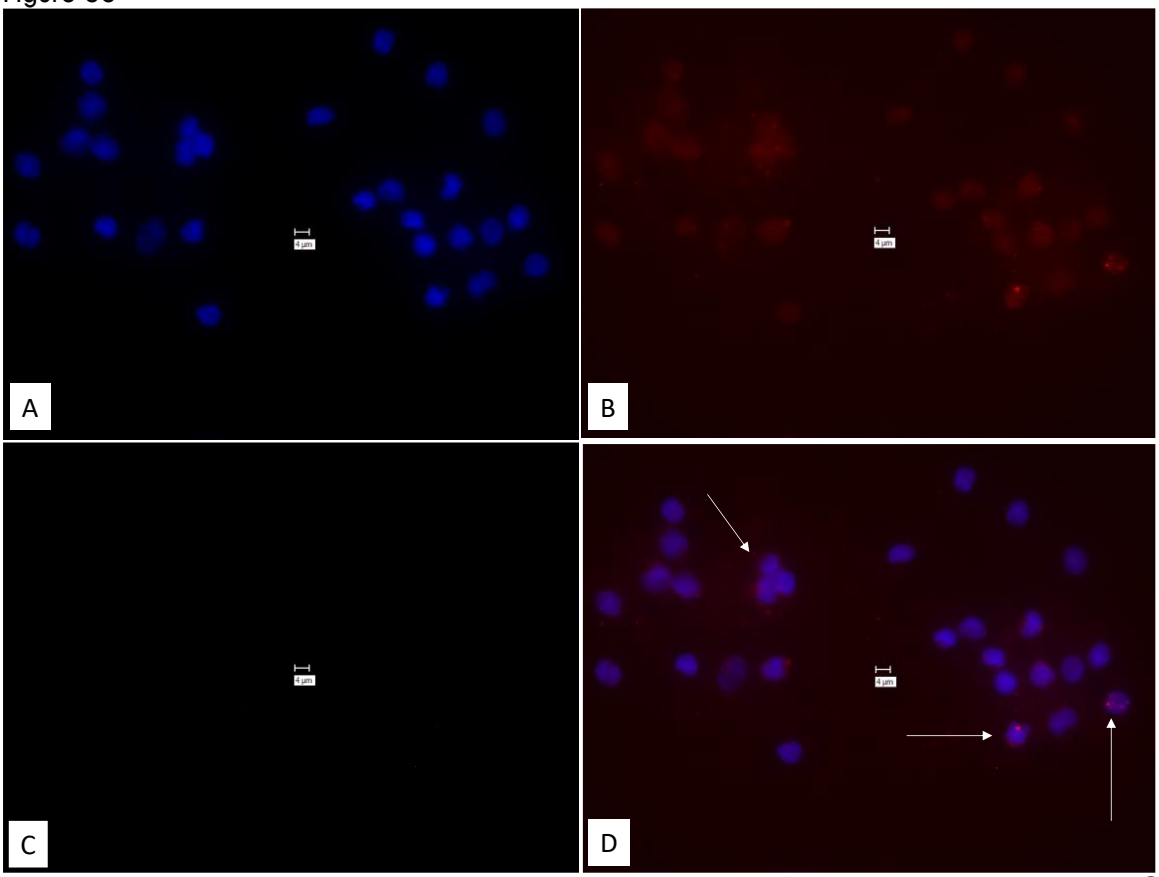
Figure 35



Legend to Figure 35. No-Probe control for Figure 34. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood left at room temperature for one hour. Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note, in this Cell assay Thermo Fisher Catalog #: QVT 000, there is virtually no spurious signal in Panel B & D and zero spurious signal in Panel B. Indicating that the signal in Figure 34 may be reliable.

Figure 36



Legend to Figure 36. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at 4°C for 24 hours prior to centrifuging for Ficoll gradient. Probes were two human house-keeping genes: Human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (Type 6 = Green.) Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

