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Analysis of Villin Expression and Free Radical Load of Butyrate-Treated HT29 Cells

Ella Bakker

s4280474

Department of Biomedical Engineering

Period: 17/04/2023 - 01/07/2023

Bachelor's Project

1st Examiner: dr. R. Schirhagl, Professor, Schirhagl Group

2nd Examiner: dr. P. K. Sharma, Sharma Group

Contents

Abstract

The use of diamond magnetometry for detection of free radical load is an innovative technique allowing for analysis on a single-cell level. This study examined the relationship between the expression of villin and free radical load over the course of 13 days of enterocytic differentiation. The results showed a gradual increase in villin expression during differentiation, consistent with previous studies, demonstrating the enhancing effect of butyrate on enterocytic differentiation. However, a decline in villin expression was observed after day 8, raising questions about the longer-term effects of butyrate treatment and potential sources of error in the immunostaining process. T1 values were evaluated in control and butyrate-treated groups, as well as in mixed samples, consisting of half butyrate-treated cells and half green fluorescent protein expressing control cells, in order to compare cells in the same dish. While there was no statistically significant difference in T1 values for either pure or mixed samples on individual days, the mixed samples had a significant change when evaluated over the entire differentiation period, indicating a general change in free radical load. Overall, further testing with a higher sample size will greatly improve the results and may provide a statistically significant conclusion. This study provides valuable insight into the complex dynamics of enterocytic differentiation, and more precise testing is important for further understanding of the effect of butyrate treatment on free radical production, cellular differentiation, and gene expression.

1 Introduction

Colorectal cancer, developed in polyps in the colon's inner lining, is the third most common cancer worldwide [1]. Polyps originate as small accumulations of mucosa. As they enlarge, the combined effects of cell proliferation and traction lead to the formation of a stalk. The most common type of polyp is the adenoma, which can potentially become cancerous. When left untreated, cancerous tissue can spread to the muscle and outer layer of the colon, or to other parts of the body through the lymph nodes or blood vessels. Adenocarcinoma of the colon is both the most common malignancy of the gastrointestinal tract, and is the main cause of morbidity and mortality worldwide. Incidence of colorectal adenocarcinoma is highest in North America, the United States alone contributing 10% of cases and deaths.

Dietary factors are the principal factor contributing to risk of colorectal cancer, primarily low intake of unabsorbable vegetable fiber and high intake of refined carbohydrates and fat [2]. In terms of treatment, dietary adjustment as well as pharmacologic chemoprevention is possible. Staging of the cancer is done with respect to the depth of the tumor penetration (T-stage) into the wall of the bowel, the presence of regional lymph node involvement (N-stage), and distant metastases (M-stage), referred to as TNM staging [3]. Accurate tumor staging is crucial for defining the progression of the cancer, and is an important factor in treatment decisions. Early detection allows for well-timed intervention, significantly decreasing the chances of further spread and increasing survivability. This is particularly true in further stages with increased involvement of the lymph nodes and detection of cancerous tissue in distant metastases.

The HT-29 cell line, a human colorectal adenocarcinoma cell line with epithelial morphology, serves as a beneficial tumor model for colorectal cancer [4]. Derived from a human colon adenocarcinoma, this cell line has been widely used as a model for studying the biology of colon cancer. This cell line has been used to study the immune response when faced with bacterial infection, and the survival, adhesion, or invasion of microorganisms [5]. A 1984 study showed that when HT29 cells were treated with sodium butyrate, multinucleation, a possible indicator of differentiation, was observed [6]. This study concluded that permanently differentiated cell populations emerge after treatment with sodium butyrate.

Villin is one of the major proteins of the brush border that contributes to the organization and maintenance of the microvilli; synthesis of this protein greatly increases throughout enterocytic differentiation. Villin content was found to be 10 times lower in undifferentiated HT29 clone cells, as compared to differentiated cells [7]. The HT29 cell line is further characterized in terms of its differentiation and molecular features, particularly by the production of free radicals.

A free radical is defined as an atom or molecule with one or more unpaired electrons in the outermost valence shell [8]. An increase in free radicals leads to both protein and lipid damage, which is critical in carcinogenesis, as well as facilitation of tumor progression by affecting cell proliferation and survival. The accumulation of Reactive Oxygen Species (ROS), a highly reactive free radical containing oxygen, contributes to the conversion of healthy cells to cancer cells through enabling of genomic instability, ECM independency, and increased motility [9]. As stated, an early diagnosis of colon adenocarcinoma can be life-saving. As oxygen free radicals play a critical role in the stimulation of tumor growth, detection of altered free radical load may be a promising new method for early detection of cancerous cells.

In modern developments in biomaterial science and imaging, there has been a significant increase in the utilization of nanodiamond particles as imaging agents in biomedicine due to the optical and magnetic properties occurring in point defects in diamonds [10]. In particular, nanodiamonds containing fluorescent color centers, or Fluorescent Nanodiamonds (FNDs), are suitable for their biocompatibility and photostability at near infrared (NIR) fluorescence emission. Nitrogen is a frequently occurring defect in diamond, either naturally or synthetically with doping. The optical readout capabilities of nitrogen-vacancy (NV) centers in diamonds allow for the detection and control of the NV centers' quantum states, which in turn enables nanoscale resolution imaging. Using the diamond magnetometry technique, free radical levels can be monitored [11]. This technique is comprised of measuring the T1 relaxation time in microseconds of the NV centers. The spins of the NV centers are first polarized with a strong laser pulse, following which the NV centers will begin to decay into various magnetic states, allowing for the measurement of T1 relaxation. 'T1' refers to the process of the quantum spins returning to their equilibrium states after being disrupted by a magnetic field. Interaction between the free radicals and the NV-centers in the diamond affect the relaxation dynamics of the spins, thus allowing for a direct assessment of the amount of free radicals present in the cells.

In this study, diamond magnetometry will be used for quantification of free radical load, while immunocytochemistry can be used for detection of villin over the course of enterocytic differentiation in differentiated HT29 cells. This paper will evaluate the evolution of free radical load and villin expression of HT29 cells, as well as analyzing their relationship, over the course of 13 days, with both untreated control group and the butyrate-treated group.

2 Materials and Methods

2.1 HT29 Cells

HT29 cells were kept in complete Dulbecco's Modified Eagle Medium at 4.5 g/L glucose (due to the cell's high glucose uptake), 10% fetal bovine serum (FBS, ScienCell, USA), and 1% Penicillin-Streptomycin (PS). Approximately 300,000 cells were kept in T25 flasks, and passaged once they reached 70-80% confluency. To prepare dishes for magnetometry and immunocytochemistry measurements, 88,000 cells were put into 35-mm dishes with 2 mL of DMEM-HG with FND's, either with or without butyrate, at a concentration of 5 millimolar butyrate. Fresh butyrate-containing medium was prepared on the day of cell-seeding. The medium was first removed from the T25 flask, the cells were rinsed with PBS, and treated with 1 mL trypsin-EDTA. The cells were then incubated for 1-2 minutes at 37 °C, 5% CO_2 in order to activate trypsin. They were then observed in the microscope and struck with the hand in order to assure the cells are detached. The cells were then collected and centrifuged for 3 minutes at 15000 RPMs. The supernatant was then removed, and the cells resuspended in 1 mL fresh DMEM-HG. A hemocytometer was then used to determine the approximate volume that contains 88,000 or 300,000 cells. If there was an insufficient amount of cells in the butyrate-treated sample, the non-butyrate flask was prepared with the same amount of cells as the treated flask. Prepared dishes were then incubated at 37 °C, 5% CO_2. Control and treated dishes were prepared each day for 13 days.

