Anti-proliferative effects induced by New Zealand honey in colorectal cancer

By

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Abstract

Colorectal cancer is responsible for nearly a third of all deaths within New Zealand and has contributed 15% of worldwide malignancies. At present, colorectal cancer treatment is inadequate, although with developing treatments, such as the front-line drug, oxaliplatin, there are new options for those battling the disease. Still, as with other cancer treatment drugs, the benefit of oxaliplatin is marginal, especially depending on the stage of the disease. Cancer treatment drugs come with many toxic side effects, which can potentially include (but are not limited to): neurotoxicity, nausea, vomiting, diarrhoea, neutropenia, ototoxicity, extravasation and hypokalaemia. Therefore, developing new types of treatment with less harmful side effects has always be the main goal through medical history. Previous studies suggested honey could be a potential candidate due to its antioxidant effect, anti-inflammatory effect and possibly anti-tumour effect. The number of honey anti-tumour research projects are increasing and quite a lot of them have indicated effective reduction in cancerous cells. Although the anti-tumour effect of honey is limited to in vivo studies, a lot of researchers have hypothesised that using honey as a treatment for cancer could provide an outcome with less side effects as a result.

Existing drug treatments for colon cancer are associated with toxic side effects, while the potential for less side effects from using honey against colon cancer is promising. In this respect, honey treatments would rival those offered with existing drugs (e.g., Avastin, Bevacizumab, oxaliplatin, etc.). We hypothesize that honey induces an anti-proliferative effect through apoptotic pathways in colon cancer cell lines. The primary objective of this research was to investigate the anti-proliferative effects of Manuka honey and Thyme honey on two colorectal cancer cell lines by using MTT assays. The secondary objective would be to measure the apoptotic properties of these honey treatments by flow cytometry.

Two types of colon cancer cell lines were used in this study: LoVo (CCL-229) and WiDr (CCL-218) cell lines. Anti-proliferative effects were determined by 24 hour, 48 hour and 72 hour MTT cell viability assay. Oxaliplatin was used as a positive control in this study, and both cell lines were treated with 0.5%, 1.25%, 2.5% and 5% of Vitabeez's honey solution. The honey solution was prepared by mixing RPMI 1640 cell culture medium with honey to achieve a similar osmolarity as found in the human body.

The absorbance values were measured and the cell viability (%) was determined. The anti-proliferative effect seemed to be increased when the honey concentration was increased, as well as with increased time of treatment. Therefore, according to our statistically significant results, the tested honey show time-dependent and concentration-dependent anti-proliferative activity against the tested human colon cancer cells lines.

The secondary objective involved in this study was to determine the pro-apoptotic activity of tested honey in LoVo and WiDr cells. An increased apoptotic activity was observed when the honey concentration was increased at designated time period. Analysis for apoptotic activity indicated greatest numbers of cell death associated with treatment using the 2.5% honey solution.

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Attestation of Authorship

"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning."

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Abbreviation

AUT	Auckland University of Technology
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
CDK	Cyclin-dependent kinases
CRC	Colorectal cancers
CO_2	Carbon dioxide
COX-2	cyclooxygenase-2
CV	Coefficient variation
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
HCl	Hydrochloric acid
hr	Hour
IC_{50}	the concentration of the experimental compounds generating a 50% inhibition in cell growth
iNOS	inducible nitric oxide synthase
Log	Logarithm
LOX-2	lipoxygenase-2
mg/mL	milligram/millilitre
mM	millimoles per litre
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
NaOH	Sodium hydroxide
PARP	Poly ADP-ribose polymerase
PBS	Phosphate buffered saline
PI	Propidium Iodide
PKC	Protein kinase C
PS	Phosphatidyl serine
TNF	Tumour necrosis factor
TPK	Membrane tryosine kinase
WHO	World Health Organization
μM	Micromoles per litre

Chapter 1: Introduction & Literature Review

Overview

Cancers are a class of diseases characterised by out of control cell proliferation. They are one of the most destructive health problems worldwide. At present, over 100 types of cancer are classified by the type of cells that are originally affected. Recently, the global cancer statistics reported that ischemic heart disease has been replaced by cancer cases as the primary cause of death (especially in economically well-developed countries) around the world. The World Health Organization (WHO) announced in April 2003 that there might be a significant rise in cancer rates (over 50%) by 2020. Around 12.6 million cancer cases and 8.7 million deaths were reported by the WHO in 2012. Unfortunately, the WHO cancer statistical report also stated that after clinical treatment, only 30% of the diagnosed cancer cases are cured or prevented.

In 2012 and 2013 in New Zealand, the registered cancer cases did not significantly change for either males or females; this was also true for both adults and children, based on the age-standardised rate per 100,000 population which is standardised to the WHO world standard population. The New Zealand Ministry of Health announced that there were 21,050 new cases of cancer registered in 2011 in New Zealand, and cancer had become the most common cause of death for both males and females, accounting for nearly a third of all deaths. The total number of registered cancer cases were increased as twice as before from 1956 to 2012, especially in the age of over 75 in all adults. As for New Zealand childhood statistics, the registered cancer cases were unchanged from 1956 to 2012 (Ministry of Health, 2013). According to the data reported from the Ministry of Health (2011 to 2013), colorectal and anus, breast cancer, cervix cancer, leukaemia, melanoma, prostate cancer, lung cancer, chronic myo-proliferative disorders and myelodysplastic syndromes, Hodgkin lymphoma and non-hodgkin lymphoma were the most common causes of death in New Zealand. Cytotoxic chemotherapy, surgery and radiation therapy are the most common known methods of treatment, however, most therapeutic administrations produce a number of negative side effects such as haematological toxicity. The administration of different chemo-therapeutic agents (even with those of natural origin - for example, taxol, irinotecan and epirubicin) have been suggested to result in early bone marrow depression (Jaganathan, Mandal, Jana, Das, &

Mandal, 2010). Moreover, extensive radiotherapy which covers wide parts of the bone marrow can lead to the development of an unpredictable, dose dependent series of haematopoietic syndromes (Wang Li, White, & Lu, 2014). Enhanced sensitivity to infectious diseases with opportunistic micro-organisms is also seen to occur in parallel with progressive radiation-induced atrophy of lymph nodes, spleen, and bone marrow (Jaganathan, Mandal, Jana, Das, & Mandal, 2010). It had been recommended that radio-protective activity conferred by immune-modulators can be redounded to their capacity to increase immune functions and haematopoietic. Moreover, most of the cancer patients suffering mucositis from intensive chemotherapy treatment, experience worse side effects when combining both radiation and chemotherapy, leaving them susceptible to infection diseases (Swellam, et al., 2003). This is even more severe in patients who receive treatment for cancer of the neck and head.

The deaths of 20% of the human population are caused by various types of malignant diseases. There is no known curable treatment for the majority of malignancies, while every remedial administration produces different types of side effects, such as haematological toxicity.

Malignant Tumours

Cancer represents the abnormal cell proliferations, which have the potential to spread and invade other parts of the body. When the proliferations spread and invade, this is also known as a malignant tumour or malignant neoplasm (Kruse, 1970). When damaged cells divide uncontrollably, the process harms the body by forming lumps, or tumours (except in the case of leukemia, where cancer prohibits normal blood function by abnormal cell division in the blood stream) (Kruse, 1970).

A set of accumulative alteration, including both epigenetic and genetic alterations, occurring in a normal cell can generate cancer (Hoff & Machado, 2012) by un-regulated proliferation. Unregulated growth of these abnormal cells finally becomes a cancerous growth over time, and these tumours cells can grow into a larger size and interfere with other parts of the body. In order to invade other parts of the body, cells from the primary tumour must come off and travel through the bloodstream or lymphatic system. Since the genes, or protein structures within the tumour cells have already be mutated, release of a different hormone would change the normal bodily function in some cases. Benign

tumours and malignant tumours are the different distinctions used to describe tumours. Benign tumours are rarely life-threatening as they do not invade other parts of the body. In contrast, a malignant neoplasm describes uncontrollable survival, proliferation and differentiation of the cancer cells, and these steps are known as malignant progression. The malignancy process occurs when two things happen: First, a cancer cell manages to move throughout the body by using the blood stream or lymphatic systems, thereby destroying healthy tissues in a process named invasion; Second, angiogenesis occurs when cancerous cells manage to divide and proliferate, as well as stimulate the formation of new blood vessels to feed the growing tumour. It is said to have metastasised when a tumour has successfully spread to the other parts of the body, invading, proliferating and destroying other healthy tissues. The result is a severe situation, and is extremely difficult to treat.

The cause of cancer

The origin of every cancerous disease is thought to first begin with a single abnormal cell. What actually happens is that the process of managing cell division and multiplication is altered or damaged, likely as a result of certain vital genes. This process results in formation of abnormal cells and it may multiply into a cancerous, or malignant, tumour in an uncontrolled manner if the abnormal cell survives and continues it unchecked multiplication. There are more than 200 unique sorts of tumours, and each is classified by the kind of cell that is initially influenced. Potential causes of cancerous diseases can be covered in a wide range of situations, such as exposure to certain chemicals, aging, genetic predisposition, and infection with certain viruses and a weakened immune system, etc.

The longer we live, the higher the chance of developing cancer (Kelloff, 1999). At some point there must be various alterations to the genes within a cell prior to when it changes into a cancer cell. These changes can occur by accident during cell division, or through injury when the cell is damaged by carcinogens such as harmful chemicals. In either case, the damage is then passed onto future cells when the transformed cell divides. Therefore, the longer we live, the more opportunity there is for genetic mistakes to happen in our cells.

Genetic mutations happen at various times within the cell before it becomes cancerous. In some cases, people are born with one or more pro-cancer mutations already. This

doesn't mean that they will definitely get cancer, but statistically with one mutation from the start, people with mutated genes of this type are more likely to develop cancer within their lifetime (Warburg, 1926). For instance, women who carry mutations in the BRCA1 and BRCA2 breast cancer genes have a higher chance of developing breast cancer than the women who do not have mutations in these particular genes (Tan, 2014).

People with immune system problems are more likely to develop some type of cancer. People who receive organ transplants and must suppress their immune system to prevent organ rejection by taking various types of drugs, people who have Human Immunodeficiency Virus or the Acquired Immunodeficiency Syndrome, or people who are born with rare medical syndromes which affect their immunity, are at greater risk of developing cancer. The types of cancers that affect these groups of people can fall into two overlapping areas. First there are cancers that are induced by viruses. Examples for this type include cervical cancer, or other types of cancers of the anal or genital area, as well as stomach cancer, liver cancer or some lymphomas. The second type of cancers associated with immune system compromise are lymphomas. Transplanted organs or chronic infections can continuously stimulate cells to proliferate, and this continual cell proliferation means that immune cells are more likely to develop genetic errors and become lymphomas.

Viruses are responsible for causing some cancers by participating in genetic change within the cell, making it easier for the cell to become cancerous (Alvarez-Suarez, F., & Battino, 2013). Research has shown that the majority of cervical cancer (squamous cell cancer and adenocarcinoma) is mainly due to Human Papilloma Virus (HPV). There are currently more than 100 types of HPV identified, and at least 40 of them are passed on through sexual contact; at least 15 types are considered as high risk for cervical cancer (Hoff, 2012). In addition to viruses, there are also bacterial infections which are seen as cancer causing agents. In the past, bacterial infections were not considered as cancer causing agents, but investigations have shown that people with Helicobacter Pylori infection in their stomach have a higher risk or developing stomach cancer (Gribel, 1990). The bacterial infection is responsible for causing inflammation on the stomach lining, which in turn is linked with an increased risk of developing stomach cancer (Abubakar, Abdullah, Sulaiman, & Suen, 2012). Other research findings indicate that substances secreted or produced by specific types of bacteria within the digestive

system could increase the risk of developing bowel cancer or stomach lymphomas (Gribel, 1990).

DNA is constantly attacked by exogenous and endogenous agents causing DNA modifications or damages. If these DNA lesions are left unrepaired, they may contribute to mutagenesis and oncogenesis (Jaganathan S. K., 2010). Thus, DNA repair constitutes a first line of defence against cancer. Subtle variations in DNA repair capacity may be caused by commonly occurring polymorphisms in the DNA repair genes. The polymorphisms may thereby have an impact on individual genetic susceptibility to cancer.

Adverse side effects from cancer treatment

Cancer treatments vary and are mostly determined by the type of cancer they target. For instance, different stages of the cancer (spreading location, quantity, etc.), health status of the individual, age, and/or additional personal characteristics, all play a role in deciding on treatments. Cancer treatment usually includes multiple treatment sessions and a combination of palliative care and therapies will be given to the patient. Treatment usually falls into one of the following categories: surgery, radiation, chemotherapy, immunotherapy, hormone therapy, or gene therapy. Complete removal of the cancer without damage to the rest of the body is the ideal goal of treatment and is often the goal in practice. However, there is no known effective cancer treatment that can directly target the cancer cells without affecting the host. Therefore, adverse side effects are often seen in the patient during treatment.

Surgery

Surgical operations are the oldest cancer treatments. Provided that the cancer has not metastasised, it is possible to completely cure a patient by surgically removing the cancerous tissues from the body. Surgeons use the scalpels to remove a tumour and also remove a margin of healthy tissue just to make sure no malignant cells are left behind. Examples of this are seen in the removal of the testicle, prostate, or a breast. The tissue samples are sent and tested in the pathology lab, and this step requires the patient to remain under general anaesthetic for an extra 30 minutes. In helping to control symptoms such as spinal cord compression or bowel obstruction, surgery may also be instrumental. New developments such as iKnife are being developed to aid in the

surgical process. However, after the disease has spread, the down side of surgery as a treatment is that it is nearly impossible to remove all of the cancer cells. Additionally, it was also found that patients with a long anaesthetic time frame will be harder to recover from the surgery treatment. Therefore, by eliminating the surgery treatment, the recovery time from the treatment was reduced.

Chemotherapy

Chemotherapy utilises chemicals (e.g., cytotoxic, anti-neoplastic drugs) that disturb the cell division process by damaging the cellular DNA or proteins to force the cancer cells to commit suicide (Gerhauser, Alt, Heiss, & Gamal-Eldeen, 2002). These treatments target any rapidly dividing cells within the body, not just necessary to the cancer cells, and so normal cells are targeted as well. Chemotherapy is used widely in cancer treatment, especially after the cancer cells have metastasised or spread. It is a necessary treatment for some forms of leukaemia and lymphoma due to the medicines travelling around the entire body (Wilson, 2000). These treatments are given over set time periods so that the body can recover between individual doses. Unfortunately, adverse side effects are commonly seen in chemotherapy such as nausea, hair loss, vomiting and severe pain. Therefore, the use of chemotherapy as a front-line treatment option has not been favourable. A chemotherapy agent (often Cisplatin) is used to increase the sensitivity of cancer cells to radiation (Huang, et al., 2010). Cisplatin inhibits DNA synthesis by forming DNA cross-links, and Cisplatin combinations have been shown to produce the highest overall survival and complete remission rates (Huang, et al., 2010).

Radiotherapy (RT)

Radiotherapy, also known as radiation treatment, works by focusing high energy rays to destroy the cancer cells, or damage various vital molecules such as DNA which lead the cancer cells to commit suicide (Chiba, Idobata, Kobayashi, Sato, & Muramatsu, 1985). By using high-vitality gamma rays, emitted from metals such as radium, or high-vitality x-rays that are generated in a specific machine, attempts are made to either cure or improve the symptoms of cancer (Chiba, Idobata, Kobayashi, Sato, & Muramatsu, 1985). Radiation ionises water molecules to produce ions and high energy electrons which cause a cascade of further ionisation events (Kelloff & Boone, 1994). As the electrons lose their energy they are captured by molecules in the cell (mainly water molecules) to generate free radicals such as ROS (reactive oxygen species), superoxide,

hydrogen peroxide and free hydroxyl radicals. These metabolites damage chemical bonds in the cell's macromolecules, particularly those responsible for DNA damage (Campling, Pym, Baker, Cole, & Lam, 1991). The irreparable molecular breaks lead to chromosome damage and damage to the cell's mitotic apparatus resulting in mitotic catastrophe when the cell attempts to divide (Kelloff & Boone, 1994). Damaged cells can also die by apoptosis but this is less frequent than the mitotic death. However, adverse side effects were observed at early radiation treatments due to the damage of the normal and healthy tissue by the high energy beams when they passed through the area to reach the tumour (Campling, Pym, Baker, Cole, & Lam, 1991). Chemotherapy is often be used in combination with this therapy for cancer treatments.

Palliative care

Palliative care refers to treatment that attempts to make the person feel better and may or may not be combined with an attempt to treat the cancer. This is often used to help the person cope with their immediate needs and to increase the person's comfort. Unlike other treatments that are aimed at killing the cancer cells directly, the primary objective of palliative care is to improve the person's quality of life.

Colorectal cancer (CRC)

Colorectal cancer, also known as rectal cancer, colon cancer or bowel cancer, is a malignant tumour arising from the inner wall of the large intestine (colon or rectum). It is common in both males and females, and the risk of developing colorectal cancer rises after age 50. It is considered a multifactorial disease and is more commonly located in the sigmoid and rectum regions of the colon. Although hereditary predisposition is considered an important factor, 80% of colorectal neoplasms occur in the absence of a family history (Bedi, et al., 1995). A hypercaloric diet, low in dietary fiber and high in fat content is positively correlated with colorectal cancer occurrence. 95% of colorectal cancers are adenocarcinoma (epithelial cancers) that originated from the glandular tissue (Bedi, et al., 1995). Other rarer forms of colon and rectum cancers are squamous cell carcinoma and lymphoma. The main reason for death in connection with the presentation of primary colorectal cancer is complications associated with the onset of bowel obstruction (ileus). Ileus is a symptom seen in circumferentially-growing tumours, most often located in the descending or sigmoid colon, and patients may present with a dangerous ileus state as the initial symptom (Griffin, Haynes, & Levin, 1982). Tumours located in the cecum and ascending colon tend to grow outwards (exophytic growth) and patients are more likely to present symptoms of anaemia and abdominal discomfort before any acute life threatening condition (Griffin, Haynes, & Levin, 1982). However, as different endoscopy techniques (sigmoid- and colon- oscopy) are becoming more available and patients are referred at an earlier stage, most CRCs are discovered before they cause total bowel obstruction and are surgically resectable at presentation. Few patients die from complications related to the primary cancer. Furthermore, the frequency of local recurrence after resection for primary CRC is relative low (10-20%) compared to the frequency of recurrence after resection in distant organs (~55%) (Griffin, Haynes, & Levin, 1982). Local recurrence is more common after resection for rectum cancer than colon cancer, but the frequency of both has declined after the implementation of adjuvant chemotherapy and improved surgical techniques, especially after the introduction of neoadjuvant radiotherapy and total mesorectal excision (TME) of rectal cancer (Griffin, Haynes, & Levin, 1982).

Risk factors associated with CRC are either internal (genes/stimuli) or environmental. Some risk factors are known, but as indicated in the previous section, unidentified risk factors cannot be ruled out. Patients with inflammatory bowel diseases such as Crohn’s

disease and ulcerative colitis have an increased risk of developing colorectal cancer (Jemal, et al., 2011). Chronic inflammation is more likely to be the result of the risk rather than genetic pre-disposition. During the development of sporadic colorectal cancer, the occurrence of the progression from adenoma to carcinoma appears to be an inflammatory sequence called dysplasia. Another chronic inflammatory disease, Primary Sclerosing Cholangitis, is associated with colorectal cancer when accompanied with inflammatory bowel disease (Griffin, Haynes, & Levin, 1982). The development progress of the colonic neoplasia is contributed to by the pro-inflammatory factors of the congenital and adaptive immune systems.

Morphology and pathogenesis

Incidence of colorectal cancer cases is rising in NZ and is the second most common type of cancer case related to mortality in Europe, with approximately 150,000 deaths annually (Jemal, et al., 2011). The mainstay of treatment of advanced colorectal cancer has been chemotherapy with oxaliplatin or irinotecan and 5-fluorouracil. As most cancers are primarily due to environmental changes and genetic damages which affect normal cellular functions, including cell proliferation, DNA repair and programmed cell death (apoptosis), these drugs are preferred for their ability to interfere with the inner workings of the cell. Colorectal cancer occurs most likely due to dietary factors and is the most important exogenous factor identified up to now in the aetiology. More than 75-95% of colorectal cancer cases occur in people with no genetic risk, but who are identified as having high intake of fats, alcohol, and red meat (Griffin, Haynes, & Levin, 1982). Obesity, male gender and a lack of physical exercise also contribute to the majority of cases (Jemal, et al., 2011). People with inflammatory bowel diseases (ulcerative colitis and Crohn's disease) have an increased risk of developing colorectal cancer. Although people with inflammatory bowel disease account for less than 2% of annual cases, there are reports recorded where 16% from this group developed colorectal cancer or a cancer precursor after a decade, or a few decades (Jemal, et al., 2011).

There is some evidence for adverse associations of eating red and processed meat with higher risk of developing colorectal cancer (Griffin, Haynes, & Levin, 1982). The evaluated risk may due to an increased endogenous production of N-nitrosocompounds, which may enhance the colonic formation of DNA adduct O6-carboxymethyl guanine

(Griffin, Haynes, & Levin, 1982). Cooking meat at high temperature leads to the formation of polycyclic aromatic hydrocarbons and heterocyclic amines.

Most colorectal cancers are sporadic, but a significant proportion (5-6%) has a clear genetic background. Colorectal cancer is a multi-step process involving the inactivation of a variety of tumour suppression and DNA-repair genes and simultaneous activation of certain oncogenes (Collins, Jacks, & Pavletich, 1997). Epigenetic alterations through aberrant promoter methylation and histone modification have been found to play a major role in the evolution and progression of a large proportion of sporadic colon cancers (Collins, Jacks, & Pavletich, 1997). In general, colon carcinoma results from the cumulative effect of multiple sequential genetic alterations. These alterations can either be acquired, as happens in the sporadic forms, or be inherited, as in genetic cancer predisposition syndromes. In familial adenomatous polyposis and Lynch syndrome, the germ line mutation either provides the first mutation in a critical tumour-suppression gene in every cell from birth, or it creates a situation that can lead to accumulation of mutations at a greatly accelerated rate (Griffin, Haynes, & Levin, 1982).

Under normal circumstances, the turnover of cells within the gastrointestinal tract is very high, with the differentiating cells shed into the lumen and replaced every 2-7 days. Therefore, the lifespan of the cells is not sufficient to accumulate the mutations necessary for malignant change (Griffin, Haynes, & Levin, 1982). However, according to the immortal strand hypothesis, if the mutational changes first target the perpetual stem cell, then, there may be a retention of the template DNA strand within the stem cell located in the niche, which allows any DNA replication errors to pass into the differentiating, short-lived daughter cell, affording a mechanism of stem cell genome protection during cell replication (Griffin, Haynes, & Levin, 1982).

The Adenoma-Carcinoma sequence

There are numerous steps involved in the formation of tumour tissue from normal tissue through dysplasia during progression. It is estimated that a typical colorectal tumour contains at least 11,000 genomic alterations. Two distinct pathways have been suggested in colorectal carcinogenesis which involves chromosomal instability that is characterised by allelic losses in chromosome 5q, 17p, and 18q, and the other involves microsatellite instability (Rivlin, Brosh, Oren, & Rotter, 2011). Mutations in the adenomatous polyposis coli (APC) gene are found in 63% of sporadic adenomas and up

to 80% of sporadic colorectal tumours. Furthermore, mutations in the beta-catenin gene prevent the breakdown of the translated APC protein, promoting adenoma initiation (Rivlin, Brosh, Oren, & Rotter, 2011). The tumour suppressor gene, p53, located on chromosome 17p is frequently lost in colorectal malignancy (Rivlin, Brosh, Oren, & Rotter, 2011). The gene encodes for a DNA-binding phosphoprotein that prevents progress past the G_1-phase of the cell cycle if DNA damage has occurred. It is also characterised as a transcription factor, activating and promoting expression of gene involved in growth inhibition (Rao, et al., 1993). The protein p53 is involved in several essential cell functions including control of the cell cycle, DNA repair and apoptosis, and thus is called the "guardian of the genome" (Rao, et al., 1993). The half-life of wild type p53 protein and mutant p53 protein is approximately 20 minutes and 25 hours, respectively (Rivlin, Brosh, Oren, & Rotter, 2011). The extended half-life of mutant p53 allows it to accumulate in the nucleus and be over-expressed in tumours. Mutations of p53 are found in more than 50% of all human cancers and in more than 75% of colorectal adenocarcinomas (Jemal, et al., 2011). It is debated whether the DCC, "deleted in colorectal carcinogenesis", gene is a candidate tumour-suppressor gene. The DCC gene is deleted in more than 70% of colorectal carcinomas (Iozzo, 1984). A second candidate tumour-suppressor gene, DPC4/Smad4, located in the same region on 18q21, is deleted in up to a third of the cases (Iozzo, 1984). The SMAD protein family consists of intracellular proteins that mediate the effects of signalling from extracellular transforming growth factor beta (TGF-β) and TGF-β-related factors.