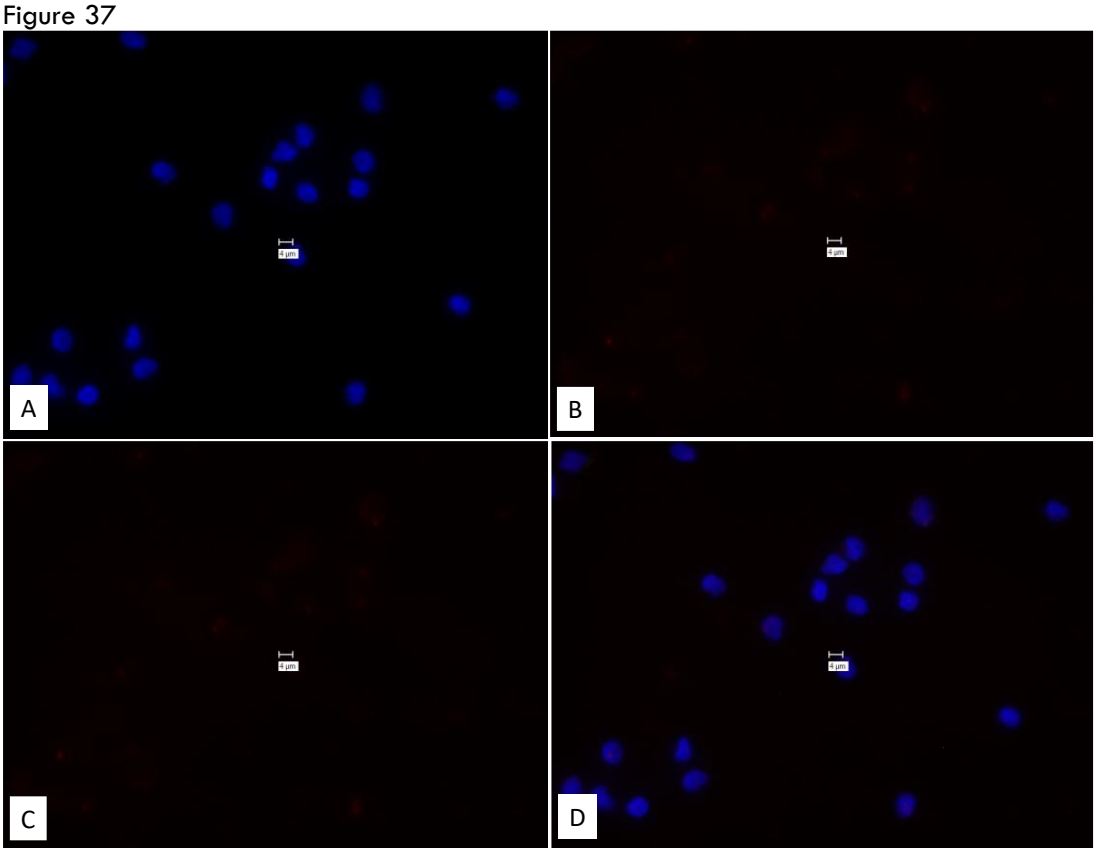
Note positive signal, samples indicated with white arrows, always associated with DAPI positive signal, in Panel D. In contrast to the one-hour period of RT storage, this signal is predominantly Human β-actin (Type 1 =Red) and should show no signal. **"No-Probe"** control for Figure 27 TISSUE assay, Filters are Texas Red (Panel B) and Cy-5 (Panel D). The false positive signal is additionally seen when TritC and "Hope" are the filters used in the identical image (Figure 31.) We conclude that using different filters, as recommended by the Affymetrix technical staff, does not eliminate false positivity. And that Cy5 (Figure 30; Panel C) gives clearer background that "Hope", that was predicted by Affymetrix technical staff

Next the TISSUE assay was evaluated with cultured eukaryotic cells (Mouse RAW264 NIH 93 cells.) "With-Probe" TISSUE Assay; both MAP and bacteria 16S are positive. Similarly, the "No-Probe" control is positive (Figure 33), indicating that the TISSUE assay cannot be used to study cultured eukaryotic cells.

A series of studies were then performed on buffy coat human WBC's using the CELL (Catalog #: QVT 0001) assay. The initial study evaluated two, human specific, house-keeping genes, (human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase; Type 6; Green). Positive signal was seen, always associate with DAPI positive regions: indicating association with cells (Figure 34.) In marked contrast, in the "No-Probe" control, limited signal is seen in panel B and zero spurious signal in panel C. (Figure 35.) We consider that this may indicate that on circulating WBC's the CELL assay may give reliable signal. Accordingly, the conditions under which this signal could be obtained was studied.

Blood was stored at 4°C for 24 hours prior to being processed **With-Probe** (Figure 36) and "No-Probe" (Figure 36.) There is zero spurious signal in the "No-Probe" control. Human β-actin may be more stable and an appropriate house-keeping gene than GAPD. Blood was then stored at room temperature (RT) for 24 hours prior to processing.