2.2 Fluorescent Nanodiamonds

In this experiment, 120-nm FNDs were used (Adamas Nanotechnologies). The stock solution was prepared by diluting FNDs with DMEM-HG medium for a concentration of 3 μg/mL. All cells were incubated in FND-loaded medium at all times, allowing for maximal uptake of diamonds. This is necessary for FNDs to be found and used for T1 measurements.

2.3 Mixed Samples

Mixed samples were prepared in order to analyze the T1 values of untreated cells and butyrate-treated cells from the same dish. The untreated, or control cells were expressing green fluorescent protein (GFP) conjugated with epithelial cell adhesion molecules (EpCAMs). The cells were given as a courtesy of Professor Ben Giepman's group. 44,000 GFP-expressing and 44,000 butyrate-treated cells were put together in FND-loaded medium before the measurement and allowed to attach. After the T1 measurements, the samples were fixed and counterstained with DAPI. Confocal microscopy was used to determine whether each of the measured cells was a non-treated one (control adenocarcinoma cells, GFP-expressing) or a butyrate-treated one (re-differentiated cells, no GFP). The results of the T1 measurements were then categorized into two respective groups. These were prepared with cells on the 1st, 7th, and 12th day of differentiation.

2.4 Immunocytochemistry

Immunocytochemistry, a laboratory technique used for staining of cell cultures using immunofluorescence, was performed in order to estimate villin expression. The method in this experiment is in accordance with Abcam's immunofluorescence protocol. The μ-Dish 35-mm dishes, high grid-50 glass bottom, containing 88,000 cells with FND-supplemented medium, either with or without butyrate,

were rinsed with 1% Phostphate-Buffered Saline (PBS) and then fixed with 4% paraformaldehyde (PFA) at pH 7.4 for 12 minutes at room temperature. The dishes were then washed with PBS three times, for 5 minutes each time. The cells were then permeabilized by incubating with 1% Trition X-100 in PBS for 15 minutes. The dishes were then washed again with PBS three times, five minutes each time. Then, in order to block unspecific binding of the antibodies, the cells were incubated in 1% Bovine Serum Albumin (BSA) in PBS (1% PBSA)for 30 minutes.

The cells were then incubated with the primary antibody, in this case Anti-Villin antibody (N-terminal, monoclonal), at a concentration of 1:500 in PBSA. Monoclonal antibodies are often used for the primary antibody due to their high specificity. The primary antibody was then removed, and once again washed three times with PBS for 5 minutes each time. The cells were then incubated with the secondary antibody, Goat Anti-Mouse IgG H&L (FITC, abcam, polyclonal), diluted in PBSA at 1:1000, and 1:100 of DAPI (D31, DNA stain) for 1 hour at room temperature. These concentrations are commonly employed concentrations for immunocytochemistry, and were based on the manufacturer's recommendations and previous optimization. The samples were stored in the dark, as the secondary antibody and DAPI is light-sensitive. The dishes were then rinsed once with PBS, and fixed with 1% PFA in PBS. The dishes were sealed and stored at 4°C in the dark until measurements were performed.

2.5 Confocal Microscopy and Image Post-processing

Confocal microscopy provides high-resolution visualization of fluorescent samples by use of pinhole aperture that excludes out-of-focus light. Laser illumination will excite the fluorophores in the sample, enabling them to emit fluorescence that is collected by a detector. The sample is then scanned by the laser, allowing for good resolution and contrast. For the dishes prepared for T1-measurements, the T1-measurements are first collected and the cells are then fixed and stained to prepare for confocal imaging. A brightfield image, blue fluorescence image for DNA staining, green fluorescence for anti-villin staining, and red fluorescence for nanodiamonds will be taken. The same images will be taken of the dishes prepared just for immunocytochemistry, where images will be taken of approximately 50 cells.

The images will be processed in Fiji (ImageJ). Each channel of the image can be viewed, and the raw integrated density can be measured in order to quantify the level of green fluorescence. Raw integrated density is a quantitative measurement which sums the pixel intensities in a certain region of interest (ROI). It provides an estimate of the total amount of signal (fluorescence, in this case) in the image. The ROI, the cell, will be manually selected.

2.6 T1 Measurements

T1 Measurements will be done with a diamond magnetometry setup in order to analyze the free radical load of the cells at different stages of differentiation. During the measurement, a strong laser pulse is shone on the NV-centers of the diamond, stimulating the system into relaxation. The NV-center will then have a dimmer fluorescent signal. An increase presence of free radicals will elevate magnetic noise, which accelerates the relaxation time of the NV-centers. The relaxation time is quantified as a T1 constant that is based on the relationship between the fluorescence of the NV-center and the time taken for the system to relax.

Two hours of measurements were done for each cell, and measurements for approximately 3-5 nanodiamonds per cell were performed. Following the measurements, cells were immediately fixed with 4% PFA and stored at 4°C, to be later immunostained.

2.7 Data Analysis and Statistics

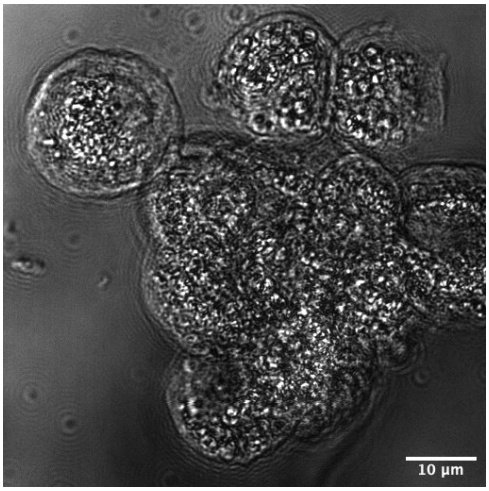
All statistical analysis and data processing was done with Python 3.0. Examples of code can be found in the Appendix. The following table displays the statistical tests and null hypotheses for the data analysis.

Table 1: Statistical Tests and Null Hypotheses

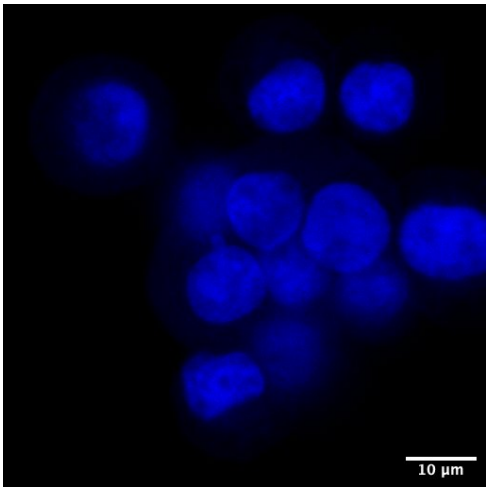
Dataset	**Statistical Test**	**Null Hypothesis**
Villin Expression in Control and Butyrate-Treated HT29 Cells	Mann-Whitney U	The control and butyrate-treated samples have identical villin expression distributions for each day.
T1 Values in Control and Butyrate-Treated HT29 Cells	Mann-Whitney U	The control and butyrate-treated samples have identical T1 values for each day, until day 13.
T1 Values in Mixed Samples: GFP-expressing cells and Butyrate-Treated Cells	Kruskall-Wallis and Mann-Whitney U	The GFPs and butyrate-treated samples have identical T1 values for each day, day 1, 7, and 12.