Microsatellite instability is explained by defects in DNA mismatch repair genes, encoding proteins involved in recognition and repair of single base lesions and larger strand slippage mismatches in DNA replication (Rivlin, Brosh, Oren, & Rotter, 2011). In sporadic colorectal cancer the described instability usually arises due to epigenetic silencing of the DNA mismatch repair gene *MutL homologue 1* (MLH1) by methylation of cytosine and guanine residues in Cp-G-rich promoter regions, which prevents the gene-regions from being transcribed (Rivlin, Brosh, Oren, & Rotter, 2011). Microsatellite instability causes Lynch syndrome primarily by a germ line mutation in the mismatch repair genes *MutS homologue 2* (MSH2) and MLH1. The lifetime risk of developing colorectal cancer is up to 75% higher in children with Lynch syndrome compared with the general population (Triantafillidis, Nasioulas, & Kosmidis, 2009). Approximately 70% of large bowel tumours in patients with Lynch syndrome arise in the right/proximal colon. On the other hand, mutation at the tumour suppressor gene

adenomatous polyposis coli (APC) impairs the function of APC protein (Triantafillidis, Nasioulas, & Kosmidis, 2009). In most of the cases, the initial mutations occur at the APC tumour suppressor gene locus (5q21-22). Loss of the APC tumour suppressor gene is thought to be one of the first genetic changes in colorectal adenoma development. APC is an essential component of a destruction complex in the Wnt pathway involved in the binding and down-regulation of beta-catenin, which prevents excessive cell proliferation, regulation of apoptosis, cell-cycle progression and chromosomal stability (Triantafillidis, Nasioulas, & Kosmidis, 2009).

Statistical data and epidemiology of colorectal cancer in New Zealand

Fifteen (15)% of worldwide cancer malignancies are due to colorectal cancer (Jemal, et al., 2011). Colorectal cancer is the third leading cause of cancer in men (10% of total) and the second cause of cancer (9.4% of total) in women after the leading cause, breast cancer. There were 21,235 total cancer cases (including colorectal cancers) registered in New Zealand in 2010, which was an increase of 18.7% from 2000 to 2010. 52.1% of the cancer registrations in 2010 were male. Cancer was the leading cause of death for both male and female in 2010 in NZ, accounting for nearly a third of all deaths (Ministry of Health, 2013). The number of people who suffered from cancer has gradually increased throughout the years. In 2011, 22,000 cancer cases were registered; in 2013, there were about 8500 new cases diagnosed with different types of cancer in NZ, and the number is still climbing in 2015 (Ministry of Health, 2013). In 2008, the Ministry of Health announced that nearly 1 in every 3 deaths were due to cancerous diseases. These data show that cancerous diseases are increasing and will slowly become the population's primary cause of death in the future (Ministry of Health, 2013).

Melanoma, lung cancer and breast cancer were the most serious types of cancer, and also the most common cause of death in registered cancer cases for both Maori and non-Maori population in NZ, as reported by The Ministry of Health in NZ in 2013. Colorectal cancer was a new type of cancer registered in NZ and incidence rates of colorectal cancer in NZ rank among the highest worldwide. In 2005 there were 2,716 colorectal cancer cases reported, and 1,222 deaths were recorded, contributing to 45% of the CRC death rates (Ministry of Health, 2013).

Table 1: Rankings of major cancer statistic count in 2004/2008 and projected 2014/18 in NZ (Ministry of Health, 2010).

Rankings of Major Types of Cancer from 2004/2008 and projected 2014/18		
Rank	Registrations	
	Male	Female
1	Prostate	Endometrium
2	Liver	Kidney
3	Oesophageal	Lung
4	Non-Hodgkin Lymphoma (NHL)	Melanoma
5	Melanoma	Breast
6	Leukaemia	Oesophageal
7	Brain	Pancreatic
8	Colorectal	Colorectal
9	Kidney	Bladder
10	Lung	Cervix

Lung cancer, colorectal cancer, and melanoma cancer were the top ten types of cancer affecting the NZ population, according to 2011 statistical data from the Ministry of Health (Ministry of Health, 2011). Cervical cancer and breast cancer are the leading types of cancerous diseases that affect the NZ female populations. Of note, these types of cancer cases are expected to be the major percentage of registered cancer cases in NZ in the near future.

Suggestion on anti-proliferative effect of honey

Honey has long been utilised for medical needs, but only recently the cancer prevention properties of honey have come into the spotlight. Anti-oxidant properties have an impact against various diseases, for example, coronary diseases, neurologic degeneration, inflammatory disorders, cancer and aging. This has prompted a quest for substances rich in anti-oxidants. In fact, chemo-prevention utilises different dietary agents which are rich in phyto-chemicals that work as anti-oxidants. With expanding interest in anti-oxidant-rich substances, honey has received more research consideration since it is rich in phenolic compounds and additional anti-oxidants, as well as amino acids, ascorbic acid, and proteins (Wahdan, 1998). Some simple and straightforward polyphenols were found in honey: chrysin, quercetin, caffeic acid, caffeic acid and phenyl esters, galangin, acacetin, pinobanksin, pinocembrin, kaempferol, and apigenin.

All of these phenolic compounds and anti-oxidants have advanced as promising pharmacologic reagents in terms of cancer treatment (Al-Waili, Salom, & Al-Ghamdi, 2011).

Chrysin has also been found in honey, and acts as a bio-active compound. Chrysin has been used as a cancer prevention agent, in a comparative manner to anastrozole, which is used to treat conditions such as inflammation and anxiety. As mentioned above, honey is also known as a dietary source for flavonoids, which have been exhibited to have anti-inflammatory and anti-carcinogenic activities (Chan, Deadman, Manley-Harris, & Wilkins, 2013). Although some authors have reported that crude honey may exhibit proliferative agents that increase proliferation of both malignant and normal cells, a promising anti-tumour reagent with emphatic anti-metastatic effect was also reported. The nutritional effect on tumour cells was suggested due to the proliferative effect of honey rather than a carcinogenic effect. Therefore, the anti-tumour effect was reported to be the result from many activities, for example, the inhibition of DNA synthesis with no sign of cytotoxicity, and the down-regulation of MMP-2 and MMP-9, both of which have been implicated in the induction of the angiogenic switch in different model systems (Othman N. H., 2012; Saxena S., 2012).

Honey has become a natural product under intense scientific investigation in recent years due to its healthcare applications and its observed active properties. Some literature suggests that honey may induce apoptosis in various types of cancer cell lines (Zhang, Zhao, & Wang, 2008). Honey was reported to reduce the mitochondrial membrane potential of breast and colorectal cancer cells. A significant increase in the ROS generation of honey-exposed colon cancer cells was found (Wang, et al., 2011). Further, levels of thiols were altered with induction of p53 and poly ADP-ribose polymerase cleavage in colon cancer cells. Honey-induced apoptosis was accompanied by cell cycle arrest in sub-G1 phase of the cancer cells (Othman N. H., 2012). Increase in the level of caspase-3 was reported in colon, breast and liver cancer cells (Othman N. H., 2012). Moreover, inflammatory cytokines like TNF-α, NF-kB and IL-1β were also involved in honey-induced apoptosis.

One of the most commonly reported phenolics of honey is caffeic acid which is known to suppress liver metastasis through inhibition of the NF-kB and MMP-9 enzyme activity (Green & Kroemer, 2004). Caffeic acid-induced apoptosis involves the

indispensable ROS-mitochondrial pathway in colorectal cancer cells; having a second example: chrysin, work as another phenolic other than caffeic acid, chrysin-generated apoptosis through caspase-3 activation, downregulation of Akt and p38-MAPK in cancer cells (Green & Kroemer, 2004). Another important constituent of honey, apigenin, resulted in the activation of Bax and Bim and downregulation of Akt in cancer cells (Gratzner & Leif, 1981). Quercet was found to inhibit the leukemia cancer cells through suppressing protein kinase C and membrane tyrosine protein kinase *in-vitro* (Gratzner & Leif, 1981). It also reduced the expression of c-Myc and K-ras oncogenes (Cunning & Noguchi, 1983). Acacetin-induced apoptosis is accompanied with activation of Bax and p53, whereas galangin resulted in activation of caspase-3 and increased accumulation of cells at sub-G_1 phase (Cunning & Noguchi, 1983; Omotayo O. Erejuwa, 2014). Pinocembrin treatment was found to increase caspase-3 and also simultaneously activated Bax and cytochrome-C in colon cancer cells (Green & Kroemer, 2004).

Honey

Honey is a natural substance produced by honey bees, usually from the nectar of flowers and honeydew excreted by scale insects (Orsolic, Bee Honey and Cancer, 2009). Honeybees make honey as a food storage to use during winter and this has been exploited by humans since ancient times (Orsolic, Bee Honey and Cancer, 2009). Beekeeping has been documented as far back as the Ancient Egyptians (2000-5000 years ago) who used honey as a medicine, as a food source and in embalming. Honey is the only known natural food which will never spoil and the only sweetening material which requires no processing to render it ready for consumption. Raw honey can be eaten straight out of the comb taken from the beehive.

Honey is a saturated solution of sugar which is made from nectar collected from flowers by bees. The nectar is mixed with enzymes in the beehives and is placed in wax cells. A ripening process follows where the enzymes (invertases) convert the sucrose into glucose and fructose and the overall water content is reduced. As well as sugars, honey contains small quantities of enzymes, amino acids, vitamins, minerals and other organic acids, the exact composition of a specific honey is variable and depends in the geographical location and floral source of the nectar.

Manuka Honey

Manuka honey is derived from the nectar of the Manuka tree, *Leptospermum scoparium*, a native of New Zealand (Erejuwa, Sulaiman, & Wahab, 2012). This nectar is produced in the flowers of the Manuka tree, which are generally white in the wild and 10 to 12 mm across in diameter. Flowering takes place between September and February. The Manuka plant is often colonised by scale insects which feed off the phloem of the tree and excrete honeydew. This encourages black sooty mould to grow on the bark, giving it a dark colouring (White, 1978). Honeybees can use this honeydew as a raw material for the production of honey and indeed, honeybees have been observed harvesting honeydew from scale insects on Manuka trees. However, it is unclear as to whether this honeydew is incorporated into Manuka honey. Honeydew honeys have a very complex oligosaccharide composition due to transglucosylation of the sugars by enzymes in the scale insects' gut. It has been shown that while Manuka honey does have higher levels of complex sugar (10%) than most floral honeys (1.5%), it clearly lacks the variety of oligosaccharides found in honeydew honeys such as Beech honeydew honey. This suggests that the sugars used to produce Manuka honey are predominantly floral in origin rather than from honeydew. Previous studies have also shown that the content of Manuka honey contains more phenolic and flavonoid antioxidants in comparison to other types of honey worldwide (White, The protein content of honey, 1978). Since the antibacterial properties are well characterised, Manuka honey has an additional antibacterial component which is referred to as the "unique Manuka factor" (UMF), now known to be "methylglyoxal" (MGO).

Thyme Honey

Thyme Honey is produced from the herb "Common Thyme" (*Thymus vulgaris*) within a small area in central Otago on New Zealand's South Island, around the town of Alexandra (Coulston, 2000). It is believed this is the main population of Thyme developed as a wild strain outside of its characteristic scope of nations circumscribing the Mediterranean. Thyme was brought to the South Island by excavators amid the gold rush of the late 1800s, who utilised it as an herb and restorative plant. As the gold mining diminished, Thyme gradually spread to the dry atmosphere of Central Otago. In spring, usually around late October and November, Thyme Honey is produced with the plant flowering. This spring honey crop tends to leads to honey being packed around the beehive's "broodnest" and in that capacity is frequently stored in close proximity to

spring pollen (Coulston, 2000). During extraction this may become a source of extraneous pollen which is not associated with the nectar producing the honey.

Thyme Honey is probably New Zealand's strongest flavoured honey. It belongs to the mint family, and the flavour and aroma of Thyme honey are pungent, distinctive and herbal. Beekeepers' honey extraction plants in the Thyme area have an unmistakable smell to them, even long after the Thyme crop has been extracted. Thyme honey is extracted from the honey frames at temperatures less than 37ºC (less than 99ºF). This is in line with the organic standard. The reason for this low temperature during honey extraction is to keep alive all the naturally occurring enzymes in the honey. This ensures truly raw and natural honey. Honey that has been heated above 45ºC (113ºF) rapidly starts to degrade, as the enzymes become denatured and other chemical reactions cause the production of toxins (Coulston, 2000). For this reason it is important to source "raw" honey that preserves the health-giving properties of that particular honey type.

Medicinal Properties of Honey

The use of honey as a physical barrier in injury treatment and anti-inflammatory effects are well studied and recorded worldwide. However, the potential knowledge of its specificity used as an anti-cancer reagent is limited. Honey contains phenolic compounds which have anti-oxidant and free radical scavenging abilities. Free radicals cause damage and prevent healing in areas of prolonged inflammation.

Flavonoids are a class of natural products derived from plants which are incorporated into honeys through propolis, nectar and pollen (Joganathan & Mandal, 2009). The flavonoids are a large and diverse group of phenolics with a basic C_6-C_3-C_6 structure (Joganathan & Mandal, 2009). The two C_6 aromatic rings are designated ring A and ring B with the C_3 unit bridging them (Joganathan & Mandal, 2009).

The main function of flavonoids in plants seems to be as a pigment, providing colour to the plant's flowers to attract pollinators. The strong light absorbance of flavonoids in the ultra-violet region also allows them to act as a protective screen against harmful UV-B radiation (Joganathan & Mandal, 2009). Flavonoids can also act as protective agents against a number of attacking organisms including viruses, bacteria, fungi, herbivorous animals (both vertebrate and invertebrate) and even encroaching plants. This protection is afforded by both allelopathic and phytoalexin mechanisms.

Allelopathic flavonoids produced by certain species of plants such as ferns, inhibit the germination and growth of encroaching plants. Plants are also known to produce flavonoid phytoalexins in response to infection (Ward, 2000). The flavonoid phytoalexins produced are antibiotic and help fight off the invading pathogen. Flavonoids are also known to alter gene expression and inhibit energy transfer in plant cells.

Table 2: The merits of honey (Al-Waili, et al., 2011).

Merit	Component	Function
Supporting the immune system	Antioxidants	Such as flavonoids and aromatic acids which reduce inflammation and cell damage.
	Unknown components	Natural honey contains raw materials which lower the production of prostaglandins which play a role as immunosuppressant and a critical role in cancer development.
	L-arginine	Enhances nitric oxide production which plays role in the immune response.
Killing factors	Hydrogen peroxide	A strong killing factor that is generated by glucose oxidase which is added by bees to nectar.
	Methylglyoxal	Non-peroxide honey such as Manuka honey contains such a unique killing factor.
	Osmolarity	High sugar content ties up water molecules so that microorganisms have insufficient water to grow.
	Acidity	In acidic environment (pH generally between 3.2 and 4.5) most microorganisms cannot grow.
Wounds healing	Physical barrier	When covering wounds with honey, a thick layer is developed which is very difficult to penetrate by environmental contaminants; this creates a moist wound healing environment.
	Nitric oxide	Cell proliferation, wound contraction and collagen formation are regulated by the inducible isoform (iNOS) of Nitric Oxide which is synthesized in the early phase of wound healing by macrophages (Witte and Barbul, 2002).
	Hydrogen peroxide	Vascular endothelial growth factor (VEGF) is an important angiogenic factor for wound healing. H_2O_2 induces the VEGF promoter in macrophage through an oxidant which results in increasing in VEGF production.
Nutrients	Glucose Fructose Vitamin Antioxidants amino acids	Honey contains such compounds which play role in its activity and potency

One of the most prominent and medically-useful properties of the flavonoids is their ability to scavenge free radicals. Free radicals are highly reactive oxidising species such as hydrogen peroxide radical and the hydroxyl radical. While these free radicals are produced during important physiological processes such as respiration and the immune

response, left unchecked they can cause extensive cellular damage (Erejuwa, Sulaiman, & Wahab, 2012). Flavonoids can scavenge these free radicals, reducing them to non-reactive species while being oxidised themselves in the process (Al-Waili, Salom, & Al-Ghamdi, 2011).

Flavonoids have been reported to process anti-inflammatory activity, oestrogenic activity, enzyme inhibition activity, anti-microbial activity, anti-allergic activity, anti-oxidant activity, vascular activity, antiviral, anti-thrombotic, anaesthetic activity and cytotoxic anti-tumour activity (Ghashm, et al, 2010). These activities give flavonoids the potential to prevent cancer formation or even the treatment of it, also with many other diseases such as cardiovascular disease, hypertension, duodenal ulcers, allergies, gastric ulcers, vascular fragility, diabetes, viral and bacterial infections. Recently, accumulating reports suggested that honey being rich in flavonoids and poly-phenols, has potential of anti-proliferative effects against various types of cancer cells (Ghashm, et al., 2010).

Table 3: Common Phenolic Compounds in Honey *(Fauzi, Norazmi, & Yaacob, 2011)***.**

Phenolic Acid	Flavonoid
Vanillic acid	Tricetin
p-coumaric acid	Isohamnetin
Gallic acid	Quercetin
Ellagic acid	Kampferol
Syringic acid	Luteolin
Chlorogenic acid	Pinocembrin
Ferulic acid	Chrysin
Caffeic acid	Fgalangin
	Pinobanksin
	Myricetin
	Apigen
	Genisten

Possible anti-cancer effects of honey

There is an increasing number of reports of natural products inhibiting tumour cell growth and metastasis, as well as those that induce apoptosis such as honey (Fukuda, et al., 2011). These reports provide chances of curing human tumours with less side effects. Various signalling pathways, including stimulation of the release of tumour necrosis

factor (TNF)-α, cell proliferative inhibition, induction of the cell cycle arrest and apoptosis, as well as the lipo-protein oxidation inhibition, intercede the beneficial effects imposed by honey and its major components such as chrysin and other flavonoids (Fukuda, et al., 2011). For instance, Tualang honey components exert anti-proliferative effects against breast cancer tissues, weakening tumour cell proliferation in MCF-7 and MDA-MB-231 cell lines (Fukuda M. , Kobayashi, Hirono, Miyagawa, & Ishida, 2009). These anti-neoplastic effects are regulated by caspase-2 and caspase-9 activation and a reduction of the mitochondrial membrane potential in cancer cells, reflecting an increase in apoptotic events. Administration of honey also induces early apoptosis in osteosarcomas in a relative dose-dependent manner and attenuates proliferation in HeLa cell lines. Apoptosis is also enhanced in oral squamous cell carcinomas following exposure to honey (Wang, et al., 2011).

As mentioned above, the induced apoptosis activity by Tualang honey was due to the increased leakage of lactate dehydrogenase and reduced mitochondrial membrane potential, however, the authors had also found that Tualang honey had exerted no cytotoxic effect in MCF-10A (a normal breast cancer cell line) (Ayyildiz, et al., 2007). This suggested the cytotoxic effect of Tualang honey is selective and specific to the breast cancer cell lines (Yaacob, Nengsih, & Norazmi, 2013). This is important because specificity and selectivity are the key characteristics of a good chemotherapeutic agent. Other studies had reported that the Indian honey also showed cytotoxic effects on a breast cancer cell line (MCF-7), and these studies reveal that honey is able to exert cytotoxic effect in both MCF-7 and MDA-MB-231 cell lines, which are oestrogen receptor positive and oestrogen receptor negative, respectively (Candiracci, et al., 2012).

Multiple proteins are involved in the adhesion of cancer cells to the extracellular matrix, and their over-expression has been linked to the initiation and progression of cancer metastasis. For instance, some studies have previously reported that apigen and quercetin, both honey-derived flavonoids, could inhibit melanoma lung metastasis through the inhibition of VCAM-1 expression (Yaacob, Nengsih, & Norazmi, 2013). Hassan *et al.* (2012) reported that nitric oxide (NO) production and decreased anti-oxidant status caused increased hepatocellular carcinoma cell proliferation, and that honey exhibited cytotoxity through a reduction in NO and restoration of anti-oxidant status (Yaacob, Nengsih, & Norazmi, 2013). Further, the authors demonstrated that a reduction in ROS activity could inhibit the Matrix metalloproteinase-9 activity, and

therefore might provide a mechanism of action for the inhibition of metastasis. Abdel Aziz *et al.* (2009) and others (Alzahrani, et al., 2012) demonstrated that honey had anti-metastatic effects in HepG2 hepatocellular carcinoma cells, due to the inhibition of Matrix metalloproteinase proteolytic and gelatinolytic activity. In addition, the honey-derived flavonoid quercetin has been shown to decrease MMP-2 and MMP-9 expression in PC3 prostate cancer cells (Bishayee, et al., 2013)(Vijayababu *et al.*, 2006). Together, these findings suggest an increased benefit of honey for the treatment of advanced or highly metastatic cancer types over early stage tumours.

Table 4: Effects of honey on the progression of cancer and tumour cells *(Erejuwa, Sulaiman, & Wahab, 2012)***.**

Type of tumour/ cancer and cancer cell type	Effects of honey (key findings)
***In vitro* studies**	
Human breast cancer (MCF- 7 & MDA-MB-231)	Antagonizes estrogen activity, inhibits cell proliferation, induces apoptosis, reduces mitochondrial membrane potential
Human Liver cancer (Hep G2)	Inhibits cell proliferation, suppresses angiogenesis, induces apoptosis, protects against mutagen- induced DNA damage
Human colorectal cancer (HT 29, HCT 15 & CT 26)	Inhibits cell proliferation, induces apoptosis, arrests cell cycle, reduces mitochondrial membrane potential, increases generation of ROS, depletes intracellular non- protein thiols, induces DNA damage, suppresses inflammation
Human prostate cancer (PC-3)	Inhibits cell proliferation, induces apoptosis
Human bladder cancer (T24, 253J, RT4 & MBT-2)	Inhibits cell proliferation
Human kidney cancer (Renal cancer cell line)	Induces apoptosis
Human oral cancer (Oral carcinoma)	Inhibits cell proliferation
Human bone cancer (Osteosarcoma)	Inhibits cell proliferation
Human skin cancer (Melanoma cells)	Inhibits cell proliferation, arrests cell cycle
Human leukaemia	Induces apoptosis
Human endometrial cancer	Inhibits cell proliferation
Human lung cancer (NCI-H460)	Inhibits cell proliferation
Human cervical cancer	Induces apoptosis, disrupts mitochondrial membrane potential
***In vivo* studies**	
Walker 256 carcinoma	Inhibits cell proliferation, arrests cell cycle, induces apoptosis
DMBA- induced breast cancer in rats	Delays the development of tumour, reduces the number of size of tumours, prevents the development of high grade cancer
Rats with DEN- induced hepatic cancer	Protects against transformation of normal liver cells to neoplastic hepatic cells, restores the PCNA and p53 expression
Mice/ rats with colon carcinoma or adenocarcinoma	Inhibits formation of metastases and tumour growth
Mice implanted with bladder cancer cells	Inhibits tumour growth
Mice with melanoma	Inhibits tumour growth, induces apoptosis

Cell cycle arrest

The cell cycle is a series of consorted events in which cell proliferation is closely regulated. Four sequential phases contribute to cell proliferation: G_1, S (DNA

replication), G_2 and M (cell division) (Wang, et al., 2000). This cell cycle event is under the control of several protein kinases and signalling checkpoints, such as the progression towards mitosis or withdrawal from the cell cycle into a quiescent stage (G_0) during G_1 phase (Wang, et al., 2000). The cell cycle is deregulated and results in uncontrolled cell proliferation in cancer cells. The application of honey-induced cell cycle arrest has been documented in some studies. For instance, cell cycle arrest in the sub-G_1 phase is recorded in bladder cancer cell lines; Aliyu and colleagues (2013) had found that the cytotoxic effect of honey against the NCI-H460 non-small lung cancer cell line was regulated through G_0/G_1 cell cycle arrest (William & Stoeber, 2012). Data suggested that cell cycle arrestment from honey is due to the different flavonoids and phenolic compounds in honey, and the viability (%) of the cancer cells depends on the levels of the honeys' phenolic content (William & Stoeber, 2012).

Every one of the occurrences in the cell cycle are overseen and controlled by several distinct proteins. The control panel of the cell cycle consists of cyclins and cyclin-dependent kinases (CDK). The G_1/ S stage transition is an essential administrative point where cell's density is bound for proliferation, quiescence, differentiation and apoptosis (Collins, Jacks, & Pavletich, 1997). Over-expression and dys-regulation of the cell cycle development components, for example, cyclin D1 and cyclin-dependent kinases are connected with tumourigenesis. The loss of this regulation is compromised to the sign of cancer. The nuclear protein, Ki-67, is a novel marker to test the "growth fraction" of cell proliferation. It is absent in the resting phase (G_0) but expressed during the cell cycle in all the proliferation phases (G_1, S, G_2, and mitosis) (Collins, Jacks, & Pavletich, 1997).

Honey and its other compounds (like flavonoids and phenolic components) are reported to be able to block the cell cycle of colon, glioma, and melanoma cancer cell lines in G_0/G_1 phase (Collins, Jacks, & Pavletich, 1997). This inhibitory effect on tumour cell proliferation resembles the down-regulation of many other cellular pathways involving ornithine decarboxylase, kinases and tyrosine cyclo-oxygenase. The results of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the trypan blue exclusion assays have affirmed that the anti-proliferative impact of honey is in a dose- and time- dependent measurement pattern (Iozzo, 1984). Honey or its other components inhibit cell growth due to its perturbation of the cell cycle. The cell cycle is also monitored by p53 which is a cell cycle checkpoint protein that is expressed as a tumour

suppression gene. Honey has been reported to be involved in the modulation of p53 regulation (Wang, 等, 2011). The anti-oxidant and anti-inflammatory properties of honey in treatment of colon cancer cells has been well explained by its anti-proliferative activity.

The Anti-proliferative effect exerted in HCT-15 and HT-29 colorectal cancer cells with honey treatment in other studies when assessed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide MTT cell viability assay (Fukuda M. , Kobayashi, Hirono, Miyagawa, & Ishida, 2009). Flow cytometry analysis showed an increasing accumulation of hypodiploid nuclei in the sub-G_1 phase of the cell cycle indicating apoptosis (Farr, 2005). Honey transduced the apoptotic signal via initial depletion of intracellular non protein thiols, consequently reducing the mitochondrial membrane potential (MMP) and increasing the reactive oxygen species (ROS) generation (Joganathan & Mandal, 2009). As a results, an increasing earlier lipid layer break was observed in the treated cells in comparison to the control.