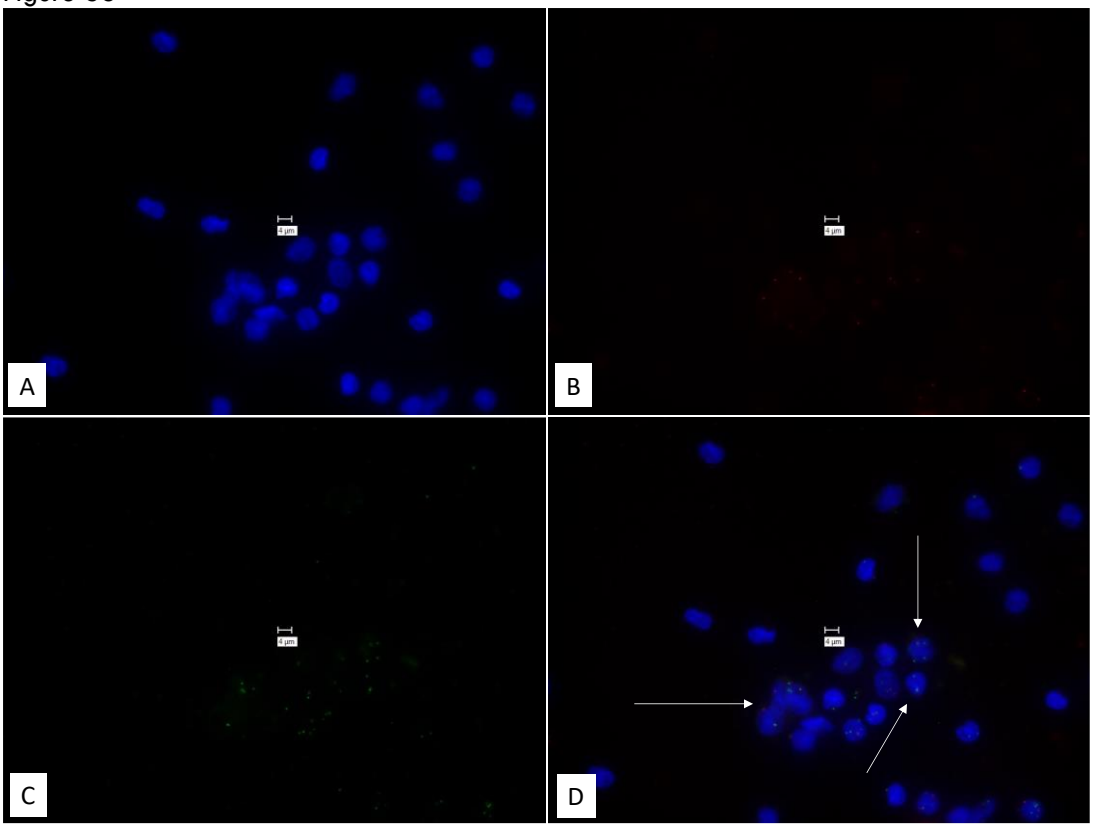
Figure 37



Legend to Figure 37. No-Probe control for Figure 36. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at 4◦C for 24 hours prior to centrifuging for Ficoll gradient. Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note, in this Cell assay Thermo Fisher Catalog #: QVT 000, there is zero spurious signal in Panels B, C & D. Possibly indicating that the signal in Figure 36 may be reliable. It may also indicate that Human β-actin may be more stable and an appropriate house-keeping gene than Human GAPD.