3 Results

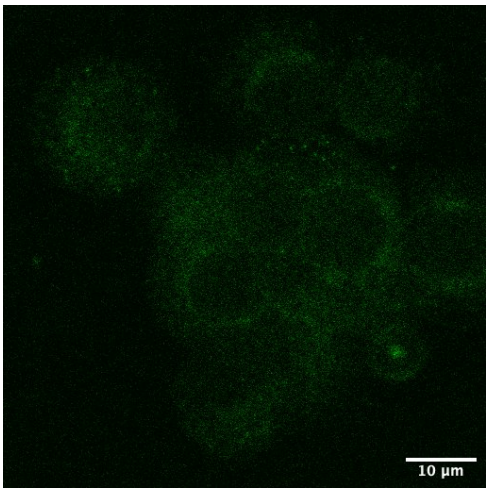
3.1 FND Uptake and Villin Expression



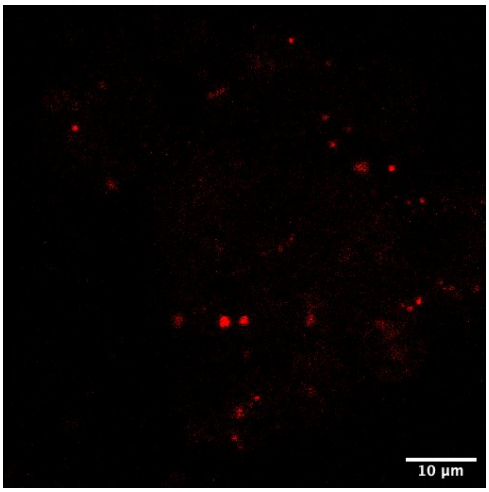
(a) Brightfield Image



(b) DNA Staining



(c) Villin Expression

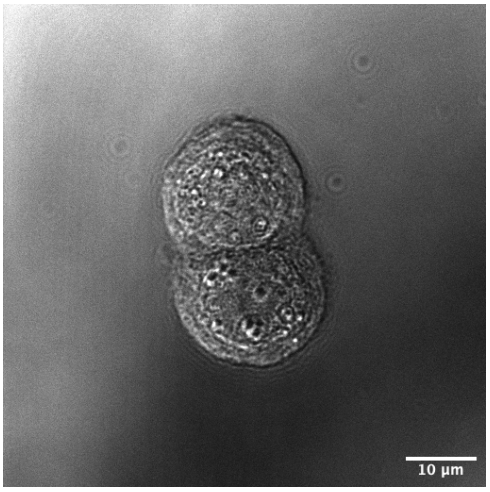


(d) FNDs

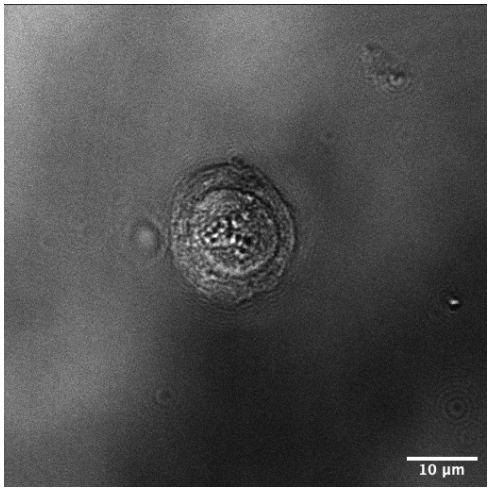
Figure 1: Channels Collected with Confocal Microscopy

The above figure shows the four channels collected with confocal microscopy. Image (a) shows the brightfield image of the cell, where the cell in its entirety can be seen. Image (b) displays the cell nuclei, a result of the DAPI staining. It can be seen here that there are four cells in this particular image. Image (c) shows the villin expression in green as a result of the primary and secondary antibody staining. Finally, image (c) shows the fluorescence of the nanodiamonds taken up by the cells in red.