Activation of the mitochondrial pathway and induction of MOMP

Cytochrome C, is an intermediate in the apoptosis processes that are a controlled form of cellular death which occur in response to infection or DNA damage (Vogelstein, Sur, & Prives, 2010). Cytochrome C is located within the intermembrane mitochondria space and is released out of the mitochondria to the cytosol to initiate apoptosis after binding with cardiolipin. Induction of mitochondrial outer membrane permeabilization (MOMP) is essential prior to the release of Cytochrome C (Warburg, 1926). These reactions are known as the intrinsic pathway, also called the mitochondrial pathway. Agents such as honey which are rich in flavonoids and phenolic compounds are capable of activating the mitochondrial pathway, and the release of cytochrome C cascades are considered as potential cytotoxic agents in some studies (Zhang, Zhao, & Wang, 2008). Other studies have shown that honey induces mitochondrial membrane permeabilisation in various cancer cell lines via reduction of the mitochondrial membrane potential.

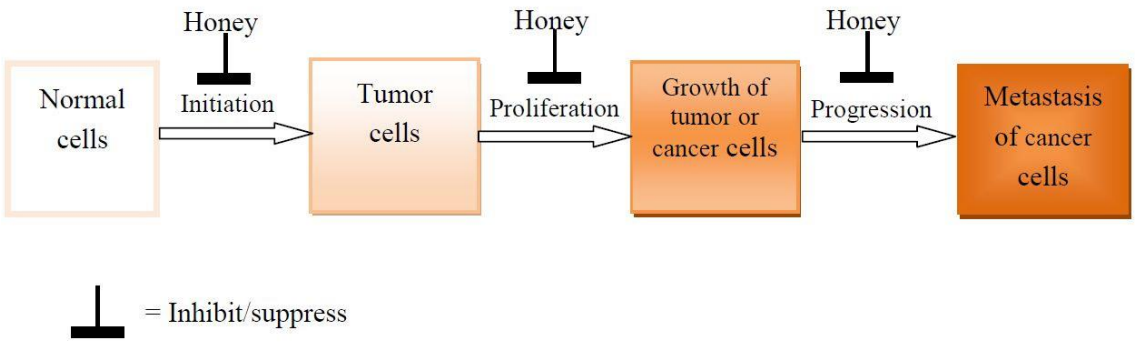


Figure 1: Honey blockage of the three stages of carcinogenesis. Honey is characterised as participating in the inhibition/suppression of initiation, proliferation, and progression of carcinogenic stages. *(Erejuwa, Sulaiman, & Wahab, Honey - A Novel antidiabetic agent, 2012).*

The mechanism of apoptosis

Apoptosis is a programmed cell death which helps to regulate cell growth and eliminate damaged cells. Several apoptotic pathway involves MOMP which then leads to the release of IMS pro-apoptotic proteins such as cytochrome C (Jaruga, Sokal, Chrul, & Bartosz, 1998). The IMS pro-apoptotic proteins then activate the caspase cascade, resulting in a mitochondrial dysfunction and eventually leads to cell death (Jaruga, Sokal, Chrul, & Bartosz, 1998). Treatment of cancer cells with honey were shown to induce cell death in an apoptotic pathway in breast cancer cells via the induction of caspase- 3/ -7 and -9 activation (Crow, Mani, Nam, & Kitsis, 2004). Honey was also recently reported to enhance tamoxifen-induced apoptosis by caspase- 3/ -7, -8 and -9 activation (Bishayee, et al., 2013). The effect of honey has also been demonstrated on several enzymes, genes and transcription factors that are related to apoptosis. Colorectal cancer cell lines HCT-15 and HT-29 treated with honey appeared to down-regulate PARP expression (Fukuda, et al., 2011). The PARP is an enzyme that plays a vital role in apoptosis and DNA repair. As a result, the inhibition of PARP activity by honey will prevent DNA repair and thereby honey contribution to the increased cytotoxicity in cancer cells (Crow, Mani, Nam, & Kitsis, 2004). The study further revealed that honey treatment induced or activated caspase-3, p53 and Bax expression while it down-regulated Bcl-2 expression (Crow, Mani, Nam, & Kitsis, 2004). Honey has also been shown to exert anti-mutagenic effect by inhibiting error-prone repair pathway (Crow, Mani, Nam, & Kitsis, 2004). Other studies have also demonstrated that the anti-neoplastic effect of honey was mediated via restoration of p53 gene expression (Collins, Jacks, & Pavletich, 1997). For instance, the apoptotic effect on cancer cells induced by

Manuka honey was due to the induction of caspase-9, which in turn activates caspase-3, an executor protein (Mandal, 2009). Apoptosis induced by Manuka honey has also involved in the induction of DNA fragmentation, activation of PARP, and loss of Bcl-2 expression (Mandal, 2009). The apoptotic property of honey makes it a very possible natural substance as an anti-cancer reagent as many of the existing chemo-therapeutics currently used are apoptosis inducers (Mandal, 2009).

As mentioned above, the apoptotic effect of honey was found to be mediated via the activation of caspase-3, caspase-9 and down regulation of the Bcl-2 expression (Crow, Mani, Nam, & Kitsis, 2004). Breast and pancreatic cancer cell inhibition were introduced by Quercetin, it induces apoptosis via down-regulation of Bcl-2 expression and up-regulation of Bax expression (Collins, Jacks, & Pavletich, 1997). Recent evidence has disclosed that chrysin is a key constituent in honey that exerts anti-metastatic effect in human breast cancer cells (Swellam, et al., Antineoplastic activity of honey in an experimentl bladder cancer implantation model: In vivo and in vitro studies, 2003). Similarly, chrysin was shown to induce apoptosis through caspase-3 and Bax activation in B16-F1 and A375 melanoma cells (Salah, et al., 1995). Bcl-2 is an anti-apoptotic protein that is commonly over-expressed in many types of cancer formations. In contrast, Bax is a pro-apoptotic protein. Characterised de-regulation or impairment of apoptosis resulting from down-regulation of pro-apoptotic proteins and/ or up-regulation of anti-apoptotic proteins have generally been found in many cancer cells (Satomi & Nishino, 2009). Therefore, these findings disclose that honey induces cancer cell death or apoptosis via activation of a caspase cascade, induction of p53, and up-regulation of pro-apoptotic proteins – such as Bax -- and down-regulation of anti-apoptotic proteins such as Bcl-2. The data also point to the role of honey flavonoids in the apoptotic effect of honey in cancer cells (Jeddar, Khassany, Ramsaroop, Bhamjei, & Moosa, 1985).

Two characteristics of cancer cells are uncontrolled cellular proliferation and inadequate apoptotic turnover. Drugs which are commonly used for cancer treatment are apoptosis inducers. Programmed cell death or apoptosis is categorised into three phases: (a) an induction phase, (b) an effector phase, and (c) a degradation phase (Jaruga, et al., 1998). The induction phase stimulates pro-apoptotic signal transduction cascades through death-inducing signals (Jaganathan S. K., Growth inhibition by caffeic acid, one of the phenolic constituents of honey, in HCT 15 colon cancer cells, 2012). The effector phase

is committed to bring cell death via a key regulator, the mitochondrion (Jaganathan S. K., Growth inhibition by caffeic acid, one of the phenolic constituents of honey, in HCT 15 colon cancer cells, 2012). The last degradation phase comprises nuclear and cytoplasmic events. Nuclear change includes chromatin and nuclear condensation, cell shrinkage, DNA fragmentation, and membrane blebbing (Jaganathan S. K., Growth inhibition by caffeic acid, one of the phenolic constituents of honey, in HCT 15 colon cancer cells, 2012). In the cytoplasm, a complex cascade of protein cleaving enzymes called caspases is activated. The cell is finally destined to become fragmented apoptotic bodies which are phagocytosed by macrophages or other surrounding cells (Jaganathan S. K., Growth inhibition by caffeic acid, one of the phenolic constituents of honey, in HCT 15 colon cancer cells, 2012).

The apoptosis usually follows the caspase-8 or death-receptor pathway and caspase-9 or mitochondrial pathway (El-Kott, Kandeel, El-Aziz, & Ribea, 2012). One of the mechanisms by which chemotherapy and radiotherapy cause cancer cell death is the activation of the mitochondrial pathway. Mitochondrial membrane permeabilization is an early event in the mitochondrial pathway, also known as intrinsic pathway (Jaruga, et al., 1998). The mitochondrial pathway involves a series of interactions between several stimuli such as nutrients, physical stresses, oxidative stress and damage, during which several protein (such as cytochrome C) usually located in the intermembrane mitochondria space (IMS) become released resulting in cell death. Therefore, compounds or agents such as honey that are rich in flavonoids are capable of activating the mitochondrial pathway and causing the release of cytochrome C. On the other hand, honey can also induce apoptosis via depolarization of the mitochondrial membrane by elevating caspase-3 activation and PARP cleavage in human colon cancer cell lines. This effect is attributed to honey's high phenolic and tryptophan content (El-Kott, Kandeel, El-Aziz, & Ribea, 2012). Honey generates reactive oxygen species resulting in the activation of p53, and p53 in turn modulates the expression of pro- and anti-apoptotic proteins like Bcl-2 and Bax (El-Kott, Kandeel, El-Aziz, & Ribea, 2012). Honey as an adjuvant therapy with *Aloe vera* boosts the expression of pro-apoptotic protein Bax and decreases the anti-apoptotic Bcl-2 expression in Wistar rats (El-Kott, Kandeel, El-Aziz, & Ribea, 2012).

Honey and its effect on tumour necrosis factor (TNF)

Tumour necrosis factor (TNF) has both positive and negative roles in inflammatory diseases. TNF-α has also been shown to be involved in host defence mechanisms as a key cytokine, however TNF also mediates tumour initiation, promotion, and progression (Jaruga, et al., 1998). The ability of TNF to activate NF-kB is part of its link to a pro-inflammatory effect in many diseases. Activation of NF-kB leads to the expression of inflammatory genes like LOX-2, COX-2, as well as cell-adhesion molecules, chemokines, iNOS, and inflammatory cytokines. As a result, TNF is considered to be a growth factor for many of tumour cells, and can play a key role in regulating important cellular processes.

Regulation of cellular processes can arise from stimulated release of TNF. Royal jelly proteins (apalbumin-1 and apalbumin-2) in honey have anti-tumour properties, and pasture, jelly bush, and Manuka honeys (at concentrations of 1% w/v) stimulate monocytes to release tumour necrosis factor-alpha and interleukin- (IL-) 1β and IL-6. The possible mechanism arising from the TNF release involves receptor binding and signal cascades which further regulate apoptosis and inflammation.

Suppressive of Tumour Necrosis Factor (TNF)

Cancer formation is linked to chronic inflammation, where prolonged and excessive inflammatory responses damage tissues by preventing it from healing. In literature reviews, honey exhibits anti-inflammatory responses when applied in cell cultures, animal models and clinical trials (Chien, et al., 2009). Various types of chemicals and biological agents induce inflammatory processes by activating pro-inflammatory cytokines or enzymes. The catabolic reaction carried out by the cyclooxygenase (COX)-2 enzyme catalyses the metabolism of arachidonic acid to prostaglandins (Chien, et al., 2009). In many cases, anomalous arachidonic acid metabolism is usually involved in inflammation and carcinogenesis. COX-2 is over-expressed in pre-malignant and malignant status. Phenolic compounds in honey are responsible for the anti-inflammatory activity. The mechanism involves the suppression of the pro-inflammatory activities of COX-2 and/ or inducible nitric oxide synthase (iNOS) through these phenolic compounds or flavonoids (Vidya Priyadarsini, et al., 2010). Therefore, honey and its components have been documented to be involved in

regulation of proteins such as ornithine decarboxylase, tyrosine kinase, iNOS, and COX-2.

Manuka honey has also been found to increase IL-1β, IL-6 and TNF-α production (Alvarez-Suarez, F., & Battino, 2013). This immune-protective and immune-modulatory activity is often linked to the anti-cancer action. It stimulates antibodies, B & T lymphocytes, monocytes, neutrophils and natural killer cells (NK-cells) production during primary and secondary immune responses in tissue culture (Abubakar, Abdullah, Sulaiman, & Suen, 2012). It has been shown that honey stimulates macrophages, T-cells and B-cells to provoke anti-tumour effects.

Chronic inflammation has been linked with the initial stages of malignant cell formation. Tumour necrosis factor (TNF) has been shown to mediate tumour initiation, promotion and progression. The pro-inflammatory effect of TNF is connected to many other diseases due to its ability to activate NF-kB, leading to the expression of inflammatory genes like lipoxygenase (LOX)-2, chemokines, cell adhesion molecules, COX-2, iNOS, and inflammatory cytokines (Abubakar, Abdullah, Sulaiman, & Suen, 2012). Therefore, the TNF factor is considered to be a growth factor for many tumour cells (Ghashm, Othman, Khattak, Ismail, & Saini, 2010). Ironically, a sub-type of TNF factor, TNF-α plays a critical cytokine role in host defence mechanisms by releasing cytokines, which is beneficial for the inhibition or induction of various inflammatory diseases (Abubakar, Abdullah, Sulaiman, & Suen, 2012).

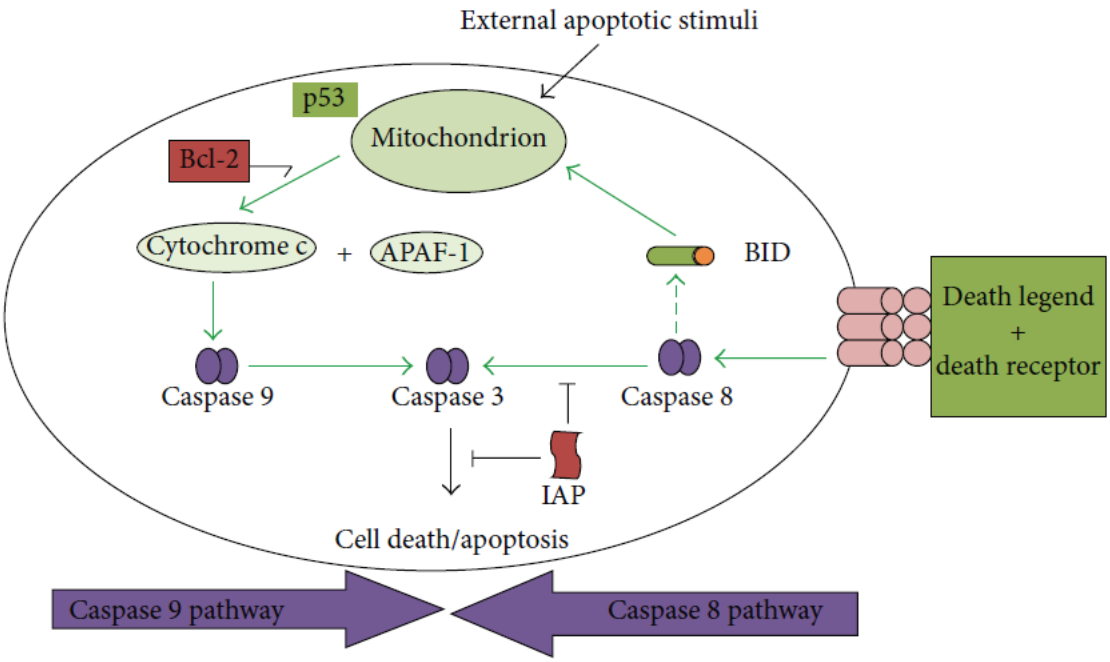


Figure 2: Induction of apoptosis through the caspase 8 and caspase 9 pathways. Whether induction of apoptosis is achieved from caspase -8 or -9 pathways, the external stimuli from honey is a possible initiator. *Bcl-2: B cell lymphoma 2 protein; Bid: Bcl-2 associated X proteins; Cyt. C: cytochrome C; Apaf-1: apoptotic protease activating factor; IAP: inhibitor of apoptosis protein; Caspase 3-caspase protein that interacts with caspase 8 and caspase 9. (Ghashm, Othman, Khattak, Ismail, & Saini, 2010)*

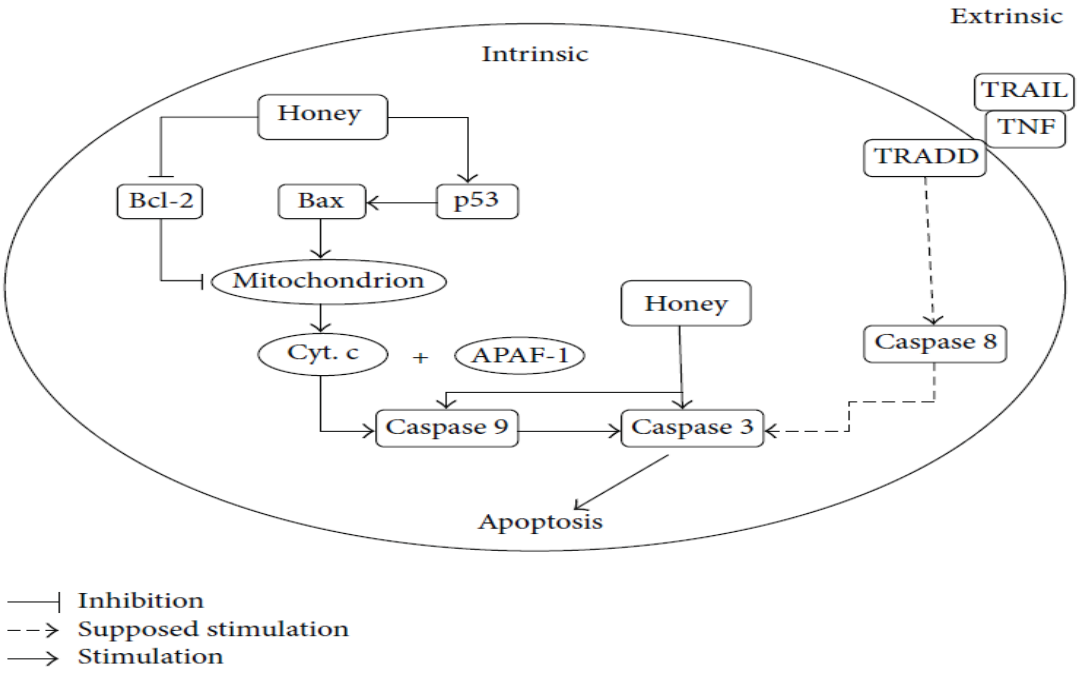


Figure 3: The effects of honey-induced apoptotic pathway. Honey exerts apoptotic effects through up-regulation and modulation of pro-apoptotic proteins (p53, Bax, caspase 3 and caspase 9) and down-regulation of anti-apoptotic proteins (Bcl-2).
Bcl-2: B cell lymphoma 2 protein; Cyt. C: cytochrome C; Apaf-1 – apoptotic protease activating factor 1: tumour necrosis factor; TRAIL: TNF related apoptosis-inducing ligand; TRADD: TNFR associated death domain protein. (Ghashm, Othman, Khattak, Ismail, & Saini, 2010)

Anti-mutagenic activity of honey

Mutagenicity, the capacity to induce genetic mutation, is closely associated with cancer-causing events in nature. Honey's anti-carcinogenic property is seen in the fact that it has been shown to be a strong anti-mutagenic agent (Rivlin, Brosh, Oren, & Rotter, 2011). The effect of honey on radiation (UV or γ types) treatment of *Escherichia coli* cells shows SOS response (SOS is an error prone repair pathway contributing to mutagenicity) (Rivlin, Brosh, Oren, & Rotter, 2011). Another study was performed to knock-out some important genes such as *recA*, *umuC* and *umuD* involved in SOS-mediated mutagenesis. These progressions are altogether restrained in the presence of honey, affirming its strong anti-mutagenic effect. Honeys from different floral origins have also exhibited inhibition of Trp-p-1 mutagenicity (Rivlin, Brosh, Oren, & Rotter, 2011).

Mutagenic substances act directly or indirectly by advancing mutations of genetic structure. During the roasting and frying of food, heterocyclic amines are built, e.g. Trp-p1 (3-amino-1,4-dimethyl-5H-pyridol [4,3-b] indole). The anti-mutagenic activity of honeys from seven different floral sources (acacia, buckwheat, firewood, soybean, tupelo and Christmas berry) against Trp-p-1 was tested via the Ames assay and compared to that of a sugar analogue and to individually tested simple sugars (Rivlin, Brosh, Oren, & Rotter, 2011). All honeys exhibited significant inhibition of Trp-p-1 mutagenicity. Glucose and fructose were found to have similar anti-mutagenic activity to honey and were more anti-mutagenic than maltose and sucrose. Stingless bee honeys from west Amazonian Ecuador showed anti-mutagenic activity assayed with Saccharomyces cerevisiae D7 strain, inhibiting back-mutation over the entire tested concentration range (Rivlin, Brosh, Oren, & Rotter, 2011).

Oestrogenic modulatory activity

Oestrogen is included as a factor in a number of malignancies. Honey modulates oestrogen by its antagonistic action. Honey may be useful in treatments of oestrogen-dependent cancers such as breast and endometrial cancers (Zhang, Yang, & Morris, 2004). Oestrogen receptors bind to oestrogens to dimerise and then translocate into the nuclei. These complexes then tie to the particular DNA base sequences called oestrogen-response elements resulting in transcription and translation of the oestrogenic effect in the targeted tissue (Sauer, Wartenberg, & Hescheler, 2011). This signalling

cascade induced by oestrogens may be modulated at any stage. Honeys from various floral sources are reported to mediate oestrogenic effects via the modulation of oestrogen receptor activity (Sauer, Wartenberg, & Hescheler, 2011). This effect is attributed to its phenolic content. Greek honey extracts exert an oestrogen agonistic effect at high concentrations (20-100 lg/mL) and an antagonistic effect at low concentrations (0.2-5 μg/mL).

Anti-oxidant activity and honey

The role of oxidative stress (including free radicals) in the cancer-causing process is well entrenched in scientific literature. Reactive oxygen species and reactive nitrogen species, such as hydroxyl radicals, superoxide, hydrogen peroxide, nitric oxide, peroxynitrite, and others, are oxidative stress agents which damage lipids, proteins, and DNA in cells (Sauer, Wartenberg, & Hescheler, 2011). Cells promote a defensive system against oxidative damage (Sauer, Wartenberg, & Hescheler, 2011). This defence system consists of anti-oxidants or oxidative protective agents such as catalase, superoxide dismutase, peroxidase, ascorbic acid, tocopherol, and polyphenols (Sauer, Wartenberg, & Hescheler, 2011). Anti-oxidants acting as a free radical scavengers, which may inhibit the cancer process in vivo. Although the exact anti-oxidant mechanism is unknown, the proposed mechanism is through hydrogen donation, free radical sequestration, metallic ion chelation, flavonoids substrates for hydroxyl and superoxide radical actions (Sauer, Wartenberg, & Hescheler, 2011). The anti-oxidant capacity of honey contributes to the prevention of several acute and chronic disorders such as diabetes, inflammatory disorders, cardiovascular diseases, and cancer. The phenolic acids and flavonoids are responsible for the well-established anti-oxidant activity of honey. The anti-tumour effect of honey may be attributed to its anti-oxidant activity. An enhanced anti-oxidant status with apoptosis has been observed in hepatocellular carcinoma cells. Daily consumption of 1.2 g/kg body weight of honey has been shown to promote the amount and the activity of anti-oxidant agents such as beta-carotene, vitamin C, glutathione reductase, and uric acid (Abubakar, Abdullah, Sulaiman, & Suen, 2012).

Possible side effects of honey

The possible side effects of honey are allergic reactions (especially to people who are allergic to bees' products), risk of having a rise in blood sugar level, and possible

interactions with certain chemo-therapy drugs. Most of the studies on different types of honey have been applied to a small number of patients. However, more studies are required to decide whether if it is safe to use or effective for various medical conditions.

Honey is relatively safe when taken by mouth in food consumption, or when recommended dosages are applied. Honey is also pretty safe when applied to the skin. Honey has the same relative sweetness and chemical backbone as table sugar, so the recommended serving size of honey is the same as it is for table sugar. One tablespoon (TBS) is considered a serving and it is not recommended that you exceed 10 TBS in the course of one day. This 10 TBS recommendation is for all added sugars, including those in packaged foods. Going over 10 TBS in a daily upper limit may introduce to gastric problems such as stomach cramps, bloating or diarrhoea. Because of the fructose content, nutrient absorption in the small intestine might be disrupted when eating too much, which may contribute to further abdominal discomfort unless the honey is fully out of the system.

Honey may also cause abnormal or absent heart rhythms, blurred vision, changes in taste, changes in white blood cell count, chest pain, diarrhoea, double vision, drowsiness, faintness, fatigue, feeling of burning or tingling on the skin, fever, heart attack, honey intoxication (sweating or weakness when honey produced from Rhododendron plants is used), hyperactivity, impaired consciousness, increased saliva, lung problems, mild paralysis, musculoskeletal problems, minor scarring, nausea, nervousness, pain, seizures, sleep problems, sweating, tooth decay, upset stomach, urinary tract infections, vomiting, weight loss, and wound dryness or infection (Coulston, 2000). Blood sugar levels may be affected, therefore, caution is advised in people with diabetes or low blood sugar, and in those who taking drugs, herbs, or supplements that affect blood sugar. Blood sugar levels may also need to be monitored by a qualified healthcare professional, such as a pharmacist, or medication adjustments may be necessary (Coulston, 2000). In addition, honey may also increase the risk of bleeding, caution is advised in people with bleeding disorders or taking drugs that may increase the risk of bleeding (Coulston, 2000). Dosing adjustments may be necessary as honey may cause low blood pressure. Caution is advised in people taking drugs or herbs and supplements that lower blood pressure.