Figure 38

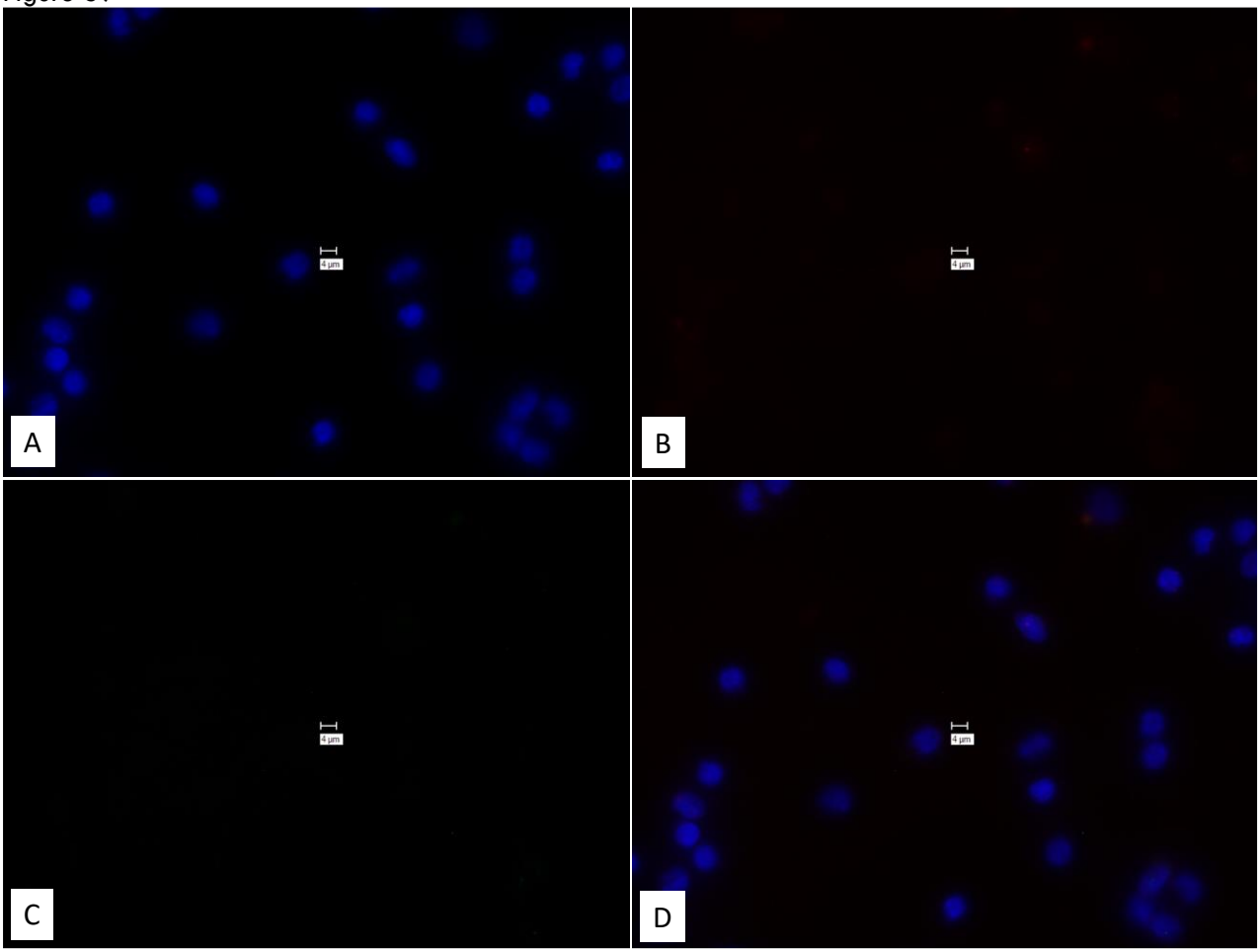


Legend to Figure 38. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at Room Temperature for 24 hours prior to centrifuging for Ficoll

gradient. Probes were two human house-keeping genes: Human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (Type 6 = Green.) Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note positive signal, samples indicated with white arrows, always associated with DAPI positive signal, in Panel D