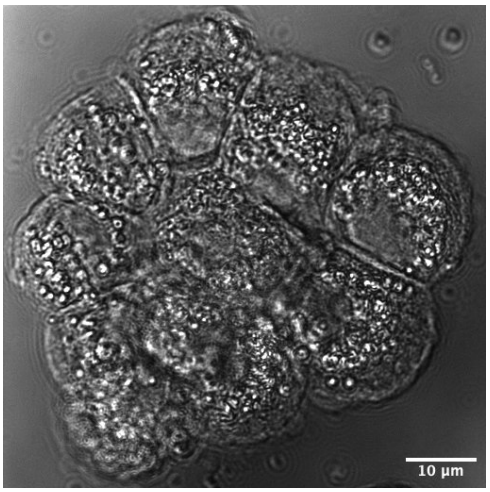
3.2 Butyrate-Induced Differentiation



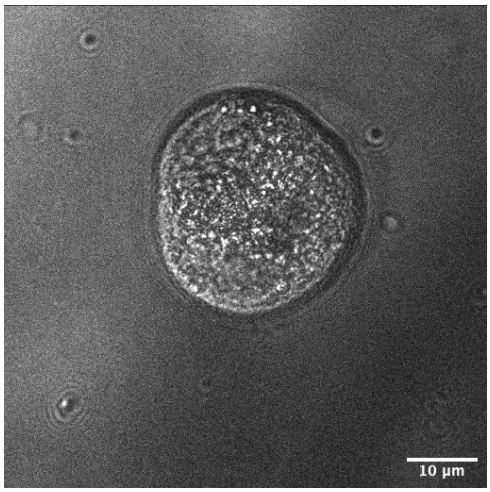
(a) Day 1, No Butyrate



(b) Day 1, Butyrate



(c) Day 13, No Butyrate



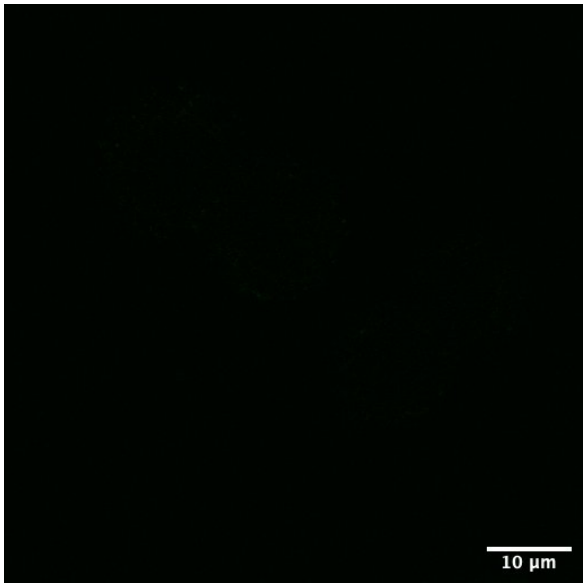
(d) Day 13, Butyrate

Figure 2: Brightfield Images of Control and Butyrate-Treated Groups

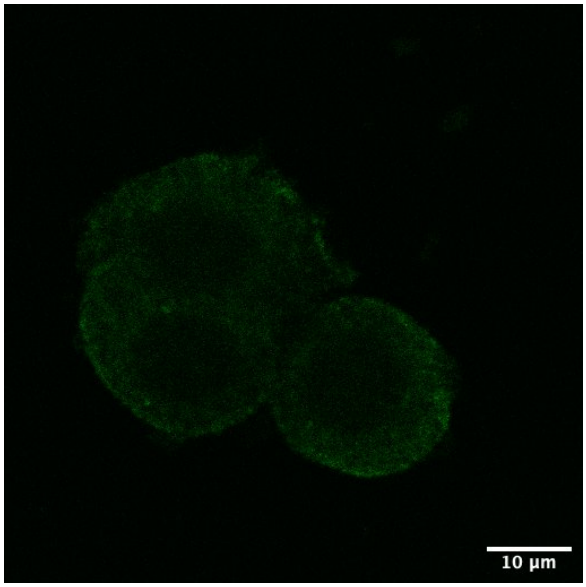
As is characterized by this particular cell line, HT-29 cells were observed to form aggregates or clusters of cells. The samples without butyrate-treatment tended to have more isolated cells, whereas the butyrate-treated samples did not, as can be seen when comparing day 1 and day 13 in Figure 2.

Over the course of differentiation, the control group cells were observed to retain their circular shape and stay in pairs or larger groups. The butyrate-treated cells were often less circular and isolated from other cells.

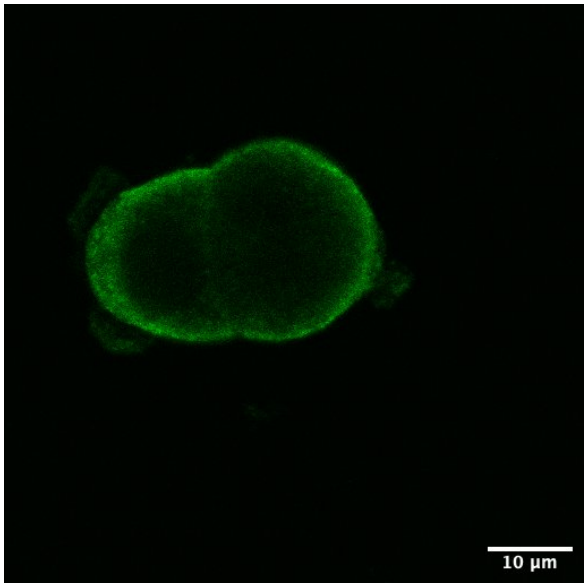
3.3 Villin Expression



(a) Day 1 Mixed



(b) Day 7 Mixed



(c) Day 12 Mixed

Figure 3: Mixed Samples at Days 1, 7, and 12

Pictured above, from left to right, are the green fluorescence confocal microscopy images taken of butyrate-treated cells, for day 1, day 4, and day 8. All are at the same scale of brightness/contrast; higher fluorescence in the picture indicates a higher level of villin expression.

3.4 Quantized Villin Expression

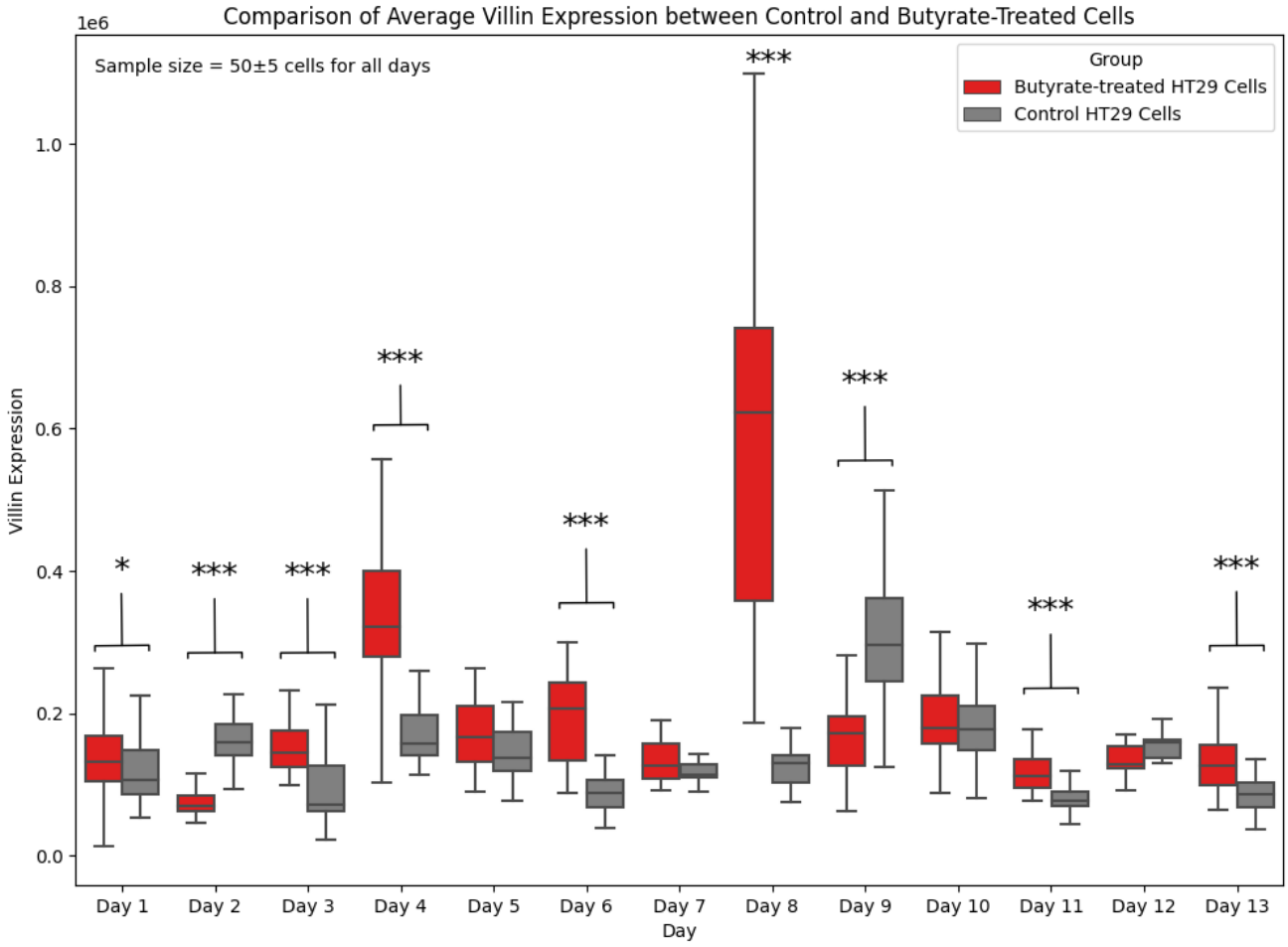


Figure 4: Average Villin Expression and Standard Deviation Boxplot

The figure above shows the fluorescence from the antibody-stained samples, both the control and butyrate-treated group. A lower value indicates a lower level of villin expression.