Perhaps the most commonly known microbial threat that comes with honey is *Clostridium botulinum*. Although an adult's intestines are usually more than capable of

handling this bacterium, dormant spores can always be activated in an infant's under-developed digestive system. The risk can easily be eliminated by simply not giving any raw honey to 1 year old children and younger. Another effect that high temperatures seem to have on honey and sugary foods in general is that it increases the amount of a particular substance called *hydroxymethylfurfural* (HMF). It's hardly present in fresh food but processed foods that undergo heat treatment have HMF in varying amounts depending on the amount of heat applied. Usually the higher the temperature, the more HMF is found. Initial research on the substance shows that it can potentially damage DNA. HMF is also found in high fructose corn syrup. Since this sweetener is also used to feed honey bees, HMF has been identified as one of the possible toxins that are killing off honey bees.

Conclusion

Cancer not only affects the quality of lives of people but is also a major economic burden. A cancer patient in a family implies not only physical trauma for the patient but also a financial encumbrance for those families. Hence, there is a big avenue for research to develop new as well as cheap chemotherapeutics which will solve this mammoth task of eliminating cancer. Although various dietary agents have been explored, honey remains one of the promising agents for cancer prevention and treatment. Honey composed of various biologically active constituents has been frequently studied by various researchers for its therapeutic potential.

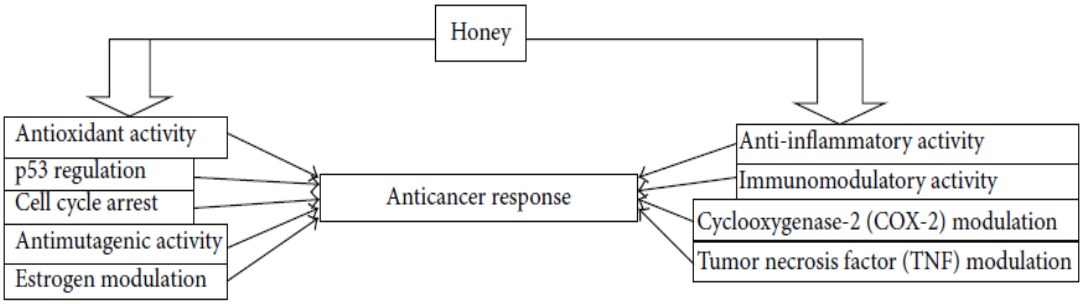


Figure 4: The schematic presentation of anti-cancer activity of honey *(Erejuwa, Sulaiman, & Wahab, 2012)***.** Honey is shown to be associated with multiple anti-cancer processes.

Evidence is developing that honey might have the possibility to be an anti-cancer reagent through several mechanisms. In spite of the fact that the full component is yet to

be described, studies have demonstrated that honey has anti-cancer impact through its impedance of multiple cell-signalling pathways, such as inducing apoptosis, anti-proliferative, anti-inflammatory and anti-mutagenic pathways. Honey modulates the body's immune system and, with different floral sources, may provide different effects.

Aims and objectives of the study

Regardless of the beneficial effects of honey, there are only limited studies of Manuka honey in terms of its target-cell speciality. Previous honey studies showed that different types of honey significantly reduced cancer cell numbers. The trial did however recommend that further multi-centre randomised trials be conducted to validate their findings.

New Zealand Manuka honey produced by Comvita Ltd is well known for its strong antibacterial activity based on its high MGO content (Ward, 2000). We hypothesized that Manuka honey would be the superior choice for testing the ability of honey to decrease the number of colon cancer cells, given its antibacterial activities, perhaps it possesses additional anti-proliferative activities. Manuka honey is of interest in scientific research regarding its potential to combat human disease. Additionally, an alternatively-sourced honey is of interest for its potential health benefits. Two types of New Zealand honey (UMF+15, a Manuka-sourced honey, and Thyme, both from Vitabeez Ltd) were designated to investigate the effects of honey's potential anti-proliferative activity against WiDr and LoVo colon cancer cell lines in order to increase the known scientific findings for these types of honey.

Aims

The aim of this study is to determine whether food grade New Zealand honey (UMF+15 and Thyme) from Vitabeez Ltd is superior to a standard best practice drug treatment in inhibiting colon cancer cell numbers by inducing apoptosis. Since the anti-tumour activities are not well characterised, this study may provide useful information on anti-tumour activities of New Zealand honey by using WiDr and LoVo colon cancer cell lines as in vitro models.

Objectives

Two main objectives for each of the two cancer cell lines were being examined.

1. To investigate the anti-tumour effects of Manuka honey and Thyme honey on two colon cell lines by using MTT assays.
2. To measure Manuka honey- and Thyme honey- induced apoptosis by using flow cytometry.

Chapter 2: Methodology
Design of this research study

This study concentrates on two human adenocarcinoma cancer cell lines: WiDr and LoVo. WiDr cells are derived from human female colon adenocarcinoma epithelial cells, and are used in cell culture studies of anti-tumour agents. WiDr cells are also tumourigenic in animal studies. LoVo cells are derived from a human male metastatic lymph node tumour in a patient suffering adenocarcinoma of the colon. LoVo cells are also used in cell culture studies of anti-tumour agents. Both cell lines are used to describe mechanisms of apoptosis. This study consists of two main parts: First, measuring the percentages of inhibitory effects from honey solutions by using cell viability (MTT) assays, and second, measuring the honey-induced apoptotic effects by using flow cytometry. Firstly, the anti-proliferation effects were determined after culturing cells with Manuka or Thyme honey solutions for 24, 48 and 72 hours within 96-well plates. The quantitative data for anti-proliferative effects was calculated to determine the half maximal inhibitory concentration (IC_{50}). Inhibitory data will be statistically analysed by "Prism" software, and "Kaluza Analysis 1.3" software will be used for analysis of flow cytometry results.

In this study, oxaliplatin will be used as a positive control to ensure the effectiveness of the honey solution towards the inhibition encounter to the cancer cells while doing the MTT assays. Once the assay is completed, the second part of this experiment involved the measuring of the apoptotic levels of the cancer cells by using flow cytometry.

Cell viability assay

Cell-based assays are frequently used for screening accumulation of compounds in order to figure out: First, whether the test molecules have any outcomes in terms of cell proliferation or second, show any direct cytotoxic impacts that in the long run which lead to cell death. Cell-based assays also are broadly utilised for measuring receptor binding and a variety of signal transduction events that may involve the expression of genetic receptors, trafficking of cellular components, or monitoring organelle function (Batumalaie, et al., 2013). Despite of the kind of cell-based assay being used, it is crucial to know the rate of viability of the cells through time toward the end of the experiment.

The primary homogenous cell viability assay that was developed was the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. It was created for a 96-well plate format that was suitable for a high throughput screening (HTS) (Spinner, 2001). The MTT substrate is prepared in a physiologically adjusted arrangement, by adding to the cells in culture, usually achieve a final concentration of 0.2 – 0.5 mg/mL, in which incubated for 1 – 4 hours. The insoluble purple formazan was generated during the incubation period as the quantity of it is a result of a direct proportional to the number of viable cells. The volume of formazan is then measured in absorbance at 540 nm using a plate reading spectrophotometer. A reference wavelength (λ) of 680 nm is always used, but not necessary for most assay conditions.

Apoptosis effects measured by the flow cytometry

Apoptosis is an intentionally-regulated procedure of cell death that happens as a common process of development in evolutionary history. Some disease states are sometimes correlates with improperly-directed apoptosis, for example, Alzheimer's disease and malignant tumour formation. Apoptosis is distinct from undesigned cell death (e.g., necrosis), by identified morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm, and loss of membrane asymmetry (Sherr, 1994). Phosphatidyl serine is an important phospholipid membrane component which is located on the cytoplasmic surface of the cell membrane in normal living cells (Bedi, et al., 1995). However, in apoptotic cells, phosphatidyl serine is translocated from the inner to the outer leaflet of the plasma membrane, therefore, exposing phosphatidyl serine to the external cellular environment. In leukocyte apoptosis, phosphatidyl serine on the outer surface of the cell marks the cell for identification which leads to phagocytosis by macrophages. The human anti-coagulant, annexin V, is a 35 – 36 kDa Ca^{2+} - dependent phospholipid binding protein that has a high affinity for phosphatidyl serine. Annexin V labelled with a fluorophore or biotin can be used to recognize the apoptotic cells by binding to the phosphatidyl serine which is exposed on the outer leaflet (Bedi, et al., 1995).

The Alexa Fluor® 488 annexin V / or Dead cell apoptosis kit with Alexa Fluor® 488 annexin V and propidium iodide for flow cytometry can offers a fast and convenient assay for apoptosis. The kit contains a collection of the annexin V that is conjugated to a fluorophores. The Alexa Fluor® 488 dye provides the maximum sensitivity. Alexa

Fluor® 488 dye is a near-spectral match to fluorescein (e.g., FITC), but it produces brighter and more photo-stable conjugates (Bedi, et al., 1995).

The kit has also includes a ready-to-use red fluorescent solution of propidium iodide, which is a nucleic acid binding dye. Although propidium iodide is an impermeable dye to living cells and apoptotic cells, it stains dead cells with red fluorescence by tightly bind to the nucleic acids within the cell (Rieger, Nelson, Konowalchuk, & Barreda, 2011). After staining a cell population with Alexa Fluor® 488 annexin V and propidium iodide in the given binding buffer, apoptotic cells will show a green fluorescence, dead cells will show a red and green fluorescence, and living cells will show a little or no fluorescence. All of these different populations can easily be distinguished by using a flow cytometer with the 488 nm line of an argon-ion laser for excitation (Rieger, Nelson, Konowalchuk, & Barreda, 2011).

A MoFlo^{TM} XDP (Beckman Coulter, Inc., Brea, CA) flow cytometer was used to analyse the single cell and study their viability and scatter properties. Both WiDr and LoVo cells were cultured in petri dishes at a concentration of 1 x 10^4 cells/well. Similar to the MTT assay, different concentrations of honey (0.5% - 2.5%) or oxaliplatin (20 µM) were added after 5 – 24 hours incubation, cells were then harvested, washed and stained followed by a 24, 48 and 72 hours interval.

The design of this experiment is to identify whether the anti-proliferative effects of honey (both UMF+15 and Thyme) are carried out by apoptotic pathways. Oxaliplatin was used as a positive control for apoptosis in colorectal cancer cells. Alexa Fluor® 488 annexin V / Dead cell apoptosis kit was purchased from InvitrogenTM (Life Technologies).

Cell lines information, materials and reagents

WiDr and LoVo human colon adenocarcinoma cell lines were purchased from ATCC (Cryosite Ltd, NSW, AU). Tissue culture flasks and RPMI 1640 culture medium were purchased from Gibco, Life Technologies. 25 cm^2 tissue culture flasks were used for cells culturing, with addition of complete growth medium (5 mL), which consisting of RPMI 1640 culture medium (REF: 21870-076, Gibco, by Life Technologies), supplemented with 1% L-glutamine, 1% Penicillin Streptomycin and 10% fetal bovine serum. All cell lines were incubated in a tissue culture incubator at 37°C, supplied with 5% carbon dioxide humidified air. A summary of all material sources is found in Table 6.

Table 5: Cell lines that were used in this study.

Cell Line Designation	ATCC Catalog No.	Cell Line Description
WiDr	CCL-218	WiDr; Colon Adenocarcinoma; Human
LoVo	CCL-229	LoVo; Colon Adenocarcinoma; Human

Table 6: Materials and reagents that are involved in this experiment.

Main Materials involved in the 1st half of this study	
Material	**Provider**
Cell Lines (WiDr, LoVo)	ATCC (Cryosite Ltd, NSW, AU)
RPMI 1640 Cell Culture Medium	InvitrogenTM (Life Technologies)
TrypLE ™ Express	InvitrogenTM (Life Technologies)
Fetal Bovine Serum Sterile Filtered (FBS)	Meregate AUS&NZ
Penicillin Streptomycin	InvitrogenTM (Life Technologies)
L-Glutamine 200mM (100X)	InvitrogenTM (Life Technologies)
Falcon® 96-Well Cell Culture Plates	In Vitro Technologies (Serving Science & Medicine)
Thiazolyl Blue Tetrazolium Bromide (MTT Powder)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Culture flasks (T75, T25)	InvitrogenTM (Life Technologies)
Dead cell apoptosis kit with Alexa Fluor® 488 annexin V and PI	InvitrogenTM (Life Technologies)

Table 7: Apoptosis cell kit information

Material	Amount V13241	Composition	Storage*	Stability
Alexa Fluor® 488 annexin V (Component A)	250 µL	Solution in 25 mM HEPES, 140 mM NaCl, 1 mM EDTA, pH 7.4, 0.1% bovine serum albumin (BSA)	2.6°C Protect from light DO NOT FREEZE COMPONENT A	When stored as directed this kit is stable for 1 year from the date of receipt.
Propidium iodide (PI, Component B)	100 µL	1 mg/mL (1.5 mM) solution in deionized water		
5X annexin binding buffer (Component C)	15 mL	50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4		
*The Alexa Fluor® 488 annexin V and propidium iodide are light sensitive and may be handled in normal room light, but avoid prolonged exposure to light.				
Number of assays: Sufficient material is supplied for 50 (Cat. No. V13241) flow cytometry assays based on a 100 µL assay volume.				
Approximate fluorescence excitation / emission maxima: Alexa Fluor® 488 annexin V: 488 / 499 in nm; propidium iodide: 535 / 617 in nm, bound to DNA.				

Materials and reagents preparation

Preparation of completed medium

Gibco, Roswell Park Memorial Institute (RPMI) 1640 culture medium (REF: 21870-076) was purchased from Life Technologies. Fetal Bovine Serum, L-glutamine, and Penicillin-Streptavidin were purchased from suppliers as noted in Table 6. Complete medium was prepared by supplementing RPMI 1640 with 1% L-glutamine, 1% Penicillin-Streptomycin, and 10% fetal bovine serum. Complete cell culture medium is used for all cell culture work as well as all dilutions for drug preparations throughout the studies to ensure that osmolarity does not exceed 330 milliosmol/L.

Preparation of phosphate buffered saline (PBS), pH 7.2

PBS (10x) stock solution is prepared with components described in Table 8. Prior to use in experiments, the stock solution is diluted 10-fold to a 1x solution, and adjusted to pH 7.2. The pH is adjusted by adding 1 M sodium hydroxide (NaOH) or 1 M Hydrochloric acid (HCl). The solution is autoclaved at 121°C for 20 minutes and stored at 4°C before use.

Table 8: The components of the phosphate buffered saline.

Components (Inorganic salt)	Molecular Weight (g/mol)	Concentration (mg/L)	mM
Potassium Phosphate Monobasic (KH_2PO_4)	136	1440	10.59
Sodium Chloride (NaCl)	58	90000	1551.72
Sodium Phosphate Dibasic ($Na_2HPO_4 \cdot 7H_2O$)	268	7952	29.66

Preparation of 12 mM MTT Solution

MTT powder (0.05 g) and 1x PBS (10ml) are combined and mixed in a 15ml centrifuge tube, to prepare a 12 mM MTT stock solution. Upon usage, a sterile Millex GV 0.22 µm syringe filter is used for sterilization, and for filtering off any spontaneously formed formazan crystals and undissolved MTT powder. The MTT solution is then be wrapped in aluminium foil and stored at 4^{o}C.

Preparation of the 1x annexin binding buffer and 100 µg/mL working PI solution

Prepare 1x annexin binding buffer by adding 1 mL of 5x annexin binding buffer (Component C) into 4 mL of deionized water. Prepare 100 µg/mL working propidium iodide (PI) solution by diluting 5 µL of 1 mg/mL PI stock solution (Component B) in 45 µL of 1x annexin binding buffer.

Preparation of the honey solutions

Aseptically prepare 10% (v/v) stock solution of honey using complete culture medium immediately prior to use. Filter the stock honey solution with a sterile Millex GV 0.45 µm syringe filter. Prepare serial dilutions from the filtrate: 1%, 2.5%, 5%, and10%. Add 100 µL of appropriate honey solution into each cell culture well, bringing total volume of each well to 200 µL. Once added to cell culture wells, the honey concentrations are 0.5%, 1.25%, 2.5% and 5%.

Osmolarity of honey solution

Osmolarity, also known as osmotic concentration, is the measurement of solute concentration. The plasma osmolarity within humans is roughly at 300 ± 30 (270 – 330) milli-osmoles per kilogram. The water content is usually less than 10% in honey to

prevent decomposition in NZ honey industry standard. Since honey is a concentrated solid, concentration of the honey solution is crucial and has to fall within the accepted osmolarity range (less than 300 mOsm/L) to prove the effect of honey towards cancer cells is biological but not physical.

20µM oxaliplatin preparation

Oxaliplatin Actavis® 100 powder for injection (100 mg) was purchased from Actavis Australia Pty Ltd. Initially prepare 12.6mM solution of oxaliplatin in milli-Q water. Prepare stock solution of 20 µM oxaliplatin in completed medium. Sonicate the oxaliplatin solution for at least 20 minutes before usage.

Basic cell culture

Initiating cell cultures from frozen stocks

Cell were thawed rapidly by briefly immersing the vial in a 37ºC water bath for 2-3 minutes with constant agitation. Upon thawing, immediately wipe the outside of the vial with 70% ethanol, then transfer the contents of the vial to a T25 flask containing 1 mL of pre-warmed completed medium. An additional 3-6 mL of medium was added to the flask. After gently swirling the flask to distribute cells evenly over the growth surface, the culture was placed in a 37ºC, 5% CO_2, humidified incubator. On the following day, the cells were examined under a microscope for adherence. Healthy cells display a flat morphology and adhere well to the plate. The medium was aspirated and replaced with fresh, warm growth medium. Cell cultures should be split when they reach about 70-80% confluence.

Split the cells

To split the cells, the medium was removed and the cells were washed once with pre-warmed sterile PBS (containing no Ca^{2+} and Mg^{2+}). An aliquot of 1-2 mL of TrypLE™ Express solution was added and the cell monolayer was treated for 1-2 minutes, or longer, until cells detach. To stop trypsinisation, 5-10 mL of growth medium was added and then the cells were re-suspended gently but thoroughly. After counting cells using a haemocytometer, the viable cells (1-2 x 10^4 / cm^2) were then transferred to a new culture flask containing an appropriate volume of growth medium. The flask was gently swirled to evenly distribute the cells.

Preparing frozen stocks of cells

Once the cells have been established in culture, the frozen stock should be prepared from an early passage to ensure a renewable source of cells. To trypsinise the cells, the medium was removed and the cells from the desired number of flasks were washed once with pre-warmed sterile PBS (containing no Ca^{2+} or Mg^{2+}). An aliquot of 1-2 mL of TrypLE™ Express solution were added into each flask and the cell monolayer was treated for 1-2 minutes, or longer, until cells detach. To stop trypsinisation, 5-10 mL of growth medium was added into each flask and then the cells were re-suspended gently but thoroughly. Pooled cell suspensions were counted and total viable cell number calculated. After centrifuging cells at 1200 rounds per minute (rpm) for 7 minutes, the

supernatant was aspirated. The cell pellet was re-suspended at a density of at least 1-2 x 10^6 cells/mL in freezing medium (Invitrogen). Aliquots of cell suspension (0.5-1 mL) were then dispensed into sterile cryovials. Freeze slowly (1^{o}C per minute) by placing vials in a thick-walled Styrofoam container at -20^{o}C for 1 hour before storage at a -80^{o}C freezer overnight. Cryovials were removed from Styrofoam container the following day and placed in liquid nitrogen for storage. Two or more weeks later, confirm the viability of the frozen stocks by starting a fresh culture from frozen cells as described above (Folkman, 1995).

MTT Cell Viability Assay

MTT linearity assay

A Cell linearity standard curve is required prior to each cell line experiment. The absorbance value is referred to on the y-axis and cell number per mL on the x-axis. The cell number should fall within a linear portion of the curve.

The absorbance value at 540 nm vs the cell number/mL can be used to determine the cell proliferation under various living conditions. As the cell number is decreased in a 1:2 dilution across the plate, the cell number should fall within a linear portion of the curve. Since there is similarity between the MTT linearity assay and the experiment, the MTT linearity assay can be used as a reference to show the experiment data are valid and also ensures the skill of handling various cell lines by the operator.

Table 9: The serial dilution plan for construction of the cell linearity standard curve (See Figure 5).

Preparation tube Number	Cells/mL	Cell number per well	Cell Culture Volume preparation
1	640,000	64,000	600 µL of 6.4 x 105 cells / mL stock
2	320,000	32,000	600 µL of 3.2 x 105 cells / mL stock
3	160,000	16,000	600 µL of 1.6 x 105 cells / mL stock
4	80,000	8,000	600 µL of 8.0 x 104 cells / mL stock
5	40,000	4,000	600 µL of 4.0 x 104 cells / mL stock
6	20,000	2,000	600 µL of 2.0 x 104 cells / mL stock

Table 10: Experiment design of the 96 well-plate.

96 Well-Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water
B	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
C	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
D	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
E	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
F	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
G	Blank (Medium Only)	Control (Cells Only)	Cells + Oxaliplatin	UMF+15 0.5%	UMF+15 1.25%	UMF+15 2.5%	UMF+15 5.0%	THYME 0.5%	THYME 1.25%	THYME 2.5%	THYME 5.0%	Water
H	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water	Water

Trypsinisation

Carefully discard the old culture medium into a beaker. Add-in 5 mL of pre-warmed sterile PBS for cell washing and discard it once it is finished. Approximately 1-2 mL of TrypLE^{TM} Express solution was added for cell detachment and placed within the cell incubator for no more than 2 minutes. Addition of 5-10 mL of completed medium to stop trypsinisation and is transferred to a 15 mL centrifuge tube for centrifugation (1200 rpm at room temperature for 7 minutes). Carefully remove the supernatant and gently re-suspend the cells by adding 1 mL of new completed medium thoroughly.

Cell Counting

10 µL of Trypan Blue and 10 µL of cell suspension were mixed thoroughly on a piece of parafilm. 10 µL of this mixture was placed onto a haemocytometer, followed by cell counting under the microscope (at least four squares). The total number of cells in 1 mL of culture medium can then be calculated by using the formula below:

Average no. of cells (per square) x 2 (Dilution Factor) x 10^4 = No. of cells/mL

Trypan Blue has been utilised for quite a long time to survey cell viability. It is negatively charged, and it only binds to the cell when the cell membrane is compromised.

Cell Seeding

Depending on the cell concentration, aseptically mix properly by adding sufficient amount of completed medium and cultured solution into the cell reservoir. By using a multi-channel pipette, carefully seed 4,000 cells/well (relatively to 40,000 cells/mL) into the 96 well-plates horizontally. The optimized seeding cell density was 4,000 cells per well for WiDr and LoVo. Each well contained 100 µL of cancer cells. At the time of all assays, cell confluence is at 60-80%.

Removal of the supernatant and re-suspension

Before the quantification step, removal of the supernatant is critical as we do not know the reaction between the drug and the MTT reagent. Therefore, carefully remove the supernatant and resuspend the cells by adding 100 µL of completed medium into each well.

Quantification

Add an aliquot of 10 µL of MTT reagent into each well, followed by another 4 hours incubation at 37^{o}C within the cell incubator. Purple precipitate should clearly visible under microscope, carefully remove 85 µL of the supernatant and followed by adding 150 µL of DMSO into each well to dissolve all precipitate. The 96 well-plate is then transferred to the orbital shaker for 10 minutes, incubate for 2-5 minutes and a further 5 minutes on the orbital shaker before the absorbance measurement. The UV absorbance value is measured by a plate reader (Multiskan Go, Thermo Scientific) at the wavelength, λ = 540 nm with reference wavelength, λ = 680 nm.

Drug/ honey treatment

After at least 5 hours (to a maximum of 24 hours) of incubation from the seeding step, an aliquot of a 100 µL of culture medium containing UMF+15 or Thyme honey from 0.5% to 5% concentrations were added into each well. Followed by another 24, 48 and 72 hours of incubation depending on the study procedure. Three 96-well plates were used as one set of experiments per cell line, followed by Day 1, Day 2 and Day 3, respectively. Each individual cell line has to be repeated at least twice to achieve the statistical requirements. Therefore, there were a total of four sets of experiments (two from WiDr and two from LoVo cell lines). MTT assays were performed at designated times, followed by 4 hours of incubation, removal of the supernatant, additional of the DMSO and plate reading at 540 nm.

Statistical Analysis

The data points from the MTT assays were examined by the statistics software "GraphPad Prism 6". One-way ANOVA non-parametric Bonferroni multiple comparisons test was carried out to examine the quantitative anti-proliferative data for anti-proliferative effects, in terms of their percentage of cell viability for each cancer cell line, by comparison to the control group (Cells only). The anti-proliferation effects were statistically demonstrated if the *p*-values were less than 0.05. The control groups between different hours were also compared by One-way ANOVA non-parametric Dunnett multiple comparisons test to determine their significance.

Apoptosis assay measured by flow cytometry

Preparation

The cellular apoptosis experiment results were determined by using the Annexin V-FITC Apoptosis Detection Kit and the testing procedure was carried out by following the manufacturer's protocol. Both colorectal cancer cells lines (WiDr and LoVo) were cultured at a 1 x 10^4 cells/mL density and seeded in a 22.5 cm^2 tissue culture dish. The cells were then treated with completed medium (RPMI 1640), each containing different concentrations of UMF+15 or Thyme honey for 24, 48 and 72 hours. The entire apoptosis assay was repeated twice, therefore, 2 sets of the data were obtained for analysis.

Propidium Iodide

Propidium iodide is an impermeant dye which is excited by 488nm laser light. Cells are considered dead when the cells take up the dye. It works by binding to DNA, intercalating between bases with no specificity, approximately one dye molecule every 4-5bp.