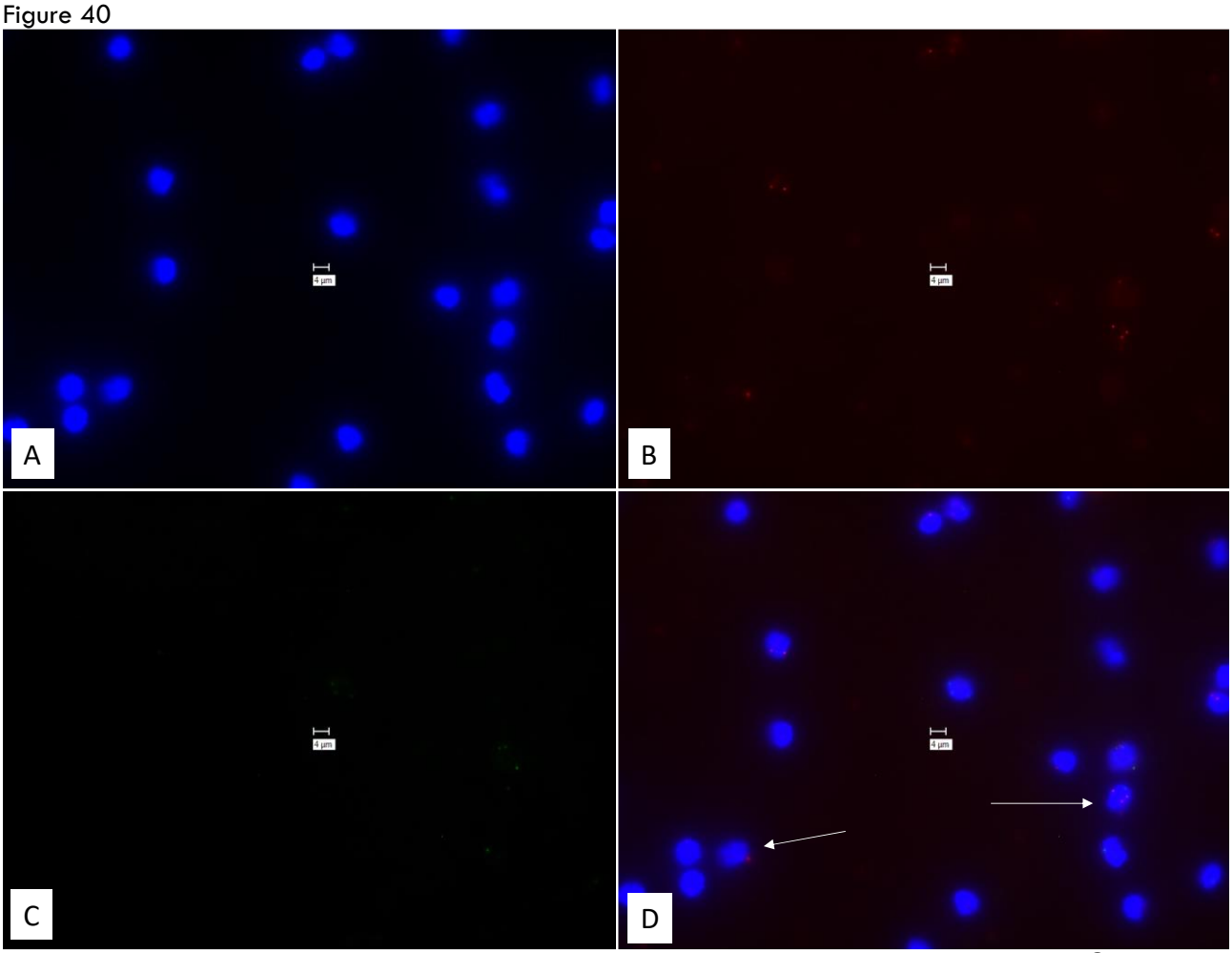
Figure 39



Legend to Figure 39. "No-Probe" control for Figure 38. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at Room Temperature for 24 hours prior to centrifuging for Ficoll gradient. Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note, in this Cell assay Thermo Fisher Catalog #: QVT 000, there is zero spurious signal in Panels B, C & D. Possibly indicating that the signal in Figure 38 may be reliable. Intriguingly, human GAPD is also present (see Figure 38, Panel D, white arrows, green signal.) It may be possible that transportation of blood samples may be possible at ambient temperature for 24 hours.