Though there is a statistically significant difference between most of the days individually, there is a slight increase in villin expression until day 8, where the increase is substantial. The control samples remain at relatively low values compared to the butyrate-treated samples. Interestingly, the fluorescent signal of the butyrate-treated group is then observed to decrease in days 10-13. This could be due to a variety of reasons that will be further explored in the Discussion.

3.5 T1 Values

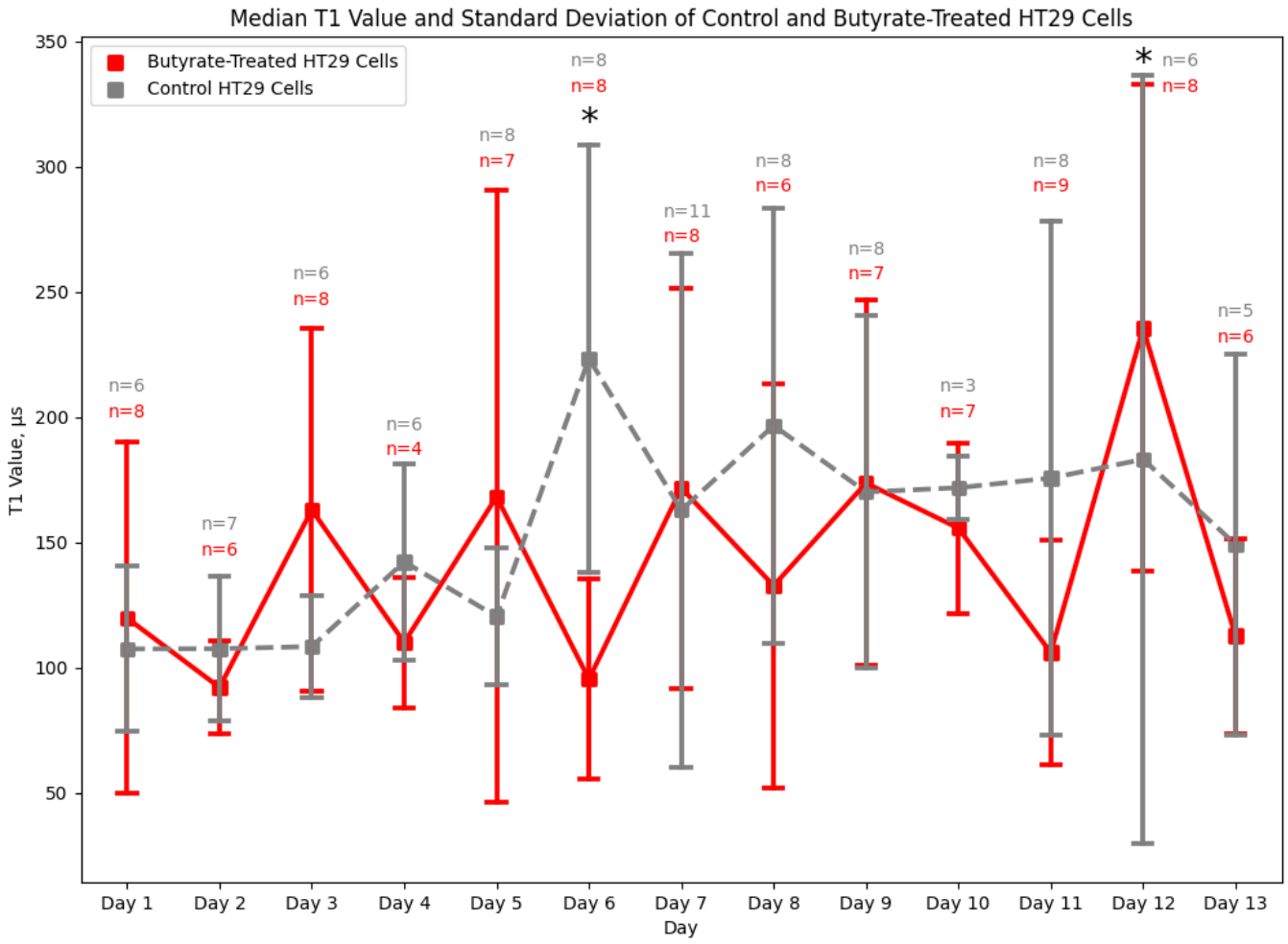


Figure 5: Median T1 Values Lineplot with Standard Deviation

The T1 values of the control and butyrate-treated samples during differentiation are depicted in this figure. A difference of statistical significance was observed on days 6 and 12, while no distinct trend was evident in either sample, except for a general median value range of 50 to 250.

These results differ from previous research, which suggests that significant differences typically manifest around day 8-9. Additionally, both datasets exhibit a large standard deviation, and there is no discernible trend beyond day 9. Overall, these findings highlight the complexities and variability in the T1 values during the differentiation process, and the high cell-to-cell variability.

The Mann-Whitney U test indicates a difference of statistical significance between the control and treated samples on days 6 and 12, so the null hypothesis can be rejected only for these particular days. For the datasets as a whole, the null hypothesis is failed to be rejected, with a p-value of 0.22.