Detection by flow cytometry

The supernatant was carefully removed after the incubation period, and the cells harvested after trypsinisation. 5-10 mL of growth medium was added to stop the trypsinisation after the cells were detached. The solution is then be transferred into a 15 mL centrifuge tube, followed by centrifugation at 1200 rpm, for 7 minutes. The supernatant was carefully removed and cells resuspended in 5 mL of cold PBS. After centrifugation again for 7 minutes at 1200 rpm, the supernatant was discarded and the cells resuspended in a 100 µL of 1x annexin binding buffer. An aliquot of 5 µL of Alexa Fluor® 488 annexin V (Component A) and 1 µL of 100 µg/mL PI working solution was then added into each of the 100 µL of cell suspension. After incubation at room temperature for 25 minutes, 400 µL of 1x annexin binding buffer was added into individual sample. After mixing gently, the sample was kept on ice shortly before analysis by flow cytometry. The annexin V-FITC was detected with the fluorescence 488 nm excitation/ 530 nm emission, and PI with 488nm excitation/ 575 nm emission. Analysis is done by the Beckman Coulter MOFLO XDP flow cytometry machine.

Chapter 3: Results of anti-proliferative effect of honey

Cell Viability MTT assay

Initial experiments were performed to study the physiochemical characteristics of Vitabeez honey. Different dilutions of Vitabeez were prepared directly in tissue culture medium in which the colorectal cancer cells are routinely cultured and tested. The potential effect of Vitabeez on cancer cell proliferation was illustrated in the in vitro cell viability assay. According to our data, the results shown a significant decrease in cell number with increased concentration of honey applied throughout 24, 48 and 72 hours. Therefore, the data show a time- and concentration- dependent effect towards the colorectal cancer cells. In this MTT cell viability assay, the higher absorbance value indicates higher numbers of living cells, as a large amount of MTT can be reduced to purple formazan by mitochondrial dehydrogenase of living cells. Inhibitory effects were found in both cancer cell lines in dose and time dependent manners. The cell viability was largely decreased with significantly increased doses and prolonged treatment. The assay was repeated twice; there was a concentration dependent anti-tumour effect in colon cancer cell lines. Both LoVo and WiDr cells showed a significant retardation with the increase in honey concentration from 0.5 to 5% ($P < 0.05$). We observed that the anti-neoplastic activity of honey is concentration and cell type dependent. In both cell lines, cells without Vitabeez honey treatment showed an exponential curve, which is considered as the normal cell growth phenomenon. However, when the cells treated with 2.5% and 5% honey for 24, 48 and 72 hours, the cells rounded up and showed reduction in number. In contrast, cells treated with 0.5% and 1.25% Vitabeez honey showed an opposite effect after 24, 48 and 72 hours. Proliferation was observed after 24 and 48 hours. All of these cytotoxicity experiments were undertaken using corresponding cell density of 60-80% at the time of the assay.

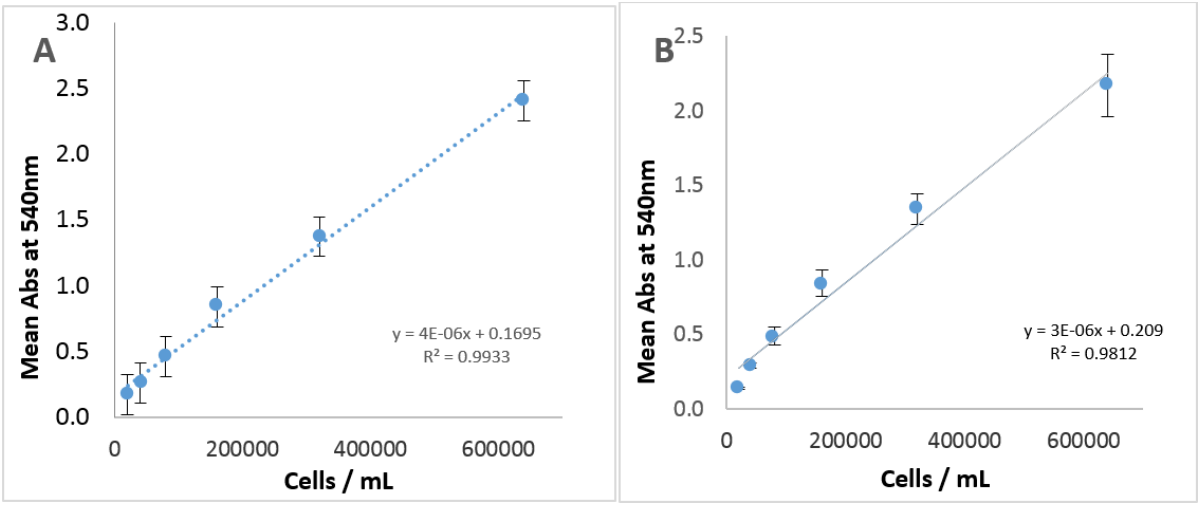


Figure 5: The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay linearity for LoVo (Graph A) and WiDr (Graph B) cell lines.

Both cell lines were plated at 6 different concentrations (2000, 4000, 8000, 16000, 32000 and 64000 cells per well) and incubated for 24 hr. At the end of the incubation period, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was performed and the absorbance values at 540nm were obtained. R^2 values are an indication of linearity. The closer the value is to 1, the better the data linearity. R^2 = 0.9933 (Graph A) and R^2 = 0.9812 (Graph B). Both R^2 were relatively linear within the cell density ranges.

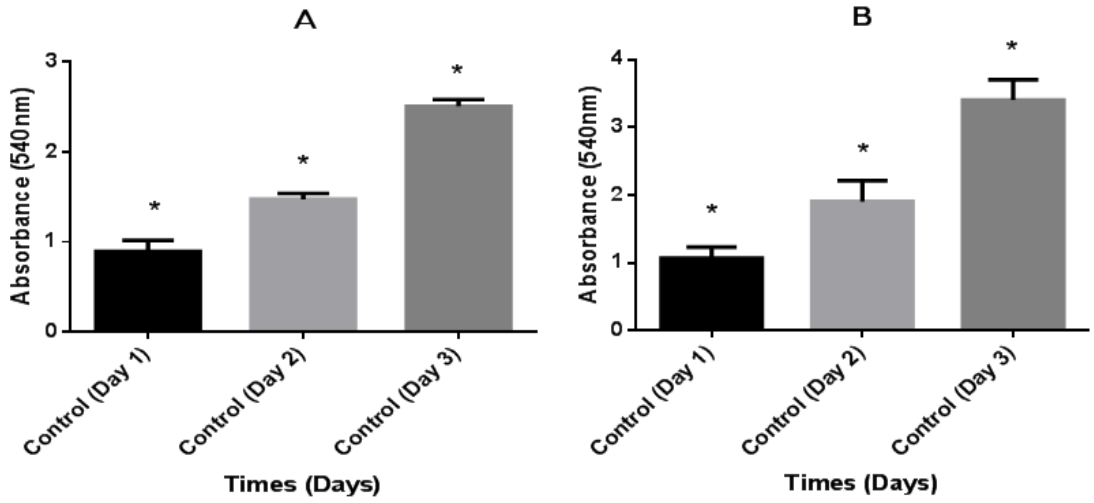


Figure 6: Control comparison of absorbance at 540nm vs Times in days (24 hr, Day 1; 48 hr, Day 2; 72 hr, Day 3).

LoVo cells were plated at $4x10^3$ cells per well in the absence of honey treatment (Graph A). WiDr cells were plated at $4x10^3$ cells per well in the absence of honey treatment (Graph B). At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as absorbance values in controls vs 24 hr, 48 hr and 72 hr. One way ANOVA statistically differences in viability of experimental groups in control comparison among 24 hr, 48 hr and 72 hr (*****, $p < 0.05$). An increase of proliferative effect was found in both colorectal cancer cell lines.

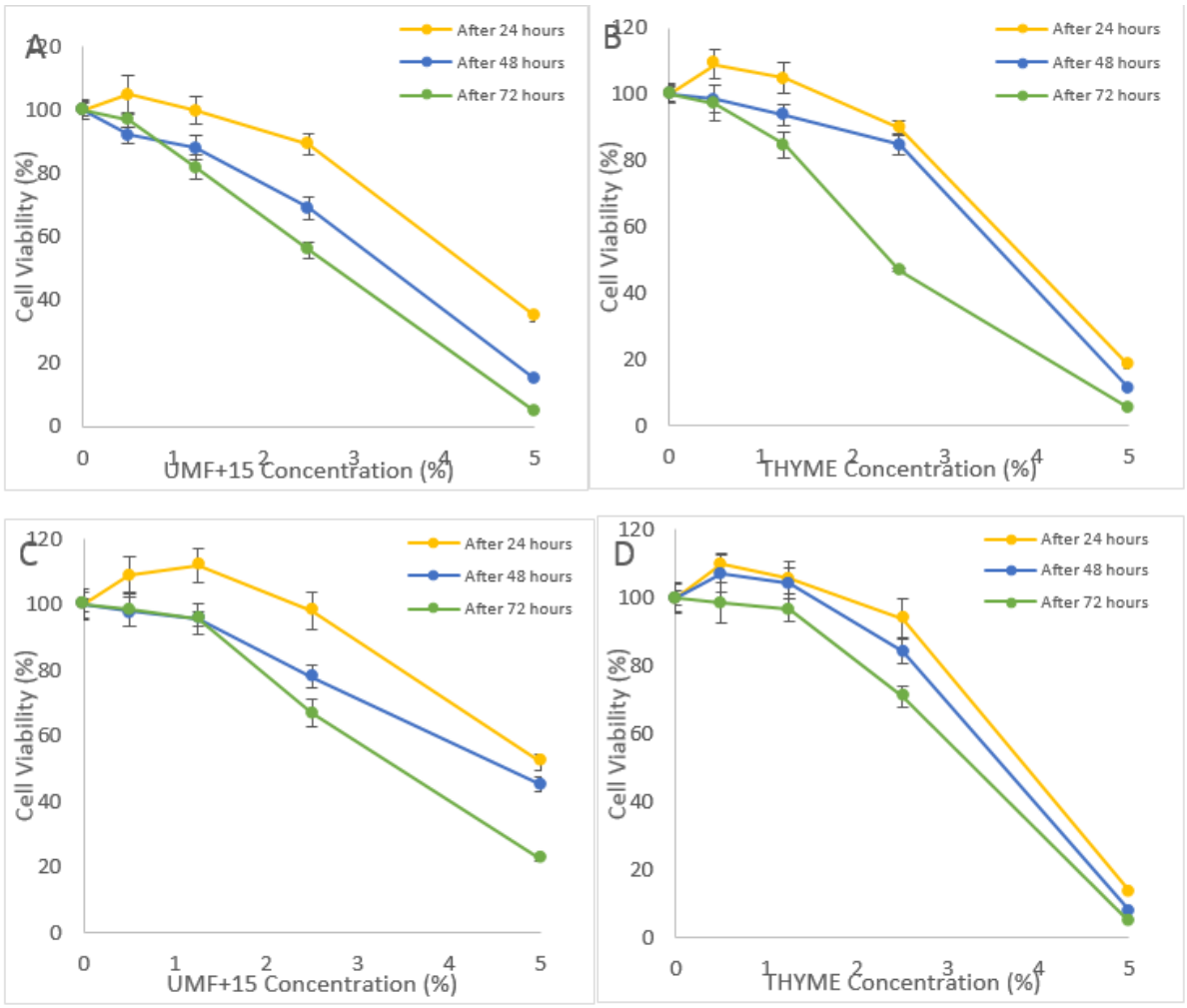


Figure 7: The trends of cell proliferation among 24 hr, 48 hr and 72 hr incubations. LoVo cells with UMF+15 honey treatment (Graph A); LoVo cells with Thyme honey treatment (Graph B); WiDr cells with UMF+15 honey treatment (Graph C); WiDr cells with Thyme honey treatment (Graph D). All concentration cell viability curves fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability.

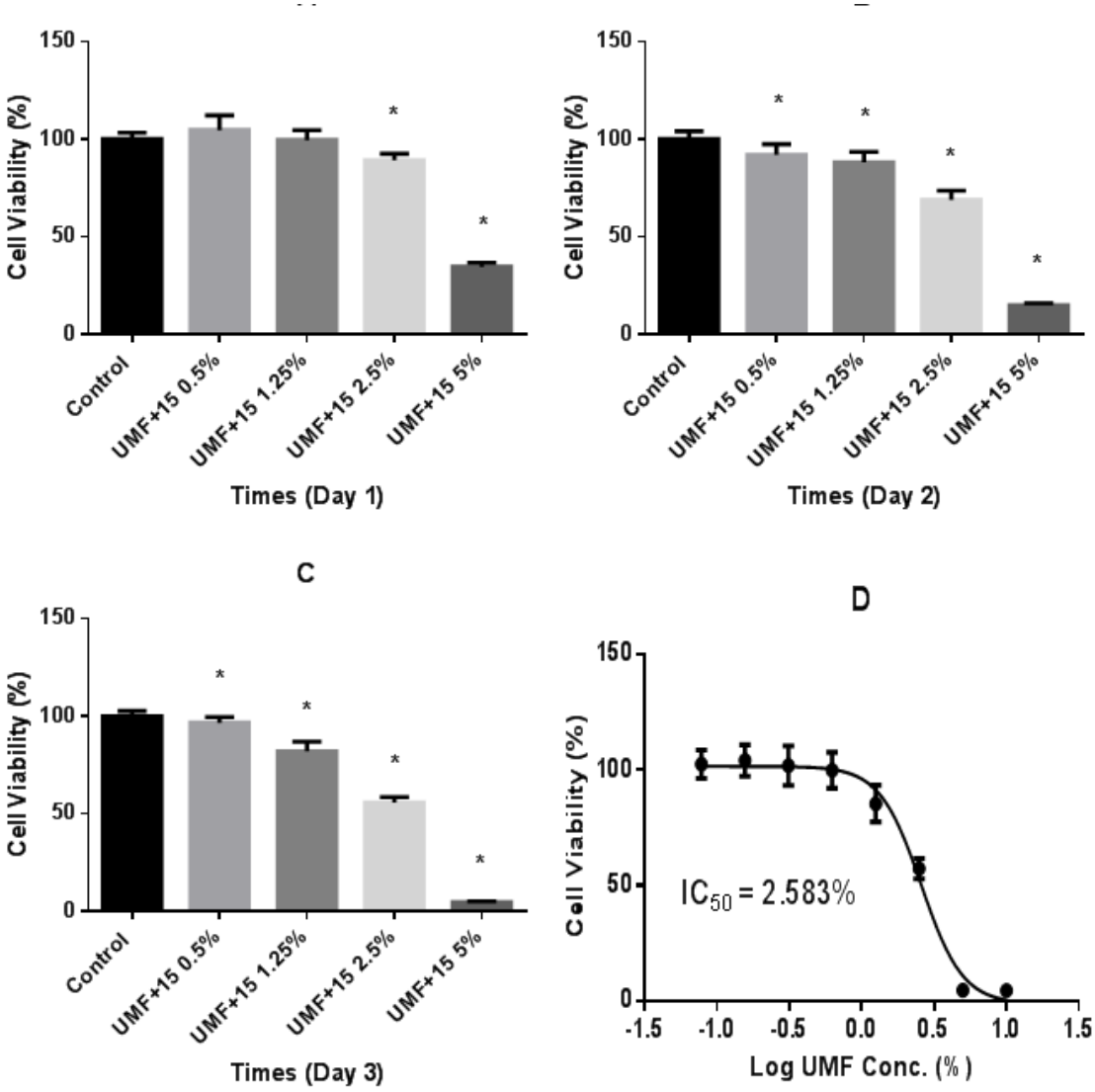


Figure 8: Inhibition of LoVo cancer cells proliferation by UMF+15 Manuka honey.

Graphs A-C detail results of cells plated at $4x10^3$ cells per well and incubated for 24 hr (graph A), 48 hr (graph B) and 72 hr (graph C) in the presence or absence of the indicated concentrations of UMF+15 Manuka honey (range 0.5% to 5%), or control, respectively. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability in treated cultures compared to control. One way ANOVA statistically differences in viability of experimental groups compared to control (*****, $p < 0.05$). Graph D was plated at 8 different concentrations of UMF+15 Manuka honey (0.078125%, 0.15625%, 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10%) and incubated for 72 hr. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability (%) vs Log UMF concentration (%). The IC_{50}=2.583% was calculated by the statistic software "GraphPad Prism 6".

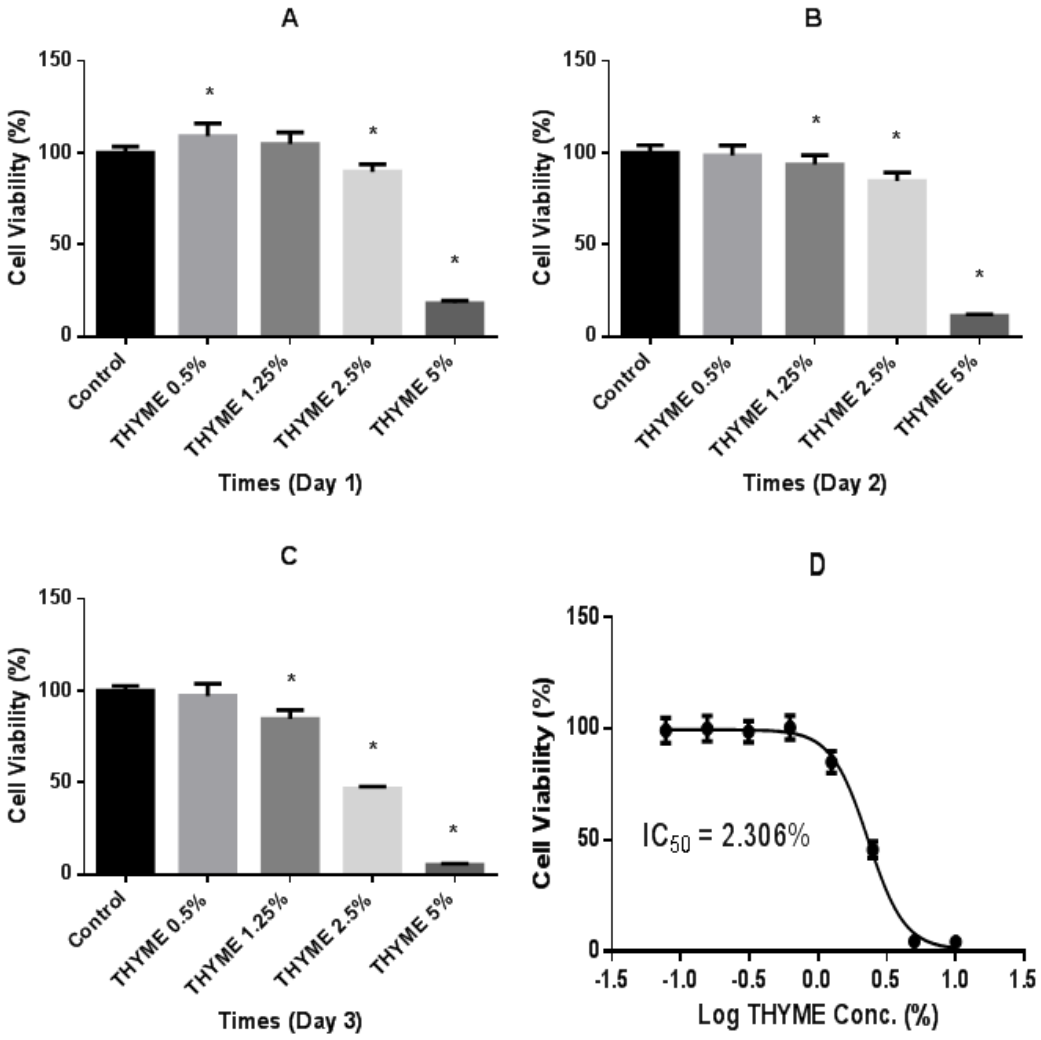


Figure 9: Inhibition of LoVo cancer cells proliferation by Thyme honey.

Graphs A-C detail cells plated at $4x10^3$ cells per well and incubated for 24 hr (graph A), 48 hr (graph B) and 72 hr (graph C) in the presence or absence of the indicated concentrations of Thyme honey (range 0.5% to 5%), or control, respectively. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability in treated cultures compared to control. One way ANOVA statistically differences in viability of experimental groups compared to control (*****, $p < 0.05$). Graph D was plated at 8 different concentrations of Thyme honey (0.078125%, 0.15625%, 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10%) and incubated for 72 hr. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability (%) vs Log Thyme concentration (%). The IC_{50}=2.306% was calculated by the statistic software "GraphPad Prism 6".

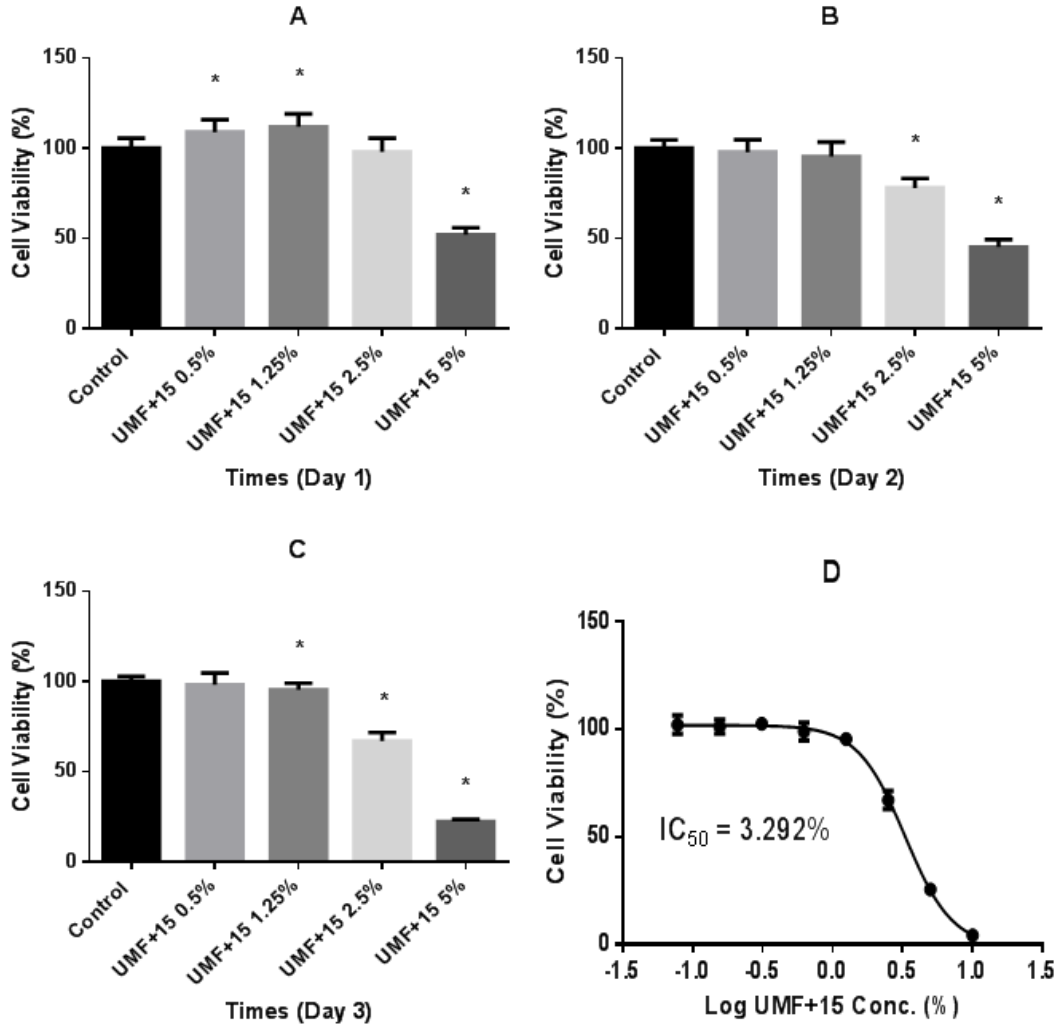


Figure 10: Inhibition of WiDr cancer cells proliferation by UMF+15 Manuka honey.

Graphs A-C detail cells plated at $4x10^3$ cells per well and incubated for 24 hr (graph A), 48 hr (graph B) and 72 hr (graph C) in the presence or absence of the indicated concentrations of UMF+15 Manuka honey (range 0.5% to 5%), or control, respectively. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability in treated cultures compared to control. One way ANOVA statistically differences in viability of experimental groups compared to control (*****, $p < 0.05$). Graph D was plated at 8 different concentrations of UMF+15 Manuka honey (0.078125%, 0.15625%, 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10%) and incubated for 72 hr. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability (%) vs Log UMF concentration (%). The IC_{50}=3.292% was calculated by the statistic software "GraphPad Prism 6".