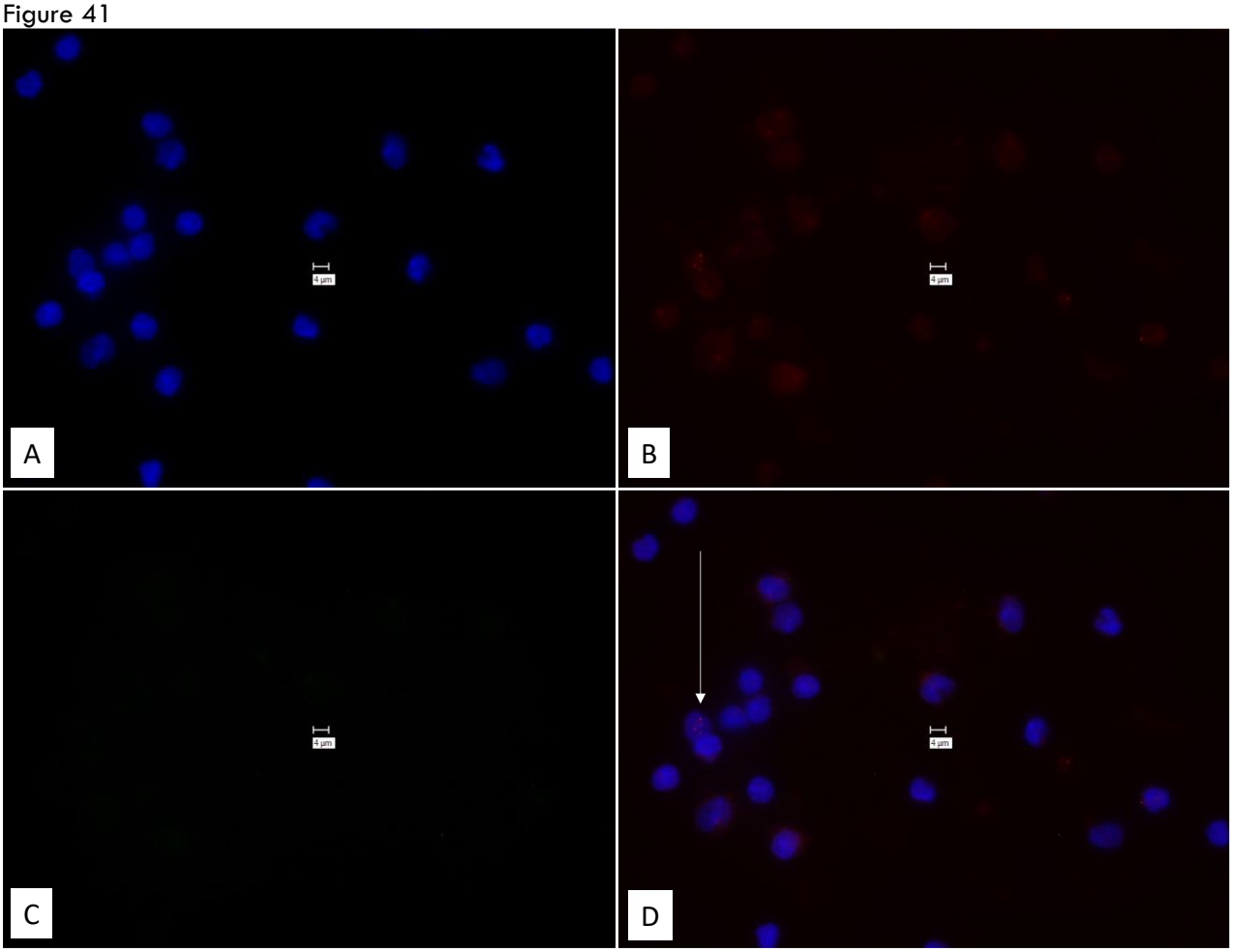
Figure 40



Legend to Figure 40. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at 4◦C for 74 hours prior to centrifuging for Ficoll gradient. Probes were two human house-keeping genes: Human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (Type 6 = Green.) Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note the limited positive signal, (Human β-actin (Type 1 =Red) samples indicated with white arrows, always associated with DAPI positive signal, in Panel D.

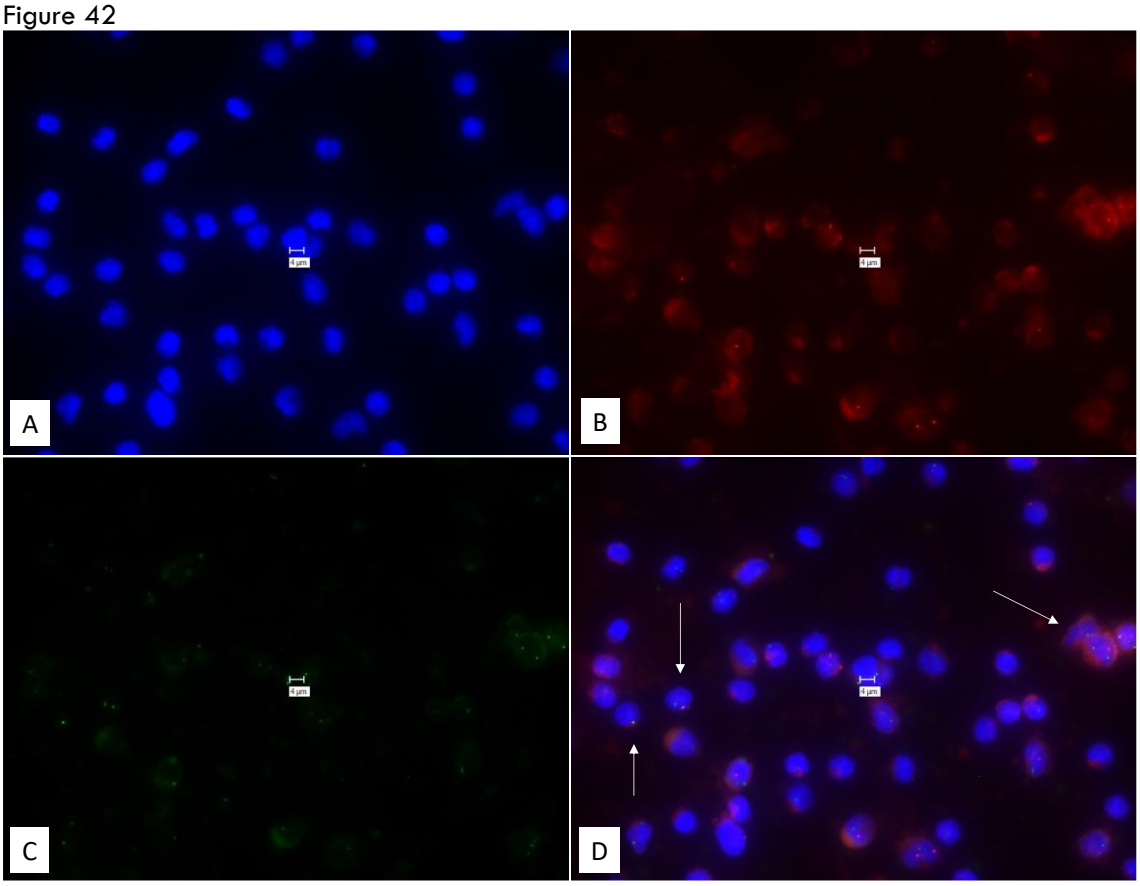
Figure 41



Legend to Figure 41. "No-Probe" control for Figure 40. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at 4°C for 74 hours prior to centrifuging for Ficoll gradient. Temperature for 74 hours prior to centrifuging for Ficoll gradient. Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100. Note, in this Cell assay Thermo Fisher Catalog #: QVT 000, there is minimal signal in Panels B & D (see Figure 41, Panel D, white arrows, Red signal.) This may indicate that storage at 4°C for 74 hours prior to centrifuging for Ficoll gradient may not result in reliable signal.