3.6 Comparison of Villin Expression and Free Radical Load

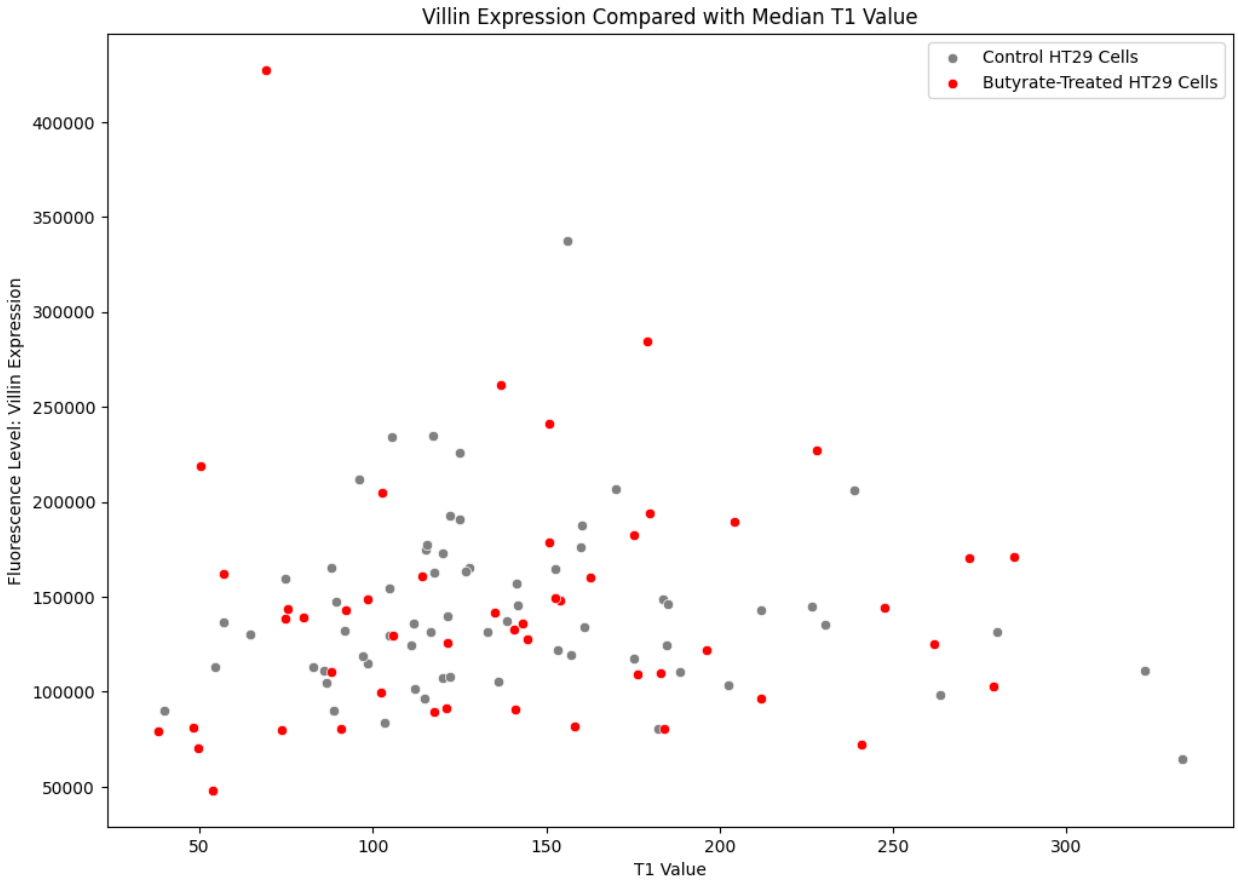
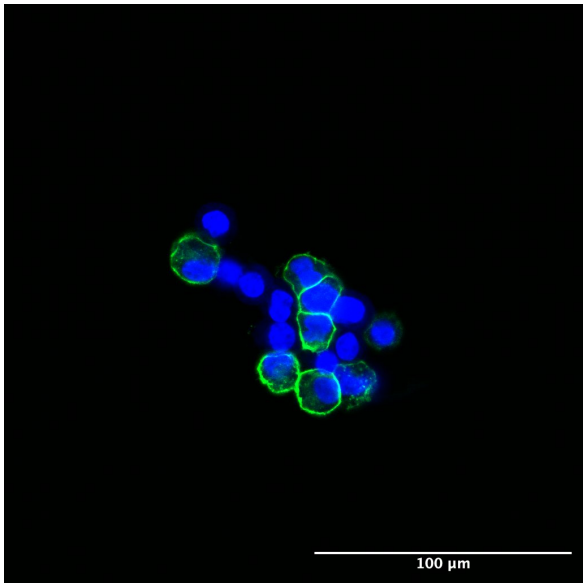


Figure 6: Average Villin Expression and Median T1 Values Scatterplot

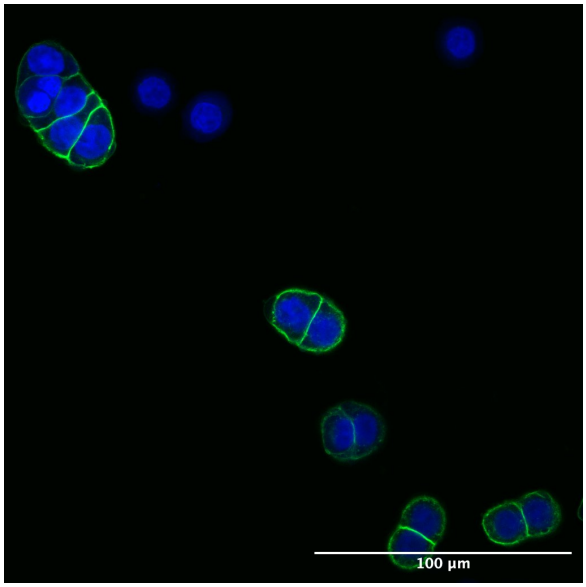
The above graph compares the T1 value of the samples with its corresponding villin expression. Each dot on the graph represents a specific cell in which magnetometry was performed, and the sample was then stained to collect the fluorescence of the same cell.

The T1 values, which reflect the level of free radical load within the cells, do not exhibit any significant changes throughout the observation period. This is due to the lack of observable or statistically significant trend for either the T1 values or the villin expression. Therefore, it is to be expected that no meaningful relationship can be derived from a graphical comparison.

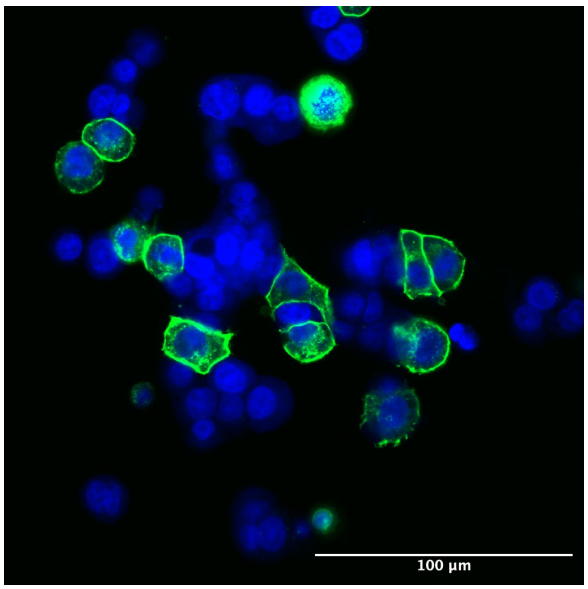
3.7 Mixed Cells



(a) Day 1 Mixed



(b) Day 7 Mixed



(c) Day 12 Mixed

Figure 7: Mixed Samples, GFP-EpCAM (green) and DAPI (blue) at Days 1, 7, and 12

Pictured here are three confocal images of the mixed samples, containing GFP-expressing cells and DAPI-stained butyrate-treated HT-29 cells. All images appear to have successful integration of two cell populations.