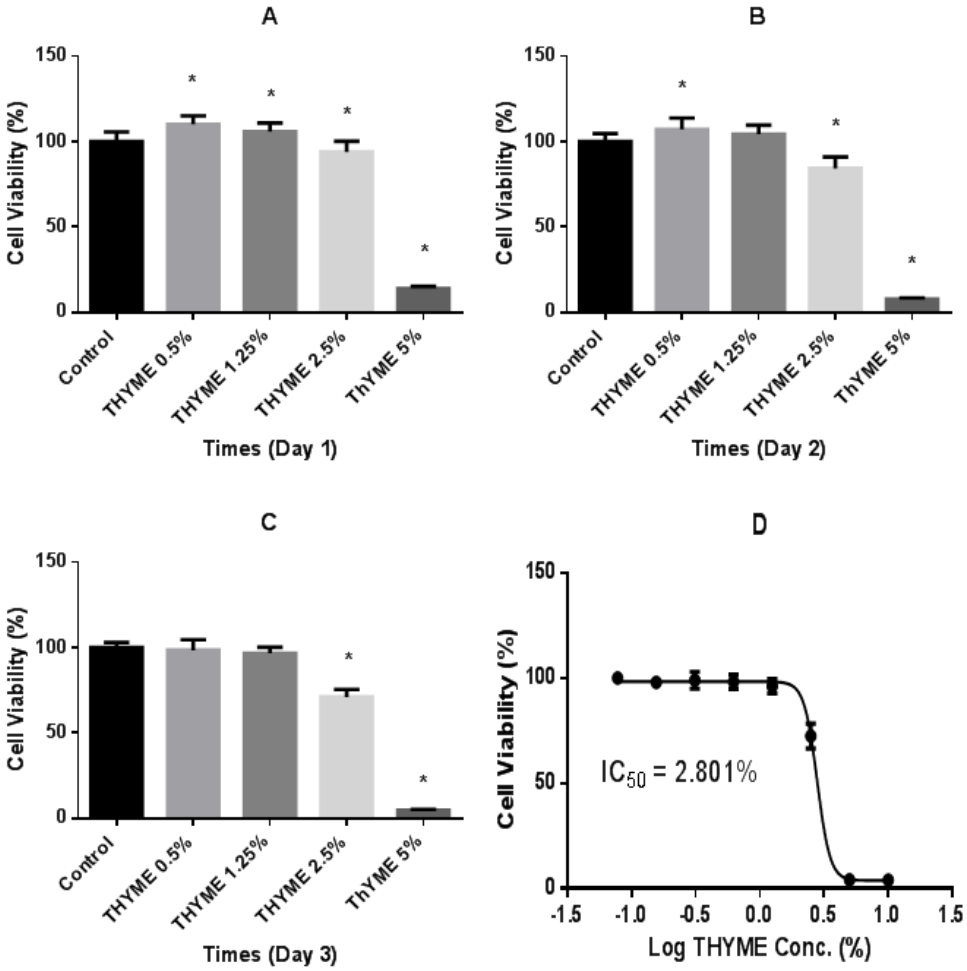


Figure 11: Inhibition of WiDr cancer cells proliferation by Thyme honey.

Graphs A-C detail cells plated at $4x10^3$ cells per well and incubated for 24 hr (graph A), 48 hr (graph B) and 72 hr (graph C) in the presence or absence of the indicated concentrations of Thyme honey (range 0.5% to 5%), or control, respectively. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability in treated cultures compared to control. One way ANOVA statistically differences in viability of experimental groups compared to control (*****, $p < 0.05$). Graph D was plated at 8 different concentrations of Thyme honey (0.078125%, 0.15625%, 0.3125%, 0.625%, 1.25%, 2.5%, 5% and 10%) and incubated for 72 hr. At the end of the incubation period, cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. Results are expressed as percentage viability (%) vs Log Thyme concentration (%). The IC_{50}=2.801% was calculated by the statistic software "GraphPad Prism 6".

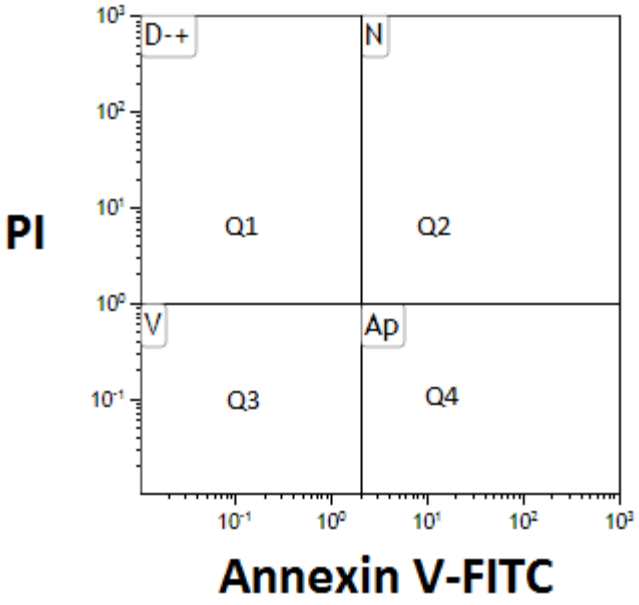
Chapter 4: Results of apoptotic effect induced by honey

In honey-treated cell lines, at time points of 24, 48 and 72 hours, different morphological variation were observed under microscope at high honey concentration. For example, chromatin condensation, fragmented nuclei and nuclear shrinkage were observed. These morphological alterations are characteristic of an apoptotic cell. Cells with blebbed membranes could also be identified. These are the morphological changes typically seen in apoptosis (Pichichero, Cicconi, Mattei, & Canini, 2011). These changes suggested that the Vitabeez honeys have the capacity to induce apoptotic cell death in both WiDr and LoVo cell lines.

Flow Cytometric Analysis

Colorectal cancer cells were cultured in cell culture dishes at a concentration of 1 x 10^4 cells/well with different concentrations of Vitabeez (0.5% to 2.5%) and oxaliplatin (20 µM). After 24, 48 and 72 hours incubation, cells were collected, washed and stained with Annexin V-FITC apoptosis detection kit following manufacturer protocol, and analysed on a Beckman Coulter MOFLO XDP flow cytometry machine.

The dual parameter fluorescent dot plots (Figure 12) shows the viable cell population in quadrant 3, which is negative to the annexin-FITC and negative to the PI. The cells at the early apoptosis are shown in quadrant 4, which is positive to the annexin-FITC and negative to the PI. Quadrant 2 represents the late apoptosis which is positive to the annexin-FITC and positive to the PI where population at quadrant 1 is considered as non-sense or cells debris. Untreated control cells were mostly alive whereas when the applications of honey treatment were applied, the percentage of the early apoptotic cells were increased in correlation to honey concentration. The box plot percentages of the cell population disclosed that the effects of honey were time and dose dependent.



Q1: quadrant 1 (Non-sense, or cells debris area); Q2: quadrant 2 (Late Apoptosis, or necrosis); Q3: quadrant 3 (Viable cells); and Q4: quadrant 4 (Early Apoptosis). The setting of this dot plot applies to Figure 13 to Figure 16.

Figure 12: An example of the dual parameter fluorescent dot plot.

Table 11: The parameters from the apoptosis assay in LoVo cells Set 1 experiment.

Apoptotic Cell (%)				Necrotic Cell (%)			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	1.11	2.10	0.00	**Control**	0.95	2.01	0.00
Oxa	10.43	15.35	21.02	**Oxa**	2.56	12.20	9.09
UMF 0.5%	1.94	0.89	0.06	**UMF 0.5%**	3.46	1.34	0.11
UMF 1.25%	6.21	1.21	2.79	**UMF 1.25%**	2.89	0.98	1.32
UMF 2.5%	6.75	8.33	30.02	**UMF 2.5%**	6.63	10.04	1.69
Thyme 0.5%	4.79	3.94	1.90	**Thyme 0.5%**	3.23	0.37	1.83
Thyme 1.25%	2.15	1.40	2.28	**Thyme 1.25%**	3.94	0.12	1.35
Thyme 2.5%	4.91	27.90	30.04	**Thyme 2.5%**	10.38	1.85	2.39
Viable Cell (%)				**Dead Cell (%)**			
	Day 1	**Day 2**	**Day 3**		**Day 1**	**Day 2**	**Day 3**
Control	95.89	93.00	98.85	**Control**	2.06	2.89	1.15
Oxa	82.48	72.05	65.63	**Oxa**	4.53	0.39	4.26
UMF 0.5%	93.50	97.10	98.57	**UMF 0.5%**	1.10	0.67	1.26
UMF 1.25%	90.46	97.12	95.50	**UMF 1.25%**	0.43	0.68	0.39
UMF 2.5%	82.89	79.91	68.29	**UMF 2.5%**	3.73	1.71	0.00
Thyme 0.5%	90.84	95.38	95.62	**Thyme 0.5%**	1.14	0.31	0.65
Thyme 1.25%	92.27	97.38	95.69	**Thyme 1.25%**	1.63	1.10	0.68
Thyme 2.5%	75.09	70.25	67.38	**Thyme 2.5%**	9.62	0.00	0.18

Table 12: The parameters from the apoptosis assay in LoVo cells Set 2 experiment.

Apoptotic Cell (%)				Necrotic Cell (%)			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	2.38	0.83	0.84	**Control**	0.96	1.76	0.89
Oxa	8.16	16.37	18.57	**Oxa**	4.85	14.60	12.86
UMF 0.5%	2.93	0.83	2.80	**UMF 0.5%**	3.74	1.76	0.35
UMF 1.25%	6.38	0.63	3.80	**UMF 1.25%**	3.85	0.63	0.90
UMF 2.5%	7.18	10.53	25.67	**UMF 2.5%**	7.66	2.63	3.98
Thyme 0.5%	4.97	1.52	2.09	**Thyme 0.5%**	0.99	1.45	0.97
Thyme 1.25%	5.67	2.47	2.93	**Thyme 1.25%**	1.67	1.93	1.37
Thyme 2.5%	14.79	28.25	28.53	**Thyme 2.5%**	8.03	1.41	2.26
Viable Cell (%)				**Dead Cell (%)**			
	Day 1	**Day 2**	**Day 3**		**Day 1**	**Day 2**	**Day 3**
Control	96.32	93.42	97.70	**Control**	0.34	3.99	0.56
Oxa	84.40	69.03	64.29	**Oxa**	2.60	0.00	4.29
UMF 0.5%	91.54	93.42	96.83	**UMF 0.5%**	1.79	3.99	0.03
UMF 1.25%	89.46	98.42	95.10	**UMF 1.25%**	0.31	0.32	0.20
UMF 2.5%	81.75	83.04	70.11	**UMF 2.5%**	3.41	3.80	0.23
Thyme 0.5%	93.74	96.19	96.14	**Thyme 0.5%**	0.29	0.84	0.81
Thyme 1.25%	92.27	94.59	94.82	**Thyme 1.25%**	0.39	1.00	0.87
Thyme 2.5%	76.62	70.06	68.85	**Thyme 2.5%**	0.56	0.28	0.36

Table 13: The parameters from the apoptosis assay in LoVo cells (average of both sets).

	Apoptotic Cell (%)				Necrotic Cell (%)		
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	1.75±0.90	1.47±0.90	0.42±0.59	**Control**	0.96±0.01	1.89±0.18	0.45±0.63
Oxa	9.30±1.61	15.86±0.72	19.80±1.73	**Oxa**	3.71±1.62	13.40±1.70	10.98±2.67
UMF 0.5%	2.44±0.70	0.86±0.04	1.43±1.94	**UMF 0.5%**	3.60±0.20	1.55±0.30	0.23±0.17
UMF 1.25%	6.30±0.12	0.92±0.41	3.30±0.71	**UMF 1.25%**	3.37±0.68	0.81±0.25	1.11±0.30
UMF 2.5%	6.97±0.30	9.43±1.56	27.85±3.08	**UMF 2.5%**	7.15±0.73	6.34±5.24	2.84±1.62
Thyme 0.5%	4.88±0.13	2.73±1.71	2.00±0.13	**Thyme 0.5%**	2.11±1.58	0.91±0.76	1.40±0.61
Thyme 1.25%	3.91±2.49	1.94±0.76	2.61±0.46	**Thyme 1.25%**	2.81±1.61	1.03±1.28	1.36±0.01
Thyme 2.5%	9.85±6.99	28.08±0.25	29.29±1.07	**Thyme 2.5%**	9.21±1.66	1.63±0.31	2.33±0.09
	Viable Cell (%)				**Dead Cell (%)**		
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	96.11±0.30	93.21±0.30	98.28±0.81	**Control**	1.20±1.22	3.44±0.78	0.86±0.42
Oxa	83.44±1.36	70.54±2.14	64.96±0.95	**Oxa**	3.57±1.36	0.20±0.28	4.28±0.02
UMF 0.5%	92.52±1.39	95.26±2.60	97.70±1.23	**UMF 0.5%**	1.45±0.49	2.33±2.35	0.65±0.87
UMF 1.25%	89.96±0.71	97.77±0.92	95.30±0.28	**UMF 1.25%**	0.37±0.08	0.50±0.25	0.30±0.13
UMF 2.5%	82.32±0.81	81.48±2.21	69.20±1.29	**UMF 2.5%**	3.57±0.23	2.76±1.48	0.12±0.16
Thyme 0.5%	92.29±2.05	95.79±0.57	95.88±0.37	**Thyme 0.5%**	0.72±0.60	0.58±0.37	0.73±0.11
Thyme 1.25%	92.27±0.00	95.99±1.97	95.26±0.62	**Thyme 1.25%**	1.01±0.88	1.05±0.07	0.78±0.13
Thyme 2.5%	75.86±1.08	70.16±0.13	68.12±1.04	**Thyme 2.5%**	5.09±6.41	0.14±0.20	0.27±0.13

Dose-dependent and time-dependent apoptosis was induced by UMF+15 and Thyme honeys (Table 13). An increase of the apoptotic percentage (Annexin V-positive) in LoVo cells were found after treatments with the NZ honeys.

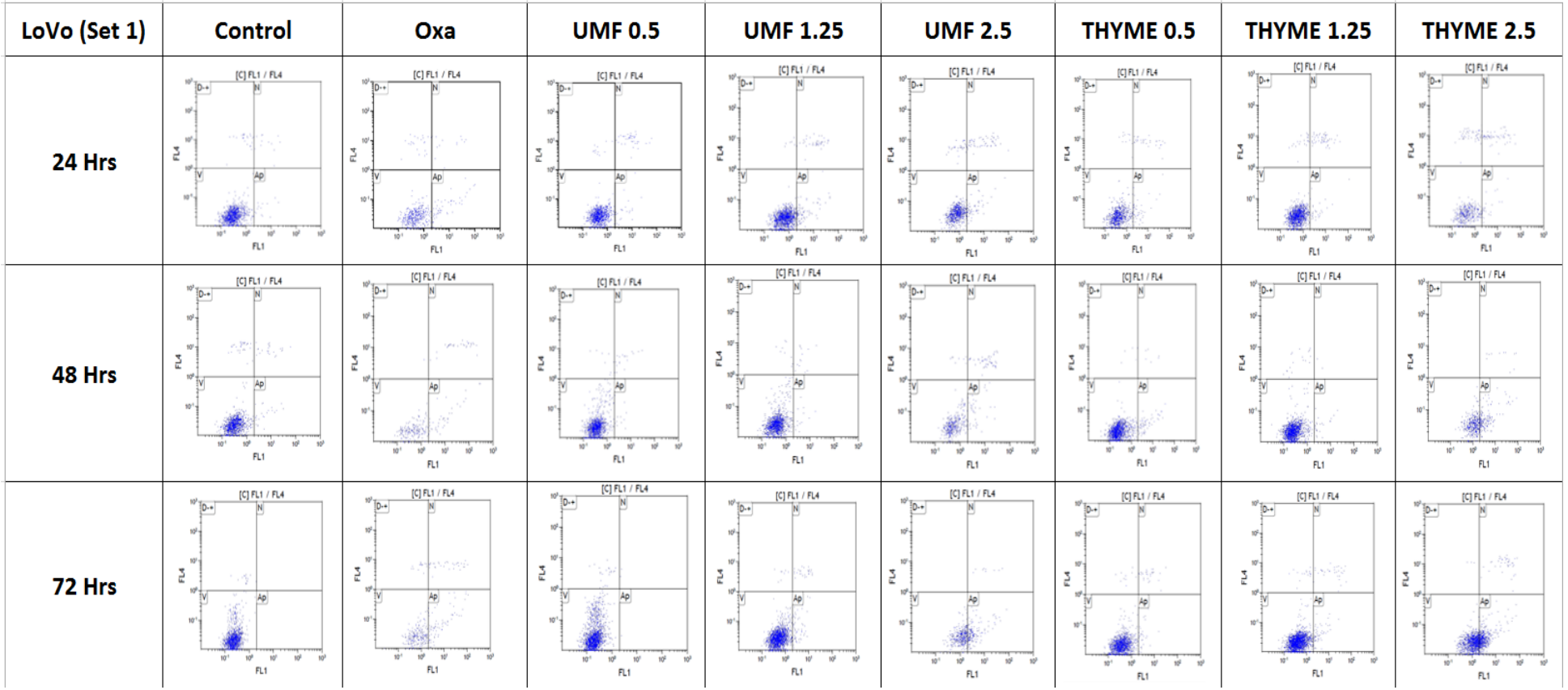


Figure 13: Vitabeez honey induces apoptosis in a dose-dependent manner (Set 1).

LoVo cells were treated for 24 hours (1st row), 48 hours (2nd row) and 72 hours (3rd row) with varying concentrations of Vitabeez (0.5% to 2.5%) honey, Oxa (Oxaliplatin 20 µM as positive control). At the end of the incubation period, cells were harvested and stained with Annexin V and PI, and analysed by flow cytometry. Data obtained from **Table 11**.

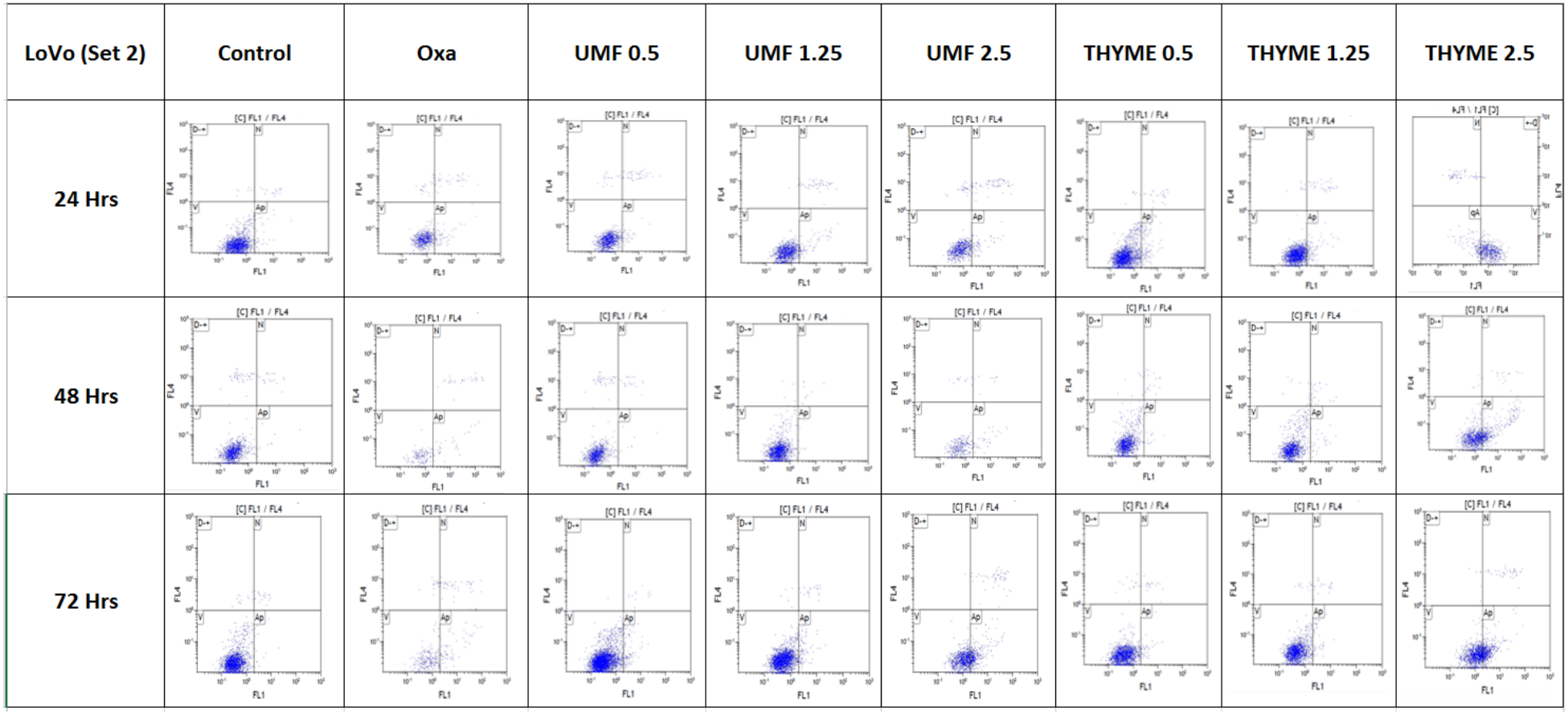


Figure 14: Vitabeez honey induces apoptosis in a dose dependent manner (Set 2).

LoVo cells were treated for 24 hours (1st row), 48 hours (2nd row) and 72 hours (3rd row) with varying concentrations of Vitabeez (0.5% to 2.5%) honey, Oxa (Oxaliplatin 20 µM as positive control). At the end of the incubation period, cells were harvested and stained with Annexin V and PI, and analysed by flow cytometry. Data obtained from **Table 12**.

Table 14: The parameters from the apoptosis assay in WiDr cells Set 1 experiment.

Apoptotic Cell (%)				Necrotic Cell (%)			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	0.14	0.27	0.23	**Control**	0.07	0.27	0.00
Oxa	7.13	4.89	21.31	**Oxa**	2.28	12.21	53.01
UMF 0.5%	1.02	0.31	1.91	**UMF 0.5%**	1.93	2.00	0.09
UMF 1.25%	1.43	1.69	0.14	**UMF 1.25%**	2.75	1.10	0.00
UMF 2.5%	6.27	9.71	27.48	**UMF 2.5%**	1.44	3.55	36.04
Thyme 0.5%	0.70	1.45	1.87	**Thyme 0.5%**	0.75	0.87	0.26
Thyme 1.25%	1.73	0.23	0.42	**Thyme 1.25%**	0.21	0.08	0.80
Thyme 2.5%	8.06	12.10	47.33	**Thyme 2.5%**	4.00	5.17	18.42
Viable Cell (%)				**Dead Cell (%)**			
	Day 1	**Day 2**	**Day 3**		**Day 1**	**Day 2**	**Day 3**
Control	99.21	98.41	99.17	**Control**	0.57	1.06	0.61
Oxa	88.32	77.16	25.68	**Oxa**	2.28	5.75	0.00
UMF 0.5%	95.29	96.38	97.68	**UMF 0.5%**	1.76	1.31	0.32
UMF 1.25%	95.31	97.07	99.10	**UMF 1.25%**	0.51	0.14	0.76
UMF 2.5%	91.70	86.30	36.49	**UMF 2.5%**	0.59	0.44	0.00
Thyme 0.5%	97.47	97.46	97.61	**Thyme 0.5%**	1.08	0.22	0.26
Thyme 1.25%	95.29	98.84	98.20	**Thyme 1.25%**	2.77	0.85	0.58
Thyme 2.5%	87.35	81.92	34.07	**Thyme 2.5%**	0.59	0.80	0.18

Table 15: The parameters from the apoptosis assay in WiDr cells Set 2 experiment.

Apoptotic Cell (%)				Necrotic Cell (%)			
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	0.00	0.05	0.49	**Control**	0.00	0.09	0.21
Oxa	4.19	2.26	19.11	**Oxa**	7.29	8.13	50.00
UMF 0.5%	0.78	0.14	4.13	**UMF 0.5%**	2.78	0.00	0.08
UMF 1.25%	1.38	0.09	0.75	**UMF 1.25%**	2.86	0.09	0.40
UMF 2.5%	5.27	10.65	30.89	**UMF 2.5%**	1.34	4.29	29.59
Thyme 0.5%	0.00	0.10	0.00	**Thyme 0.5%**	0.00	0.00	0.00
Thyme 1.25%	0.85	0.17	1.10	**Thyme 1.25%**	0.28	0.25	0.28
Thyme 2.5%	6.15	11.52	40.74	**Thyme 2.5%**	3.66	7.18	15.79
Viable Cell (%)				**Dead Cell (%)**			
	Day 1	**Day 2**	**Day 3**		**Day 1**	**Day 2**	**Day 3**
Control	99.47	98.97	98.25	**Control**	0.53	0.89	1.05
Oxa	83.57	79.46	27.49	**Oxa**	4.96	10.16	3.40
UMF 0.5%	94.89	96.60	95.46	**UMF 0.5%**	1.55	3.26	0.33
UMF 1.25%	94.99	98.60	98.09	**UMF 1.25%**	0.77	1.22	0.75
UMF 2.5%	92.73	84.61	39.52	**UMF 2.5%**	0.67	0.45	0.00
Thyme 0.5%	98.88	98.92	99.12	**Thyme 0.5%**	1.12	0.98	0.88
Thyme 1.25%	96.72	97.90	97.07	**Thyme 1.25%**	2.15	1.68	1.55
Thyme 2.5%	89.21	81.08	39.38	**Thyme 2.5%**	0.98	0.21	4.09

Table 16: The parameters from the apoptosis assay in WiDr cells (average of both sets).