Next blood was stored at 4°C for 74 hours: the **"With-Probe"** (Figure 40) whereas there is zero spurious signal in the **"No-Probe"** control (Figure 41.) There is minimal Texas Red signal in the "No-Probe" control (see Figure 41, Panel D white arrow.)

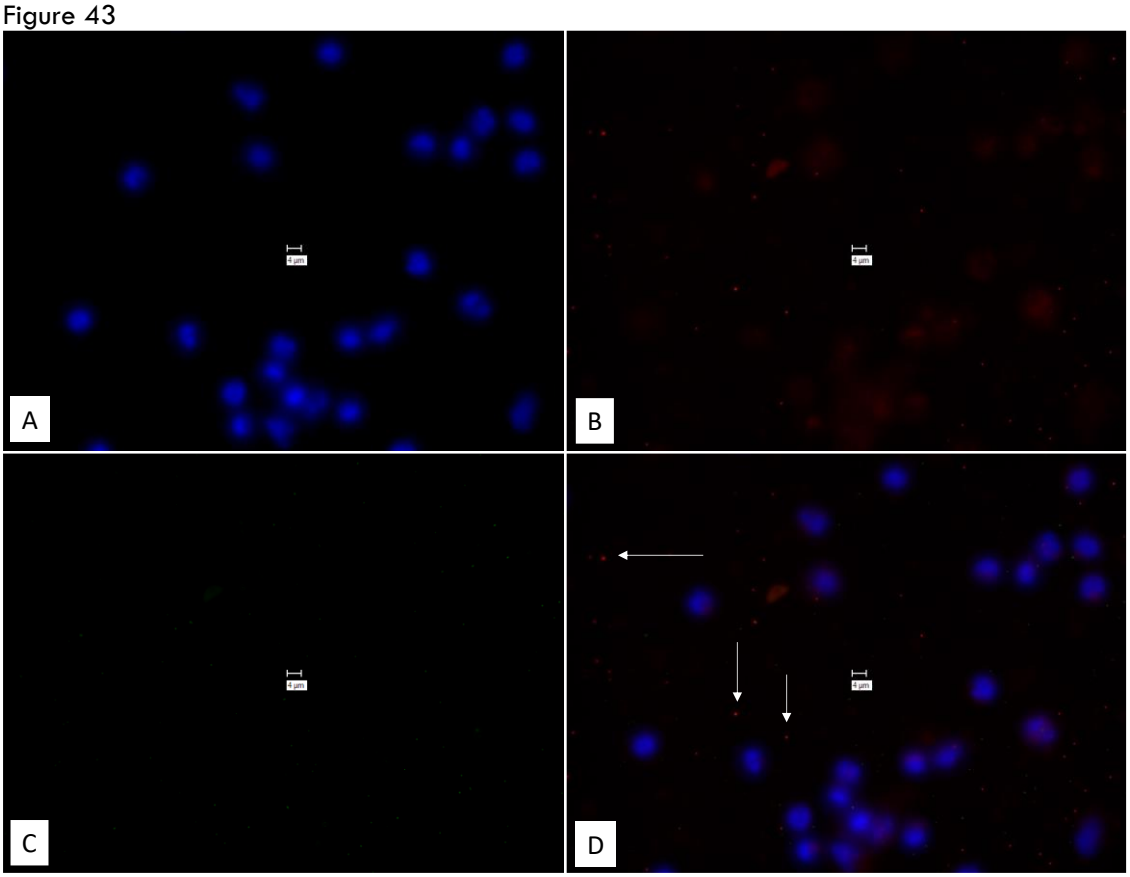
Figure 42



Legend to Figure 42. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at RT for 74 hours prior to centrifuging for Ficoll gradient. Probes were two human house-keeping genes: Human β-actin (Type 1 =Red) and Human GAPD (glycaraldehyde-3-phosphate dehydrogenase) (Type 6 = Green.) Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note positive signal for both Human β-actin (Type 1 =Red) and Human GAPD (Type 6 = Green.) See Figure 42 Panel D white arrows.

Figure 43



Legend to Figure 43. "No-Probe" control for Figure 42. Studies on Ficoll gradient buffy coat human WBC's. Affymetrix RNA View® ISH CELL Assay Kit: Thermo Fisher Catalog #: QVT 0001. Blood stored at Room Temperature for 74 hours prior to centrifuging for Ficoll gradient. Panel A= DAPI; Panel B = Texas Red; Panel C = Cy-5. Panel D = composite of A, B & C. Marker bars in µm indicates magnification of x 100.