3.8 Comparison of Villin Expression and Free Radical Load in Mixed Samples

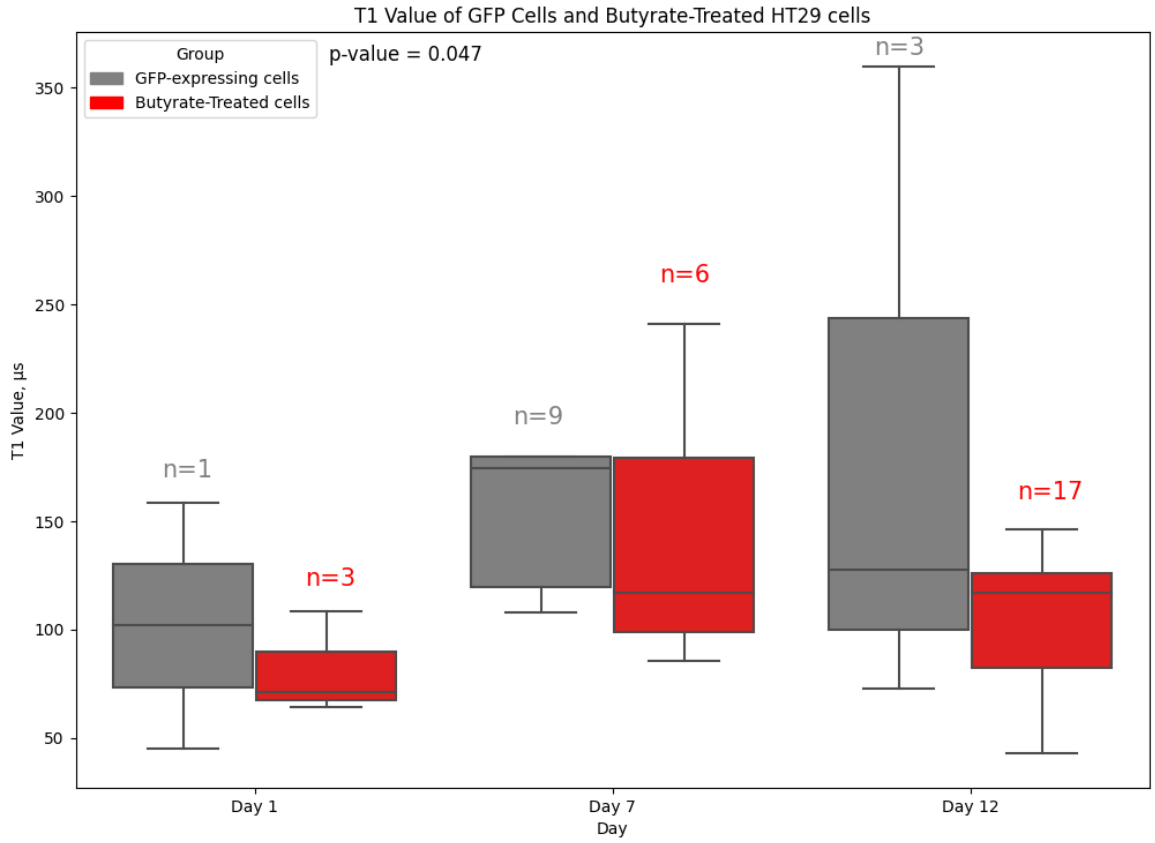


Figure 8: T1 Values Mixed Samples Boxplot

Table 2: Statistical Analysis of Mixed Samples

Groups	Median		Mann-Whitney U Statistic	P-value
	GFPs	Butyrate-Treated		
All Days	142.98	110.69	252.0	0.047*
Day 1	128.85	71.04	5.0	0.400
Day 7	174.46	117.12	33.0	0.516
Day 12	127.36	117.19	33.0	0.479

Figure 8 shows the T1 value of the GFPs and butyrate-treated cells on day 1, day 7, and day 12. On Day 1, the GFPs have a slightly higher median, indicating lower free radical load. On Day 7, the two groups share a very similar distribution. Finally, on day 12, the butyrate-treated cells contain more smaller values, indicating higher free radical load. It is important to note that the medians are very similar. As can be seen in Table 2, analysis reveals a statistically significant difference between the GFPs and Butyrate-Treated groups when considering data from all days. However, no significant differences were observed between the groups on specific individual days. This indicates a change over the total differentiation period, but not on any particular day. Reasons as to why this is the case will be further explored in the discussion.

4 Discussion

4.1 Immunostaining Images

An important aspect of this study was the expression of villin during enterocytic differentiation. To visualize the changes in the proteins expression, immunocytochemistry was performed, wherein the cells were stained with a green fluorescent marker specific to villin. The resulting images provide insights into the dynamics of the proteins expression during the differentiation process. As can be seen in Figure 3a, the fluorescent signal was relatively weak, indicating low expression levels. However, as the differentiation progressed, a gradual and significant increase in green staining intensity was observed, indicating a rise in villin expression. This trend continued in 3b, showing a more vibrant and pronounced green fluorescence. There were other samples that were expected to have high levels of expression, but rather resembled that of an earlier day, which may be due to certain sources of error, which will be further discussed. The quantified villin expression for each day is represented in Figure 4.

4.2 Quantized Villin Expression

The observed trends in the expression of villin provides insight into the HT29 differentiation process and implications for colon adenocarcinoma. The observation of an increase in villin expression at day 8 supports previous research in butyrate enabling enterocytic differentiation. As stated in the introduction, butyrate can enhance both differentiation and gene expression. These results provide support that butyrate stimulates villin synthesis, which is indicative of differentiation in the HT29 cells.

However, following day 8, there is a decline in villin expression, observed from day 9 to to the final day of differentiation, day 13. These results raise questions about the longer term effect of the butyrate treatment. Further testing would need to be done to confirm whether this is characteristic of the HT29-cell lines differentiation process, or perhaps an error with the immunostaining process. Other reasons for the observed decrease may be due to cellular saturation; perhaps the HT29 cells were no longer able to further increase villin expression due to the continued presence of butyrate, or changes in the micro-environment; for example, the immediate surroundings of the cells, including effects due to cell-cell interactions, extracellular matrix composition, or inhibition of certain signaling pathways within the cell.

It is also important to consider that there may have been errors in the immunostaining process. It is possible that the specificity of the antibodies were not optimal for accurate villin detection. Perhaps these antibodies cross-reacted with other proteins or produced non-specific staining. In future experiments, it may be best to do a pilot study testing different antibodies with a range of antibody concentrations, in order to optimize the conditions for these particular experimental conditions, maximizing specific staining while minimizing background staining. Validation of the antibody specificity can also be done with specific controls, for example, antibody blocking experiments, to minimize the possibility of false results.

There are other areas for error in the immunostaining process, such as inadequate fixation or permeabilization of the cells, which could lead to uneven staining patterns, variability in the cell preparation, as cells were stained on different occasions, potentially allowing for different environmental factors

that affected the protocol. In post-processing, the possible presence of background noise, non-specific staining, or variations in intensity were not taken into account.

4.3 T1 Values

Figure 5 shows the distribution of the T1 values of control and butyrate-treated cells over the 13-day experimental period. There is a statistically significant difference on days 6 and 12, but on no other days, and not of the datasets as a whole. This means that in this experimental period and particular conditions, the T1 value does not significantly vary in butyrate-treated cells; the null hypothesis cannot be rejected. Based on previous literature, it is expected that a difference would be detected in butyrate-treated cells, that is, a lower T1 value, indicating higher free radical load. These results may not have been found in this experiment due to various reasons. First and most significantly is the small sample size. Each day had a varied number of cells, and thus a varied number of T1 measurements. For a two-tailed independent t-test, a sample size of at least 110 is required for a meaningful significance, and a p-value less than 0.05. In this case, each day, for the control and treated groups had approximately 6-10 cells. This is not enough to prove significance, and is thus included the report simply to display the distribution of the collected data in this study. The statistical significance in days 6 and 12 are likely accidental.

4.4 Comparison of Villin Expression and Free Radical Load

In analyzing the graph depicting the T1 decay value of cells and its correlation with villin expression over a 13-day period, it becomes evident that the results are inconclusive. Given the lack of substantial alterations in the T1 decay values and the unexpected decline in villin expression, it is challenging to draw definitive conclusions from Figure 6.

Due to the lack of statistical significance in the measured T1 values of the control and treated groups, combined with the unexpected change in villin expression past Day 8, it is not likely to extract meaningful conclusions from a comparison of the two factors. In order to do so, this experiment should be repeated with a far larger sample size.