	Apoptotic Cell (%)				Necrotic Cell (%)		
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	0.07±0.10	0.16±0.16	0.36±0.18	**Control**	0.04±0.05	0.18±0.13	0.11±0.15
Oxa	5.66±2.08	3.58±1.86	20.21±1.56	**Oxa**	4.79±3.54	10.17±2.88	51.51±2.13
UMF 0.5%	0.90±0.17	0.23±0.12	3.02±1.57	**UMF 0.5%**	2.36±0.60	1.00±1.41	0.09±0.01
UMF 1.25%	1.41±0.04	0.89±1.13	0.45±0.43	**UMF 1.25%**	2.81±0.08	0.60±0.71	0.20±0.28
UMF 2.5%	5.77±0.71	10.18±0.66	29.19±2.41	**UMF 2.5%**	1.39±0.07	3.92±0.52	32.82±4.56
Thyme 0.5%	0.35±0.49	0.78±0.95	0.94±1.32	**Thyme 0.5%**	0.38±0.53	0.44±0.62	0.13±0.18
Thyme 1.25%	1.29±0.62	0.20±0.04	0.76±0.48	**Thyme 1.25%**	0.25±0.05	0.17±0.12	0.54±0.37
Thyme 2.5%	7.11±1.35	11.81±0.41	44.04±4.66	**Thyme 2.5%**	3.83±0.24	6.18±1.42	17.11±1.86
	Viable Cell (%)				**Dead Cell (%)**		
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3
Control	99.34±0.18	98.69±0.40	98.71±0.65	**Control**	0.55±0.03	0.98±0.12	0.83±0.31
Oxa	85.95±3.36	78.31±1.63	26.59±1.28	**Oxa**	3.62±1.90	7.96±3.12	1.70±2.40
UMF 0.5%	95.09±0.28	96.49±0.16	96.57±1.57	**UMF 0.5%**	1.66±0.15	2.29±1.38	0.33±0.01
UMF 1.25%	95.15±0.23	97.84±1.08	98.60±0.71	**UMF 1.25%**	0.64±0.18	0.68±0.76	0.76±0.01
UMF 2.5%	92.22±0.73	85.46±1.20	38.01±2.14	**UMF 2.5%**	0.63±0.06	0.45±0.01	0.00±0.00
Thyme 0.5%	98.18±1.00	98.19±1.03	98.37±1.07	**Thyme 0.5%**	1.10±0.03	0.60±0.54	0.57±0.44
Thyme 1.25%	96.01±1.01	98.37±0.66	97.64±0.80	**Thyme 1.25%**	2.46±0.44	1.27±0.59	1.07±0.69
Thyme 2.5%	88.28±1.32	81.50±0.59	36.73±3.75	**Thyme 2.5%**	0.79±0.28	0.51±0.42	2.14±2.76

Dose-dependent and time-dependent apoptosis was induced by both UMF+15 and Thyme honeys (Table 16). An increase of the apoptotic percentage (Annexin V-positive) in WiDr cells were found after the NZ honeys treatment.

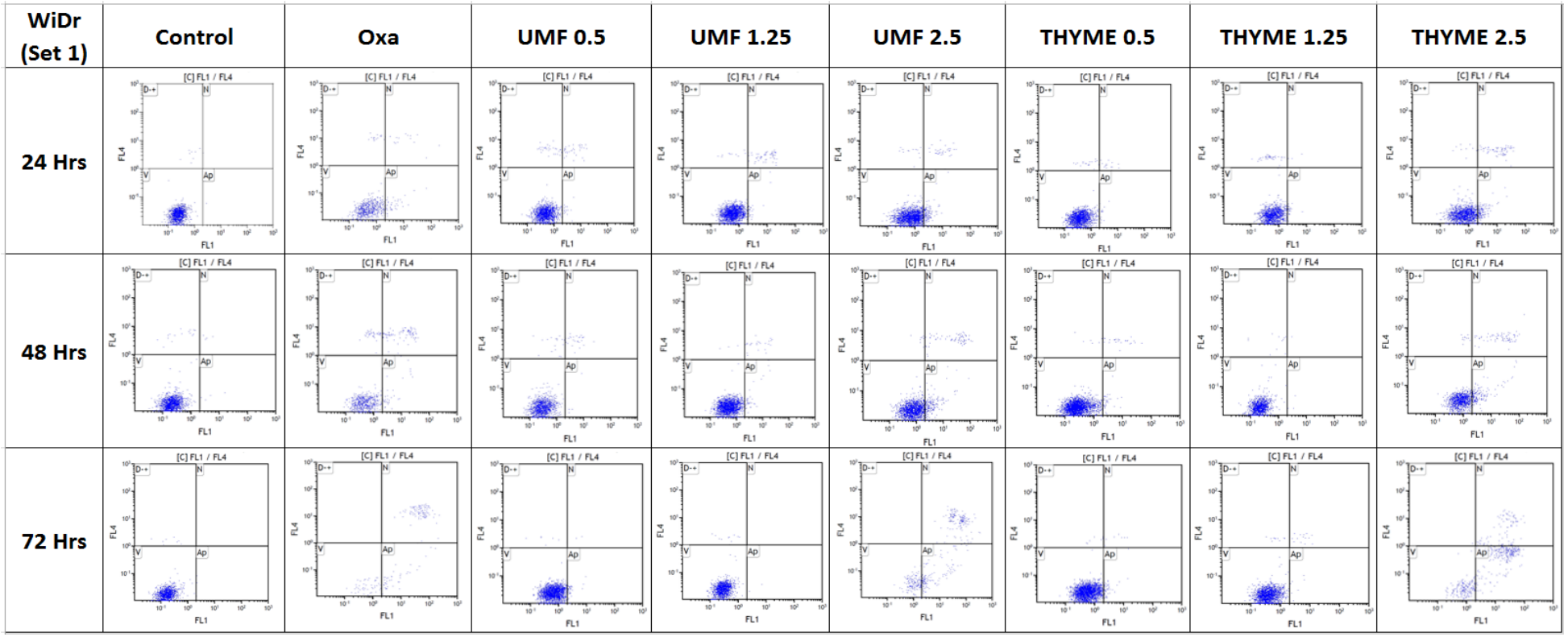


Figure 15: Vitabeez honey induces apoptosis in a dose dependent manner (Set 1).

WiDr cells were treated for 24 hours (1st row), 48 hours (2nd row) and 72 hours (3rd row) with various concentrations of Vitabeez (0.5% to 2.5%) honey, or Oxa (Oxaliplatin 20 µM as positive control). At the end of the incubation period, cells were harvested and stained with Annexin V and PI, and analysed by flow cytometry. Data obtained from **Table 14**.

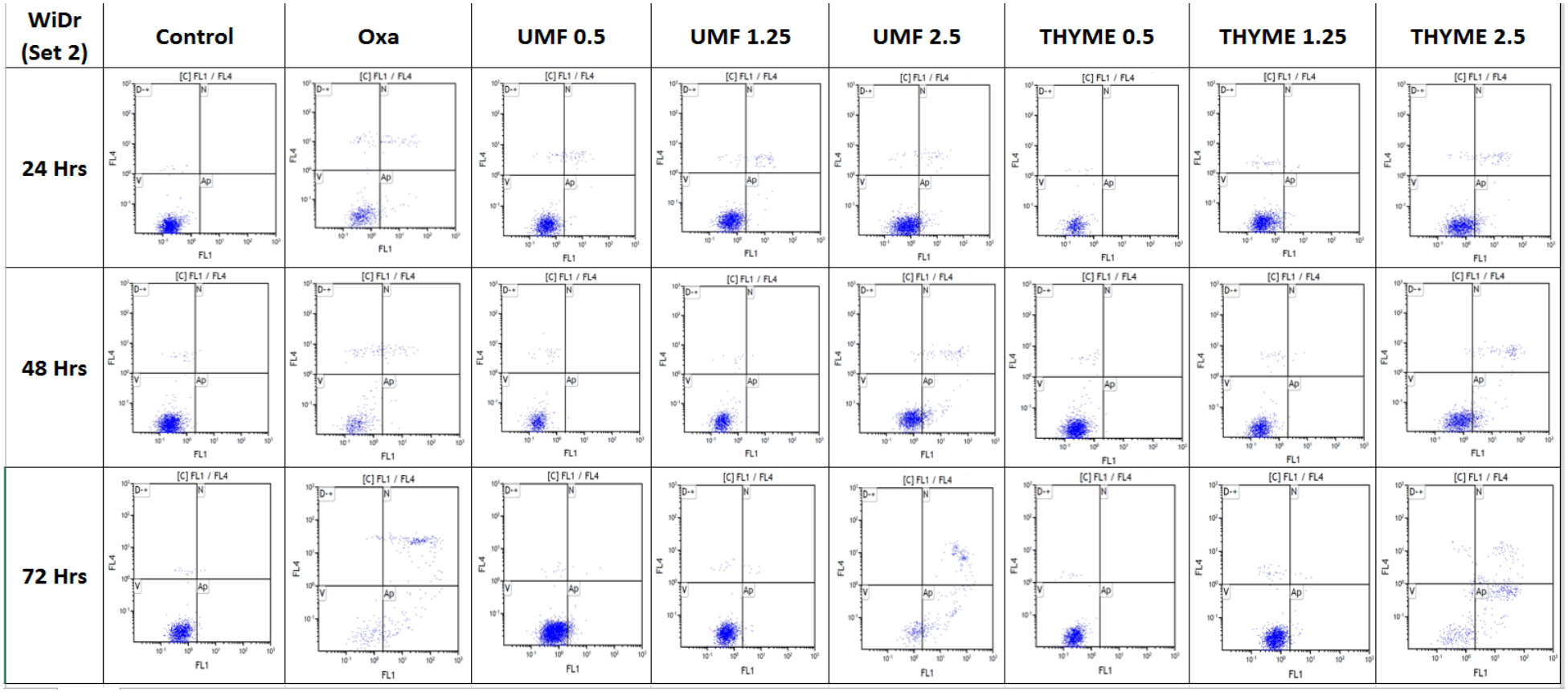


Figure 16: Vitabeez honey induces apoptosis in a dose dependent manner (Set 2).

WiDr cells were treated for 24 hours (1st row), 48 hours (2nd row) and 72 hours (3rd row) with various concentrations of Vitabeez (0.5% to 2.5%) honey or Oxa (Oxaliplatin 20 µM as positive control). At the end of the incubation period, cells were harvested and stained with Annexin V and PI, and analysed by flow cytometry. Data obtained from **Table 15**.

Chapter 5: Discussion

Reasons for this research study

This study was performed in order to present an initial in vitro model to provide novel information in terms of how New Zealand UMF+15 (Manuka) and Thyme honeys' anti-proliferative and pro-apoptotic activities affect WiDr and LoVo human colon cancer cell lines.

Significance of this study

In this study, the cytotoxic and pro-apoptotic effects of honey in both WiDr and LoVo cell lines were investigated. Our data confirmed that both UMF+15 Manuka honey and Thyme honey have cytotoxic activity against carcinomic human colon cells, indicating that these New Zealand honeys possesses anti-tumour and anti-carcinogenic activities. Although similar honey-induced anti-tumour effects which have already observed by the other researchers, the anti-tumour specificity of UMF+15 Manuka honey and Thyme honey remain unknown towards LoVo and WiDr colorectal cancer cells. Therefore, the significance of this study would provide the potency of anti-tumour activity towards this specific types of honey. In addition, the results indicate that both UMF+15 Manuka honey and Thyme honey possess not just an anti-proliferative effect, but also an apoptotic effect, as shown by the apoptosis assays. Therefore, the investigation of the cell cytotoxicity and cell apoptotic activity of a wide range of honey concentrations against human colon cancer cells (WiDr and LoVo) are significant findings in our study.

The advantages and disadvantages of the MTT assay

MTT assays are easy, cheap and relatively fast to perform and the results are reliable. The MTT assay has been used widely especially for drug screening, and could be used as a preliminary method for alternative screening purposes (Riss, 2014). It is essential to ensure that the activity is measured at the most dynamic part of the cells' proliferation stage. A growth optimisation curve is necessary for the study's cells before carrying out any drug screening. Unfortunately, the test is not delicate enough to pick up minor changes in proliferation, since it is an absorbance based assay. The assay works by measuring metabolic activity, not necessarily viability, because some cells can be perfectly viable but not necessarily possess measureable metabolic activity (Riss,

2014). It is therefore imperative to validate MTT outcomes by an additional assay that can directly measures cell apoptosis and/ or necrosis. The assay itself should not be used as primary evidence for cell cytotoxicity or the confirmation of the anti-proliferative activity, because it gauges mitochondrial metabolic activity across a set number of cells in an indirect measurement. Some compounds such as epigallocatechin gallate facilitate mitochondrial function and biogenesis, and cause artificially high absorbance readings due to the increased amount of mitochondrial metabolic activity (Riss, 2014). This situation could nullify possible decreases in cell count, or be the reason that a compound is mistaken as pro-proliferative.

Honey and its anti-proliferative activity

In terms of the anti-proliferative effect of honey, the linearity of MTT cell viability assays of both cell lines tested was presented in Figure 5. Both R^2 values are relatively close to 1 indicating that the experimental data were valid and the skill of handling different cell types by the operator is acceptable. Figure 6 represents whether the trend of the cell proliferation is linear among 24 hr, 48 hr and 72 hr populations. The statistical analyses of the *P* values from both cell lines were less than 0.05, indicated the data obtained from the experiments were statistically significant. Figure 7 illustrates the relationship of honey concentration versus time. Increases in a proliferation effect were found in graph A (Day 1 0.5%, $P>0.05$), graph B (Day 1 0.5%, $P<0.05$ and 1.25%, $P>0.05$), graph C (Day 1 0.5%, $P<0.05$ and 1.25%, $P<0.05$) and graph D (Day 1 & 2 0.5%, $P<0.05$ and 1.25%, $P<0.05$), as the percentage of honey (UMF+15 Manuka honey and Thyme) concentrations and times (24 hr, 48 hr and 72 hr) were increased, the percentage of cell viability in both LoVo and WiDr cell lines were decreased. This phenomenon of a proliferation inhibition activity of the types of honey used was time-dependent and dose-dependent against both colorectal cancer cell lines used throughout the study. Thyme honey appeared to be more potent than UMF+15 Manuka honey, as the Thyme honey inhibitory concentration at 50% were lower in both cell lines (Figure 9D; 2.306, and 11D: 2.801) when compared to UMF+15 Manuka honey (Figure 8D; 2.583, and Figure 10D; 3.292).

In Figure 8 (graphs A-C), a significant decrease of cell viability was observed when honey concentrations reached 2.5% and 5%. The inhibitory effect on cell viability appeared to be dependent on both UMF+15 Manuka and Thyme honey concentration and total incubation time. In contrast, a proliferative effect was observed in low honey

concentration (0.5%) treatments in both cell types (Figure 8A, 9A, 10A, and 11A) at 24 hours. Some studies suggested that only when honey concentrations are above 1 or 1.5% that it would stimulate the release of cytokines (e.g., TNF-α, IL-1β, IL-6, etc.), causing an anti-proliferative effect. In our results, honey concentration at 0.5% did have a proliferative effect (Figure 8-11), but most of them were not statistically significant (Tonks AJ, 2003) (C M. P., Honey as a topical antibacterial agent for treatment of infected wounds, 2001). The results would suggest that the regulatory effects of honey are related to components other than the sugar present, although the identity of the actual component(s) that mediate these effects are as yet unknown. The effects are possibly due to the phenolic compounds such as caffeic acid. Caffeic acid is a naturally occurring phenolic compound present in the honey and plays an anti-oxidant role (Ghashm, Othman, Khattak, Ismail, & Saini, 2010). An anti-proliferative effect was found in the research conducted by Hirose et al. (Rao, et al., 1993) and carcinogenicity studies complied with these phenolic compounds. Rao, et al. (1993) performed a detailed study by synthesising three caffeic acid esters, namely methyl caffeate, phenylethyl caffeate, and phenylethyl dimethylcaffeate, and examined them against the 3,2' –dimethy;-4-aminobiphenyl (DMAB, a colon and mammary carcinogen)-induced mutagenicity in *Salmonella typhimurium*. Concentration of the esters significantly inhibited the DMAB-induced mutagenicity in both strains (Abubakar, Abdullah, Sulaiman, & Suen, 2012).

Although there are numerous studies or reports suggesting that the components of honey have an anti-tumour effect, the obtained results from our study were not exactly in line with or similar to the other research studies. For example, while there is a decrease in cell proliferation associated with high honey concentrations, honey concentrations lower than 1.5% always have increased cell proliferation at 24 hours. Some reports suggested that the anti-tumour effect is observed only when honey is above 1 or 1.5% (Jaganathan, Mandal, Jana, Das, & Mandal, 2010). In addition, honey possessing higher phenolic and tryptophan content was more potent in inhibiting colon cancer cell proliferation. This could possibly be due to different types of cell lines used and different types of honey with differing concentrations. Moreover, the concentrations of different polyphenols in honey and the interactions between them have yet to be fully elucidated; therefore, more information is needed with regards to the possible role of honey in cancer prevention and therapy.

The apoptotic effect induced by honey

The capacity to cause tumour cell apoptosis is an essential property of any application of an anti-cancer drug. Much effort has been coordinated toward the investigation of the impact of honey on apoptosis and understanding the mechanisms of action (Hoff, 2012). The apoptosis evoked by honey in our study was affirmed by the Annexin-V FITC testing. Additionally, in the present study, the condition of apoptosis induced by honey was always involved in cell death. Distinctive morphologic features were also characterised for the stages of apoptosis, such as cell and nuclear shrinkage, oligo-nucleosomal DNA fragmentation, membrane blabbing and chromatic condensation as discussed by Erejuwa (2013).

The therapeutic activity is always a limitation for most of the anti-cancer drugs in clinical usage because of their association with greater toxicity. Although novel cytotoxic agents with unique and specific mechanisms of action are continuously in development, many of these developments lack tumour specificity and selectivity (Farr, 2005). Therefore, many of these development are not therapeutically useful. New targets for cancer therapy focus on interfering with specific targeted molecules needed for carcinogenesis and tumour growth in order to overcome the problems of traditional therapies. Natural or herbal products, without the side effects seen in modern medication products, are often used as alternative therapies for many cancers or other chronic diseases. Honey was used as a medicinal substance for a very long time in history. It consists of a very complex carbohydrates mixture produced from a natural environment. It is one of the best-known natural products with several biological activities. A lot of bio-active components were found in honey that have been used as a cancer preventive agents.

As shown by our flow cytometry results, when the concentration of honey was increased, the percentage of early apoptotic cells was also increased (Figure 13 to 16). For this reason, the mode of cell death appears to be the death pathway (Farr, 2005). These apoptotic changes were also visible in the morphological studies which were done using light and fluorescent microscopy where membrane blebs, chromatin and nuclear condensation, DNA fragmentation and formation of apoptotic bodies were seen (Blaser, Santos, Bode, Vetter, & Simon, 2007). A recent study was performed in order to fully understand the molecular mechanism of action of honey by applying a honey solution to colon cancer cells, and cell proliferation was inhibited. The authors had

found that the apoptosis induced by honey was accompanied by the p53 tumour suppression gene up-regulation and expressing of pro and anti-apoptotic proteins modulation (Bharti, Donato, Singh, & Aggarwal, 2003). They also reported that un-fractionated honey induced cell-growth arrest, resulting in cell cycle blockage at the sub-G_1 phase (Bishayee, et al., 2013). Furthermore, it transduced the apoptotic signal via initial depletion of intracellular non-protein thiols (GSH), consequently reducing the MMP and increasing the ROS generation (Bishayee, et al., 2013). Such findings indicate the potential pathways available for future studies to further elucidate the actions of the New Zealand honeys utilised in this study. Additionally, the use of a non-cancerous human colon cell line to act as a normal cell control for the effects of honeys will also be beneficial to building the body of knowledge in this area of research.

Limitations

The choice of honey and growth medium

As of late, significant endeavours have been made to distinguish naturally occurring and related manufactured reagents that could prevent the development and recurrence of cancer (Abubakar, Abdullah, Sulaiman, & Suen, 2012). Our investigations present an additional model to study honey as an anti-carcinogenic agent, by providing practical approaches in which to identify potentially useful novel components that could inhibit the development of colon cancer. In the past, several type of cancers were shown to be inhibited by phenols, indoles, aromatic isothiocyanates inositol-6-phosphate and dithiolethiones which were naturally occurring compounds. By illustrating the anti-proliferative and pro-apoptotic properties of two New Zealand honeys against WiDr and LoVo cell lines, there is a potential in future research to describe the components of these honeys as a means of explaining their mechanisms of action.

The hyper-osmolarity of bee honey was addressed by performing dilutions so that the concentrations used (max. 5%) in the experiment are relatively similar to the routinely used antibiotic doses (Jaganathan, Mandal, Jana, Das, & Mandal, 2010). The same guideline was applied in the choice of RPMI-1640 completed medium as the solvent because of its capacity to buffer most of the acidity from the bee honey. Thus, the acidity and hyper-osmolarity of bee honey alone cannot explain the drastic inhibitory effect on tumours. There are two reasons for us to choose the pure unfractionated honey: first, the collaboration between the different chemical constituents in honey has been reported, and second, various volatile compounds were reported to be abundant in

honey that might be lost during the fractionation process. Some of the observed biological activities of honey may be traced to its chemical constituents. Therefore, this might contribute to a limitation as pure unfractionated honey is difficult to get on hand and the choice of choosing of the growth medium has been limited.

The coefficient of variation from DMSO

DMSO was used to solubilise the formazan crystals. We have found that DMSO increased absorbance values, but also increased intra-experimental variation. Furthermore, when DMSO is used, most of the medium must be removed from the wells prior to the addition of DMSO. It would be difficult to avoid removing some cells in the process, and this could contribute to the higher standard deviations observed. Finally, since low seeding densities are used, there could be differential cell growth in the wells during the assay period, thus increasing the coefficients of variation.

Future studies and related diseases

The results of our flow cytometry experiments show that both UMF+15 and Thyme honey present an apoptotic effect towards both LoVo and WiDr colon cancer cells. Further apoptosis assays which address different markers of apoptosis are recommended for future studies, as well as cell cycle behaviour experiments. However it is notable that the results of the apoptosis studies are positive results in indicating the anti-cancer effects of the two honeys tested.

Our cell viability assay results also showed an anti-proliferative effect. Limitations of the assay have not identified all possible explanations for the observed effect. The cytotoxic effect could have been induced by honey through other additional signalling pathways. This study did not show a specific component of the honeys tested as causing the anti-proliferative or apoptotic effect. Therefore, extraction of components from the honeys, along with isolation and characterisation of these agents by experimentation and by mass spectroscopy or HPLC are required to further elucidate the mechanisms by which they exert their actions. Such studies could be future areas of interest to identify specific differences between honey components.

Erejuwa et al. (Erejuwa, Sulaiman, & Wahab, 2012) praise the anti-oxidant properties of honey as a discovery that indicates honey may decrease oxidative stress in the gastrointestinal tract, liver, pancreas, kidney, reproductive organs and plasma/serum.

The synergistic anti-oxidant impact of honey and anti-diabetic drugs in the pancreas, kidney and serum of diabetic rats are also proposed. Honey, administered alone or in combination with conventional therapy, might be a novel anti-oxidant in terms of the management of chronic diseases that are normally associated with oxidative stress. In context with our studies, the findings of honey's role in anti-proliferation and pro-apoptotic processes may provide insight into management of disease associated with oxidative stress. There is an urgent need to investigate this antioxidant effect of honey from cellular models, to animal models, and finally in human subjects with chronic or degenerative disease.

Our findings are building the pool of knowledge relative to honey being used as an anti-metastatic agent in human colon cancer cellular studies. Future studies in animals are plausible as a model for oral ingestion of New Zealand honeys. Studies in mice and rats indicated that for specified carcinoma and adenocarcinoma targets, an effective treatment of oral dosing of honey was statistically significant, and that immune system activation and metastasis prevention was a result of honey ingestion (Ghashm, Othman, Khattak, Ismail, & Saini, 2010).

Chapter 6: Summary

With a rising number of new cases of all types of cancer (and particularly hematologic malignancies), colorectal cancer contributed to a third of the cancer cases around the world. Most colorectal cancers are sporadic, but a significant portion (5-6%) have a clear genetic background. Colorectal cancer is a multi-step process involving the inactivation of a variety of tumour suppression and DNA-repair genes and simultaneous activation of certain oncogenes. Treatment of it usually comes with extremely adverse side effects, and unfortunately, effective cancer treatment that can contributed directly to the cancer cells without affecting the host is still unknown. Therefore, development of novel agents that could cure or prevent cancers and reduce adverse side effects are still very much needed.

Most anti-cancer agents act by induction of apoptosis, cell cycle arrest, as well as inhibition and proliferation of cell growth. This study indicates that both UMF+15 Manuka honey and Thyme honey have been shown to have anti-cancer activities and that they exert their effect through one of the aforementioned mechanisms. It is expected that the effects of honey on cell cycle, cell growth and proliferation, as well as induction of apoptosis in colon cancer cells, will provide clues for the prediction of novel agents that may be useful in cancer chemoprevention or chemotherapy.

Honey-induced apoptosis was associated with caspase-3 and caspase-9 activation, and PPAR cleavage. Our results shown an increase of apoptosis activity at high concentration of honey after 72 hours. The apoptosis mechanisms studied by other researchers suggested that honey-elevated caspase-3 activation and *poly polymerase* cleavages in colon cancer cells is attributed to honey's high phenolic content. Likewise, apoptosis through up-regulating the expression of pro- or anti-apoptotic proteins within the cancer cells also follows the caspase-8 or death receptor pathway and caspase-9 or mitochondrial pathway for such apoptotic activities.

Evidence is developing that honey might have the possibility to be an anti-cancer reagent through several mechanisms. In spite of the fact that the full components of honey are yet to be described, studies have demonstrated that honey has anti-cancer impact through its impedance of multiple cell-signalling pathways, such as inducing apoptotic, anti-proliferative, anti-inflammatory and anti-mutagenic pathways. Honey

modulates the body's immune system, and with different floral sources may provide different effects based on different targeted cancers.

Honey of different floral sources may give different health effects. More research is required in order to improve our understanding of the positive effects or correlation activities between honey and cancer behaviour. Results from cell culture experiments or animal testing may not apply directly to the human condition. Prospective or properly planned, randomised, and controlled clinical trials are expected to support the credibility of honey being used either alone or as an adjuvant therapy.