Note, in this CELL assay Thermo Fisher Catalog #: QVT 000, although some Texas Red signal is seen (Figure 43: Panel D white arrows), it is not associated with DAPI positive regions. This indicates that not associated with WBC's. It may be possible that transportation of blood samples may be possible at ambient temperature for 74 hours, prior to processing for Buffy coat.

Finally, blood was stored at RT for 74 hours prior to processing. Signal is seen for both Human β-actin and GAPD (Figure 42 see Panel D white arrows.) In contrast, in the "No-Probe" control any spurious signal is not DAPI associated Figure 43 see Panel D white arrows.) It is therefore possible, that reliable signal might be obtained following transportation of blood samples at ambient temperature for 74 hours, prior to processing for Buffy coat.

As previously stated, [42], we repetitively contacted the Technical staff at ThermoFisher Affymetrix in an attempt to prevent false positive signal in the No-probe controls. We have shown that this false positive "No-probe" signal cannot be ascribed to contamination of the negative control slide by probes during the post hybridization wash, by increasing the stringency or duration of the HCL pretreatment, nor using different filters (TritC for Texas-Red, and for Cy-5 a custom recommended filter set "Hope." [42] Several of these modifications were recommended by the technical staff at Affymetrix.

A proprietary FISH assay has been performed according to the recommended conditions of the vendor. Purportedly positive signal was detected for both MAP (IS900) and our eukaryotic housekeeping gene, human β-actin. However, repetitively the "No-Probe" negative control for a given experiment showed obviously false "positive" signal.

Discussion

It is concluded that when the assay is performed according to the Affymetrix ViewRNA™ ISH Tissue 2-Plex Assay recommended instructions, it cannot be used for FISH studies to identify the RNA of MAP on previously frozen human brain from individuals with and without Multiple Sclerosis or on pure bacterial cultures.

In contrast, when studying human WBC's, we observed reliable signal with no spurious background. These studies (see Figures 34-43) were performed using probes that identified human house-keeping genes, not mycobacterial pathogens. They were used as controls and were not the purpose of the study, which was to evaluate whether we could detect FISH signal of mycobacterial pathogens in human brain from subjects with MS. These arguably reliable experiments were achieved using the Affymetrix™ ViewRNA ISH "CELL" Assay Kit; Invitrogen by Thermo-Fisher Scientific: Catalog Number: QVC0001) and not the Affymetrix™ ViewRNA ISH "TISSUE" Assay Kit.

These appropriate WBC data may indicate the

possibility, in future studies, of identifying pathogens in circulating macrophages that have ingested infecting disease-causing organisms. Accordingly, we studied WBCs under a variety of conditions. We find that storage of WBCs at room temperature (but not 4°C) for up to 72 hours permitted reliable data to be achieved. These observations are the same as the timespan that we use with a phage assay that identified MAP on circulating WBCs in cattle with Johne disease.[45]

Limitations:

In this study we asked a binary question. Is MAP RNA present or absent in brains of humans with and without Multiple Sclerosis? Especially when the target is expected to be in low abundance, any background may result in a false positive interpretation and is unacceptable.

In contrast, when a change in gene expression is being quantified, for example comparing normal with inflamed tissue, a low FISH signal to noise background may be acceptable. Accordingly, our conclusions apply only to frozen human brain from individuals with and without Multiple Sclerosis. We cannot comment on other scientific investigations that employ the Affymetrix ViewRNA™ ISH Tissue 2-Plex Assay on human brain. Likewise, our conclusions only apply to frozen, not fresh human brain.

Declarations:

- Ethics approval: This study was approved by the Research & Development Committee at the VAMC Bronx NY (0720-06-038.)
- Consent to participate: Tissue is non-identifiable. No consent is necessary.
- Consent for publication: Not Applicable
- Availability of data and material: Competing interests

Dr. Greenstein has three MAP related patents based on his published work in this field.

Patents Issued

US Patent # 7,846,420: Issued: June 18, 2013 (Now lapsed). US Patent # 7,902,350: Issue Date March 8, 2011(Now lapsed.) US Patent # 8,507,251: Issue Date August 13, 2013 (Now lapsed.)

Dr. Sheldon T. Brown has the following potential Conflict of Interest

STB was a member of the National Academy of Sciences of the USA panel that issued the Report "Diagnosis and Control of Johne's Disease." ISBN 0-309-08611-6.

PSF report no potential conflict of interest.

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Authors' Contributions:

1). Conceived and designed the study: RJG.
2). Performed the study: PSF, RJG.
3). Reviewed the data: RJG, PSF, STB.
4). Wrote the manuscript. RJG.
5). Read and approved the final version of the manuscript: RJG, PSF, STB.
6). Each author agrees to be accountable for all aspects of this work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. RJG, PSF, STB.

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