4.5 Images of Mixed Samples

In addition to studying the villin expression during enterocytic differentiation, mixed samples were prepared consisting of GFP-labeled cells and butyrate-treated cells at different time points. As can be seen in Figure 7a, the mixed sample showed only two distinct GFP-labeled cells, indicating a limited presence of GFP-expressing cells within the butyrate-treated cell population. However, on day 7, shown in Figure 7b, the mixed sample exhibited a more diverse and densely populated cell population, with a noticeable increase in the number of GFP-labeled cells. This suggests that GFP-expressing cells are proliferating and becoming more prevalent within the butyrate-treated cell population as differentiation progresses. By day 12, in Figure 7b, there is a higher degree of mixing between GFPs and butyrate-treated cells, indicating extensive mixing. This is optimal for mixed cell measurements, as the T1-decay value can be taken from the same dish and directly compared.

4.6 Comparison of Villin Expression and Free Radical Load in Mixed Samples

Figure 8 shows the T1 values of the GFPs and butyrate-treated samples for days 1, 7, and 12. Overall, the comparison between the control and treated groups revealed a statistically significant difference (p-value = 0.047) in the T1 values between the two datasets.

Specifically, for day 1, there was no significant difference between the control and butyrate-treated groups, suggesting that there was no important disparity in the data. This aligns with expectations since the first day of butyrate treatment would not typically induce a significant variation in the free radical load.

Similar observations can be made for day 7 and day 12, although the p-value decreases. Visual examination of Figure 8 reveals that the values appear visually different for day 1, relatively similar for day 7, and notably higher for GFPs and lower for butyrate-treated cells on day 12. This pattern is logical as a significant change in free radical load would not be expected until day 8-9. The higher T1 values in GFP cells for day 12 indicate a lower free radical load, which is in line with expectations. Conversely, the lower values in butyrate-treated cells for this day suggest a comparatively higher free radical load, consistent with previous findings on re-differentiated HT29 cells. It is again important to note that the lack of statistical significance is very likely due to the small sample size.

A statistically significant p-value was obtained for the overall analysis of the mixed samples, indicating a significant difference between GFPs and butyrate-treated cells. This difference was not observed when analyzing the data on a day-to-day basis. It is crucial to consider the cumulative effect over the entire experimental period, which suggests a significant difference between the control and treated cells. However, based on the available data, the observable difference may not be evident in the early days of differentiation.

5 Conclusion

This paper investigated both the expression of villin and the free radical load of control and butyrate-treated HT29 cells over the course of enterocytic differentiation. Immunocytochemistry was used to analyze the level of villin production, and diamond magnetometry was used to analyze the T1 relaxation value of the cells. The immunocytochemistry revealed a gradual increase in villin expression over the course of differentiation, which is in line with previous findings that butyrate enhances enterocytic differentiation. However, there was then a significant decrease after day 8, possibly due to sources of error in the immunostaining process. It is also possible that there is a changed effect of butyrate in the longer term. In the future, a pilot study should be done to optimize antibody specificity and minimize false results.

Similarly, no clear trend was observed with the T1 values, likely due to the small sample size. However, analysis of the mixed samples showed a clearer increase in free radical load for the butyrate-treated group. If this experiment is repeated with more trials, it is likely that a difference of significance will be found. In order to potentially use the detection of T1 values for an early detection of

In general, the comparison between villin expression and free radical load was inconclusive, due to the lack of statistical significance in the T1 values, as well as the unexpected trend in the villin expression. There may be more complex occurrences over the course of enterocytic differentiation, and factors such as gene expression and free radical dynamics may have played a role in misleading the data. This possibility can again be removed with an increased sample size. This report contributes valuable information about the complexity of free radical production in HT29 redifferentiation, as well as the expression of villin. Further testing will only add to the value of this experiment, and increase specificity of the findings.

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Appendix

```
# Load T1 Files from Excel
day1_butyrate = pd.read_excel(r'day 1_butyrate.xlsx')[['filenam', 'T1_long']]

# Drop any values larger than 800 or less than 10
day1_butyrate.drop(day1_butyrate[(day1_butyrate.T1_long > 800) | (day1_butyrate.
    T1_long < 10)].index, inplace=True)

# Drop the index
day1_butyrate = day1_butyrate.reset_index(drop=True)

# Name the Columns
day1_b = pd.DataFrame(columns=['Cell Location', 'T1_value'])

# Loop through column names and find cell location
for x, y in day1_butyrate['filenam'].items():
    N = y[31:38]  # Find characters of string that contain cell location
    T1_value = day1_butyrate.loc[x, 'T1_long']  # Get the T1 value
    new_row = pd.DataFrame({'Cell Location': [N], 'T1_value': [T1_value]})
    day1_b = pd.concat([day1_b, new_row], ignore_index=True)

# If cell is R10a and R10b, for example, include a, b, c, d:
name = []
for x, y in day1_b['Cell Location'].items():
    # Split column names between underscore
    s = y.split('_')
    if s[1] == 'a' or s[1] == 'b' or s[1] == 'c' or s[1] == 'd' or s[1] == 'e':
        name.append(s[0] + s[1])
    else:
        name.append(s[0])

# Set the cell lotion to the name found in the for loop
day1_b['Cell Location']= name

# Group by cell location and take the median
d1b = day1_b.groupby(day1_b.columns[0]).median()
```

Listing 1: Loading T1 Files

```
import pandas as pd
import matplotlib.pyplot as plt
import seaborn as sns

# Load in files from the villin expression data
buty_cells = pd.read_excel('Villin Expression.xlsx', sheet_name='Butyrate')
no_buty_cells = pd.read_excel('Villin Expression.xlsx', sheet_name='No Butyrate')

comp_buty = pd.read_excel('Villin Expression.xlsx', sheet_name='Comparison_Buty')
comp_buty = comp_buty.drop(comp_buty[comp_buty.T1 > 500].index)

comp_no_buty = pd.read_excel('Villin Expression.xlsx', sheet_name='Comparison_No_Buty')
comp_no_buty = comp_no_buty.drop(comp_no_buty[comp_no_buty.T1 > 350].index)

gfps = pd.read_excel('Villin Expression.xlsx', sheet_name='GFPs')

# Concatenate the two dataframes and add a column to indicate the treatment group
buty_cells['Group'] = 'Butyrate'
no_buty_cells['Group'] = 'Control'
combined_data = pd.concat([buty_cells, no_buty_cells])

colors2 = {'Butyrate': 'red', 'Control': 'grey'}

# Reshape the combined data to long format
combined_data_long = combined_data.melt(id_vars='Group', var_name='Day', value_name='Villin Expression')

# Create the box plot with colors based on the 'Group' column
fig, ax = plt.subplots()
sns.boxplot(x='Day', y='Villin Expression', hue='Group', data=combined_data_long,
            ax=ax, showfliers=False, palette=colors2)

plt.xlabel('Day')
plt.ylabel('Villin Expression')
plt.title('Comparison of Villin Expression between Control and Butyrate-Treated Cells')

# Adjust the legend placement
handles, labels = ax.get_legend_handles_labels()
ax.legend(handles, labels, title='Group', loc='upper right')

# statistical significance

# Concatenate the two dataframes and add a column to indicate the treatment group
buty_cells['Group'] = 'Butyrate'
no_buty_cells['Group'] = 'Control'
combined_data = pd.concat([buty_cells, no_buty_cells])
```

Listing 2: Plotting Villin Expression and Statistical Tests