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Appendix

LoVo raw data section

Anti-proliferative effect of honey

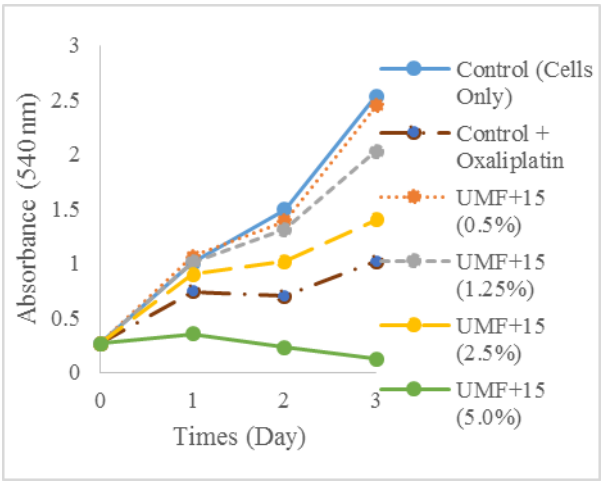


Figure 17: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on LoVo cells (Set 1).

Cells were treated with UMF+15 from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

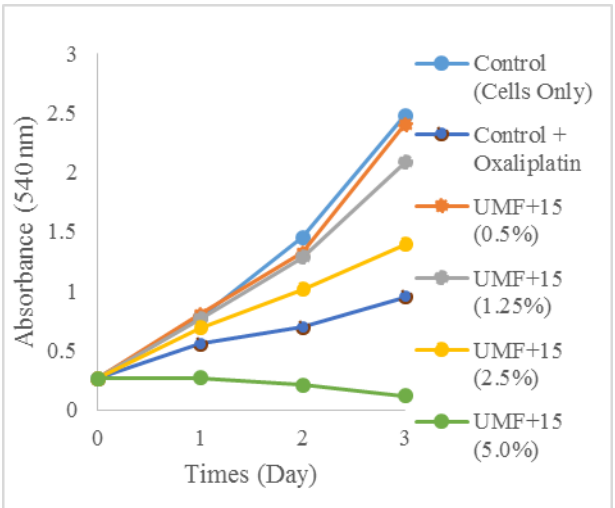


Figure 18: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on LoVo cells (Set 2).

Cells were treated with UMF+15 from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

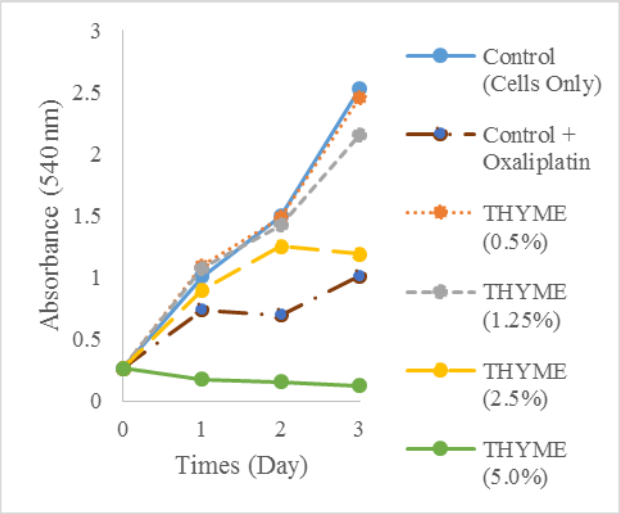
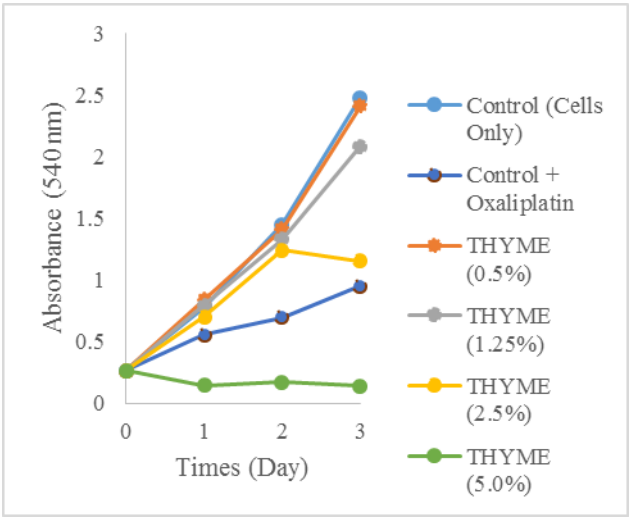


Figure 19: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on LoVo cells (Set 1).

Cells were treated with Thyme from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).



Cells were treated with Thyme from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

Figure 20: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on LoVo cells (Set 2).

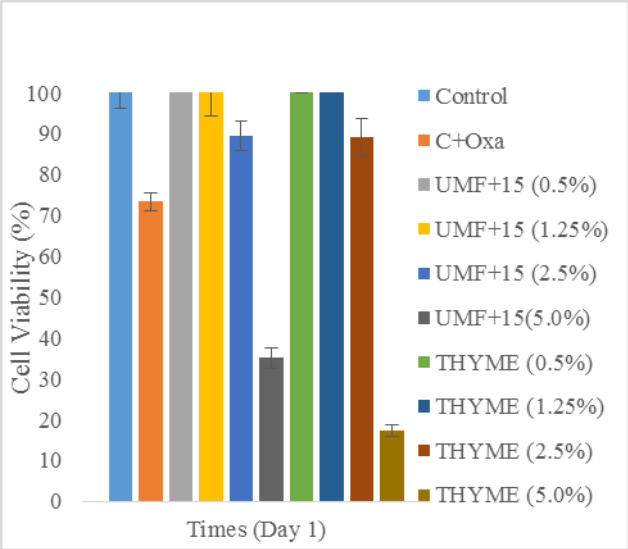


Figure 21: Cell viability (%) vs time (24 hours) on LoVo cells (Set 1).

Data were obtained as means ± standard errors (n=6).

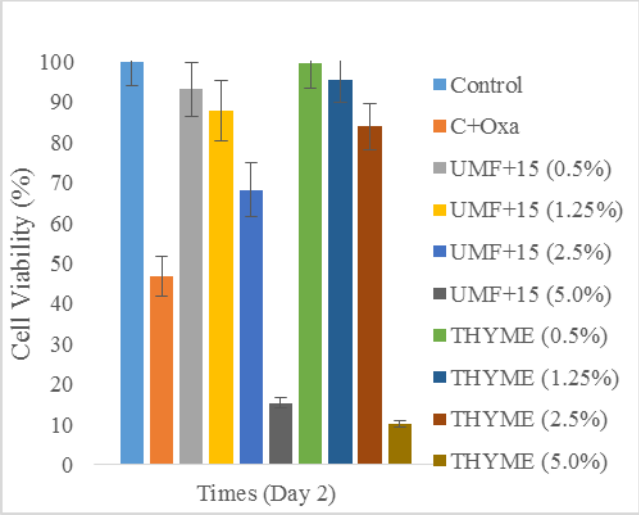


Figure 22: Cell viability (%) vs time (48 hours) on LoVo cells (Set 1).

Data were obtained as means ± standard errors (n=6).

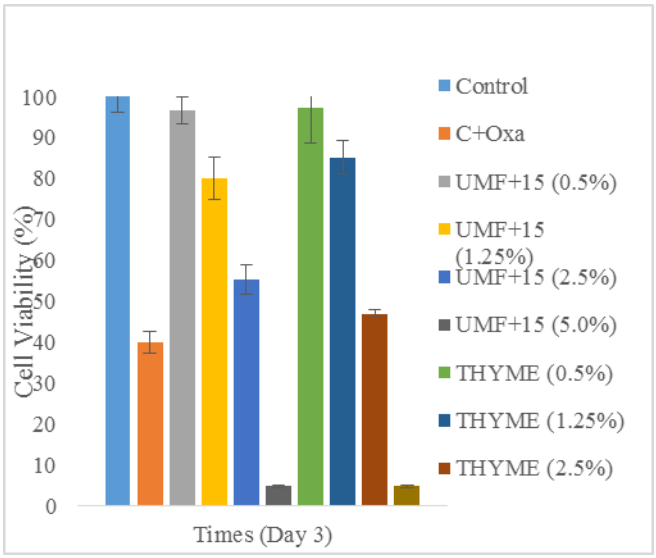


Figure 23: Cell viability (%) vs time (72 hours) on LoVo cells (Set 1).

Data were obtained as means ± standard errors (n=6).

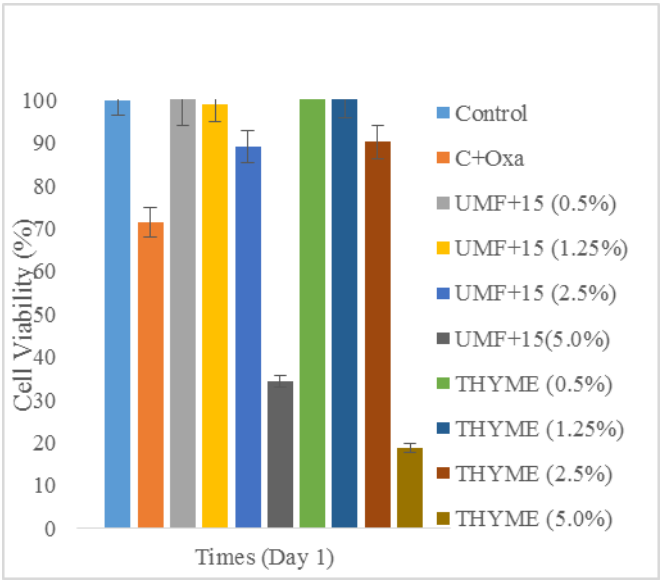


Figure 24: Cell viability (%) vs time (24 hours) on LoVo cells (Set 2).

Data were obtained as means ± standard errors (n=6).

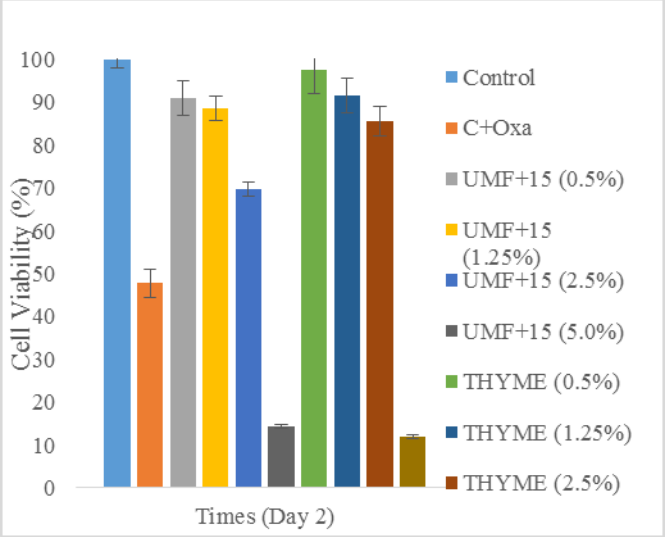


Figure 25: Cell viability (%) vs time (48 hours) on LoVo cells (Set 2).

Data were obtained as means ± standard errors (n=6).

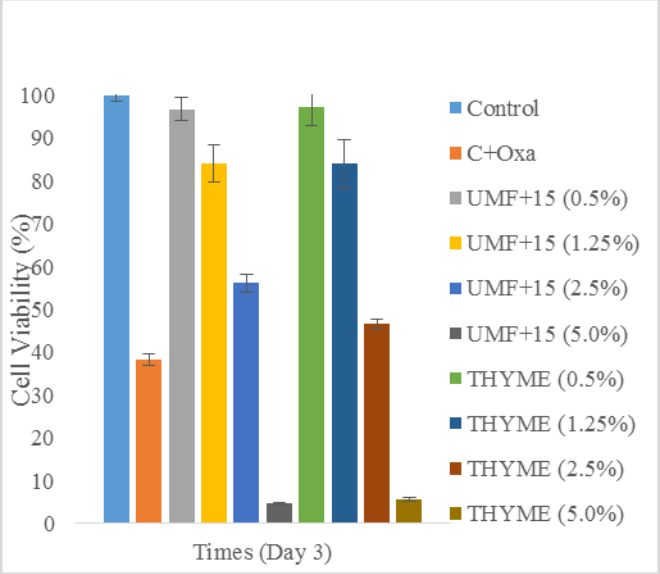


Figure 26: Cell viability (%) vs time (72 hours) on LoVo cells (Set 2).

Data were obtained as means ± standard errors (n=6).

Statistical data analysis by Prism

Table 17: Control Comparison (1way ANOVA)

Table Analyzed	Control Comparison				
ANOVA summary					
F	951.4				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.983				
Brown-Forsythe test					
F (DFn, DFd)	9.563 (2, 33)				
P value	0.0005				
P value summary	***				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	5.047				
P value	0.0802				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	16.09	2	8.045	F (2, 33) = 951.4	P < 0.0001
Residual (within columns)	0.279	33	0.008456		
Total	16.37	35			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	36				

Table 18: Control Comparison (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	2
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control (Day 1) vs. Control (Day 2)	-0.582	-0.6688 to -0.4953	Yes	****
Control (Day 1) vs. Control (Day 3)	-1.617	-1.703 to -1.530	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control (Day 1) vs. Control (Day 2)	0.8913	1.473	-0.582	0.03754	12	12	15.5	33
Control (Day 1) vs. Control (Day 3)	0.8913	2.508	-1.617	0.03754	12	12	43.06	33

Table 19: UMF+15 vs Control Day 1 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 1				
ANOVA summary					
F	462.7				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9711				
Brown-Forsythe test					
F (DFn, DFd)	2.776 (4, 55)				
P value	0.0358				
P value summary	*				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	19.97				
P value	0.0005				
P value summary	***				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	40476	4	10119	F (4, 55) = 462.7	P < 0.0001
Residual (within columns)	1203	55	21.87		
Total	41679	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 20: UMF+15 vs Control Day 1 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	-4.77	-9.701 to 0.1603	No	ns
Control vs. UMF+15 1.25%	0.2943	-4.636 to 5.225	No	ns
Control vs. UMF+15 2.5%	10.75	5.817 to 15.68	Yes	****
Control vs. UMF+15 5%	65.25	60.32 to 70.18	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	104.8	-4.77	1.909	12	12	2.499	55
Control vs. UMF+15 1.25%	100	99.71	0.2943	1.909	12	12	0.1542	55
Control vs. UMF+15 2.5%	100	89.25	10.75	1.909	12	12	5.629	55
Control vs. UMF+15 5%	100	34.75	65.25	1.909	12	12	34.18	55

Table 21: UMF+15 vs Control Day 2 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 2
ANOVA summary	
F	717.7
P value	< 0.0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.9812
Brown-Forsythe test	
F (DFn, DFd)	2.953 (4, 55)
P value	0.0278
P value summary	*
Significantly different standard deviations? (P < 0.05)	Yes
Bartlett's test	
Bartlett's statistic (corrected)	21.93
P value	0.0002
P value summary	***
Significantly different standard deviations? (P < 0.05)	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	56591	4	14148	F (4, 55) = 717.7	P < 0.0001
Residual (within columns)	1084	55	19.71		
Total	57676	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 22: UMF+15 vs Control Day 2 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	7.911	3.230 to 12.59	Yes	***
Control vs. UMF+15 1.25%	11.82	7.135 to 16.50	Yes	****
Control vs. UMF+15 2.5%	31	26.32 to 35.68	Yes	****
Control vs. UMF+15 5%	85.1	80.42 to 89.79	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	92.09	7.911	1.813	12	12	4.364	55
Control vs. UMF+15 1.25%	100	88.18	11.82	1.813	12	12	6.518	55
Control vs. UMF+15 2.5%	100	69	31	1.813	12	12	17.1	55
Control vs. UMF+15 5%	100	14.9	85.1	1.813	12	12	46.95	55

Table 23: UMF+15 vs Control Day 3 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 3
ANOVA summary	
F	1924
P value	< 0.0001
P value summary	****
Are differences among means statistically significant? (P < 0.05)	Yes
R square	0.9929
Brown-Forsythe test	
F (DFn, DFd)	4.588 (4, 55)
P value	0.0029
P value summary	**
Significantly different standard deviations? (P < 0.05)	Yes
Bartlett's test	
Bartlett's statistic (corrected)	45.37
P value	< 0.0001
P value summary	****
Significantly different standard deviations? (P < 0.05)	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	74397	4	18599	F (4, 55) = 1924	P < 0.0001
Residual (within columns)	531.7	55	9.668		
Total	74928	59			

Data summary	
Number of treatments (columns)	5
Number of values (total)	60

Table 24: UMF+15 vs Control Day 3 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	3.278	0.0001583 to 6.556	Yes	*
Control vs. UMF+15 1.25%	17.97	14.69 to 21.25	Yes	****
Control vs. UMF+15 2.5%	44.32	41.04 to 47.60	Yes	****
Control vs. UMF+15 5%	95.26	91.99 to 98.54	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	96.72	3.278	1.269	12	12	2.583	55
Control vs. UMF+15 1.25%	100	82.03	17.97	1.269	12	12	14.15	55
Control vs. UMF+15 2.5%	100	55.68	44.32	1.269	12	12	34.91	55
Control vs. UMF+15 5%	100	4.736	95.26	1.269	12	12	75.05	55

Table 25: Thyme vs Control Day 1 (1way ANOVA)

Table Analyzed	THYME vs Control Day 1				
ANOVA summary					
F	716.3				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9812				
Brown-Forsythe test					
F (DFn, DFd)	2.339 (4, 55)				
P value	0.0664				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	25.13				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	68402	4	17100	F (4, 55) = 716.3	P < 0.0001
Residual (within columns)	1313	55	23.87		
Total	69715	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 26: Thyme vs Control Day 1 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. THYME 0.5%	-9.011	-14.16 to -3.860	Yes	***
Control vs. THYME 1.25%	-4.824	-9.975 to 0.3279	No	ns
Control vs. THYME 2.5%	10.3	5.149 to 15.45	Yes	****
Control vs. THYME 5%	81.98	76.83 to 87.13	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	109	-9.011	1.995	12	12	4.517	55
Control vs. THYME 1.25%	100	104.8	-4.824	1.995	12	12	2.418	55
Control vs. THYME 2.5%	100	89.7	10.3	1.995	12	12	5.164	55
Control vs. THYME 5%	100	18.02	81.98	1.995	12	12	41.1	55

Table 27: Thyme vs Control Day 2 (1way ANOVA)

Table Analyzed	THYME vs Control Day 2				
ANOVA summary					
F	891.9				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9848				
Brown-Forsythe test					
F (DFn, DFd)	2.911 (4, 55)				
P value	0.0295				
P value summary	*				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	23.33				
P value	0.0001				
P value summary	***				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	68101	4	17025	F (4, 55) = 891.9	P < 0.0001
Residual (within columns)	1050	55	19.09		
Total	69151	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 28: Thyme vs Control Day 2 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant ?	Summary
Control vs. THYME 0.5%	1.493	-3.113 to 6.099	No	ns
Control vs. THYME 1.25%	6.428	1.821 to 11.03	Yes	**
Control vs. THYME 2.5%	15.31	10.70 to 19.92	Yes	****
Control vs. THYME 5%	88.96	84.36 to 93.57	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	98.51	1.493	1.784	12	12	0.837	55
Control vs. THYME 1.25%	100	93.57	6.428	1.784	12	12	3.604	55
Control vs. THYME 2.5%	100	84.69	15.31	1.784	12	12	8.584	55
Control vs. THYME 5%	100	11.04	88.96	1.784	12	12	49.88	55

Table 29: Thyme vs Control Day 3 (1way ANOVA)

Table Analyzed	THYME vs Control Day 3				
ANOVA summary					
F	1315				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9897				
Brown-Forsythe test					
F (DFn, DFd)	8.158 (4, 55)				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	60.58				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	78456	4	19614	F (4, 55) = 1315	P < 0.0001
Residual (within columns)	820.3	55	14.92		
Total	79276	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 30: Thyme vs Control Day 3 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant ?	Summary
Control vs. THYME 0.5%	2.78	-1.291 to 6.852	No	ns
Control vs. THYME 1.25%	15.38	11.31 to 19.45	Yes	****
Control vs. THYME 2.5%	53.24	49.17 to 57.32	Yes	****
Control vs. THYME 5%	94.77	90.70 to 98.84	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	97.22	2.78	1.577	12	12	1.763	55
Control vs. THYME 1.25%	100	84.62	15.38	1.577	12	12	9.756	55
Control vs. THYME 2.5%	100	46.76	53.24	1.577	12	12	33.77	55
Control vs. THYME 5%	100	5.228	94.77	1.577	12	12	60.11	55

Table 31: Transform of LoVo IC50 with UMF+15 and Thyme honey.

Transform of UMF+15 IC_{50}	
log(inhibitor) vs. response -- Variable slope (four parameters)	Cell Viability (%)
Best-fit values	
Bottom	-1.169
Top	101.5
LogIC50	0.4121
HillSlope	-3.037
IC50	2.583
Span	102.7
Std. Error	
Bottom	6.422
Top	2.877
LogIC50	0.03767
HillSlope	0.7675
Span	7.543
95% Confidence Intervals	
Bottom	-19.00 to 16.66
Top	93.52 to 109.5
LogIC50	0.3075 to 0.5167
HillSlope	-5.168 to -0.9066
IC50	2.030 to 3.286
Span	81.74 to 123.6
Goodness of Fit	
Degrees of Freedom	4
R square	0.9908
Absolute Sum of Squares	120.8
Sy.x	5.495
Number of points	
Analyzed	8

Transform of THYME IC_{50}	
log(inhibitor) vs. response -- Variable slope (four parameters)	Cell Viability (%)
Best-fit values	
Bottom	0.6798
Top	99.45
LogIC50	0.3629
HillSlope	-3.243
IC50	2.306
Span	98.77
Std. Error	
Bottom	3.052
Top	1.636
LogIC50	0.01951
HillSlope	0.4591
Span	3.699
95% Confidence Intervals	
Bottom	-7.793 to 9.153
Top	94.91 to 104.0
LogIC50	0.3087 to 0.4171
HillSlope	-4.517 to -1.969
IC50	2.036 to 2.613
Span	88.50 to 109.0
Goodness of Fit	
Degrees of Freedom	4
R square	0.997
Absolute Sum of Squares	38.21
Sy.x	3.091
Number of points	
Analyzed	8

Apoptotic effect of honey

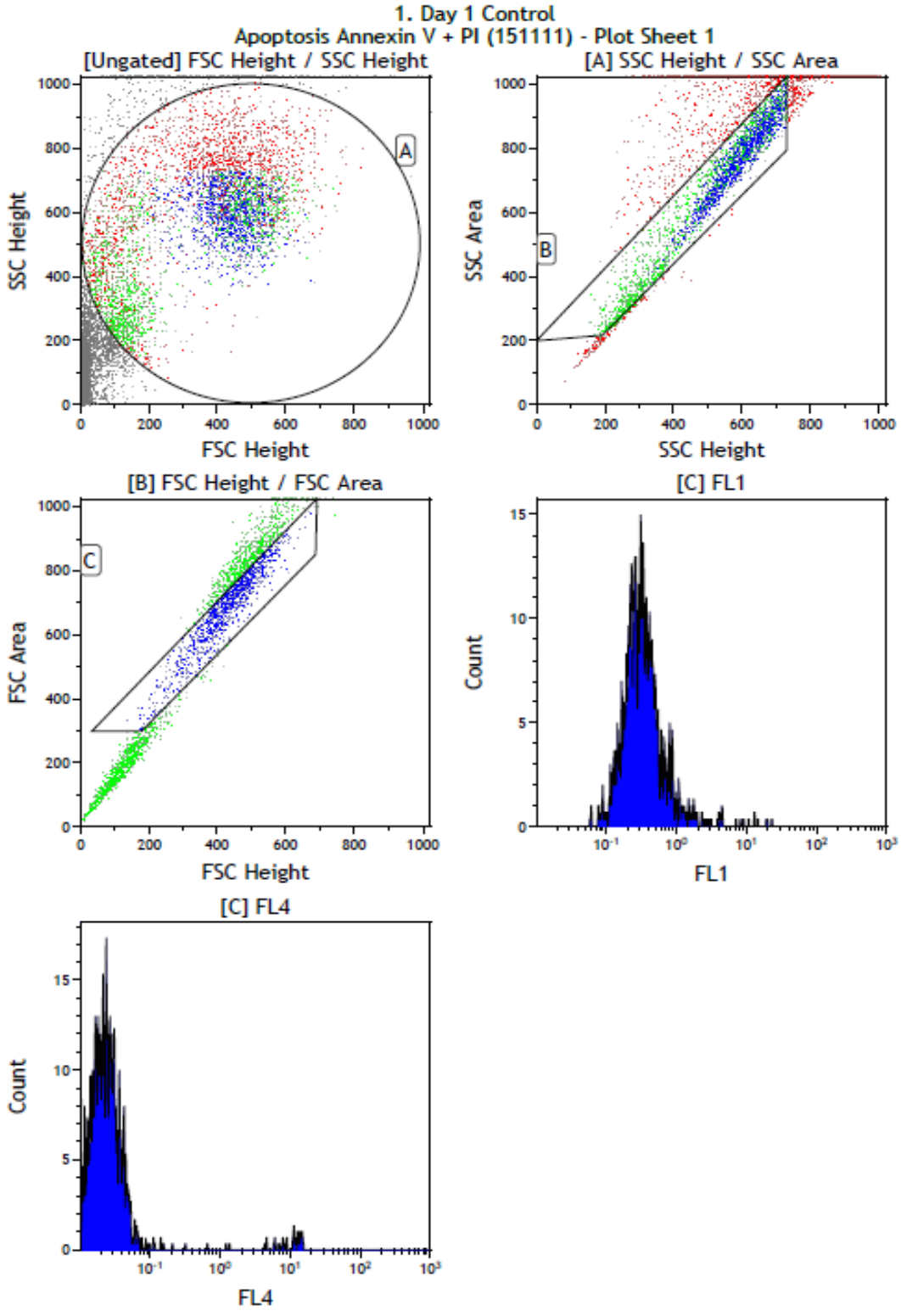


Figure 27: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 24 hours (Set 1).

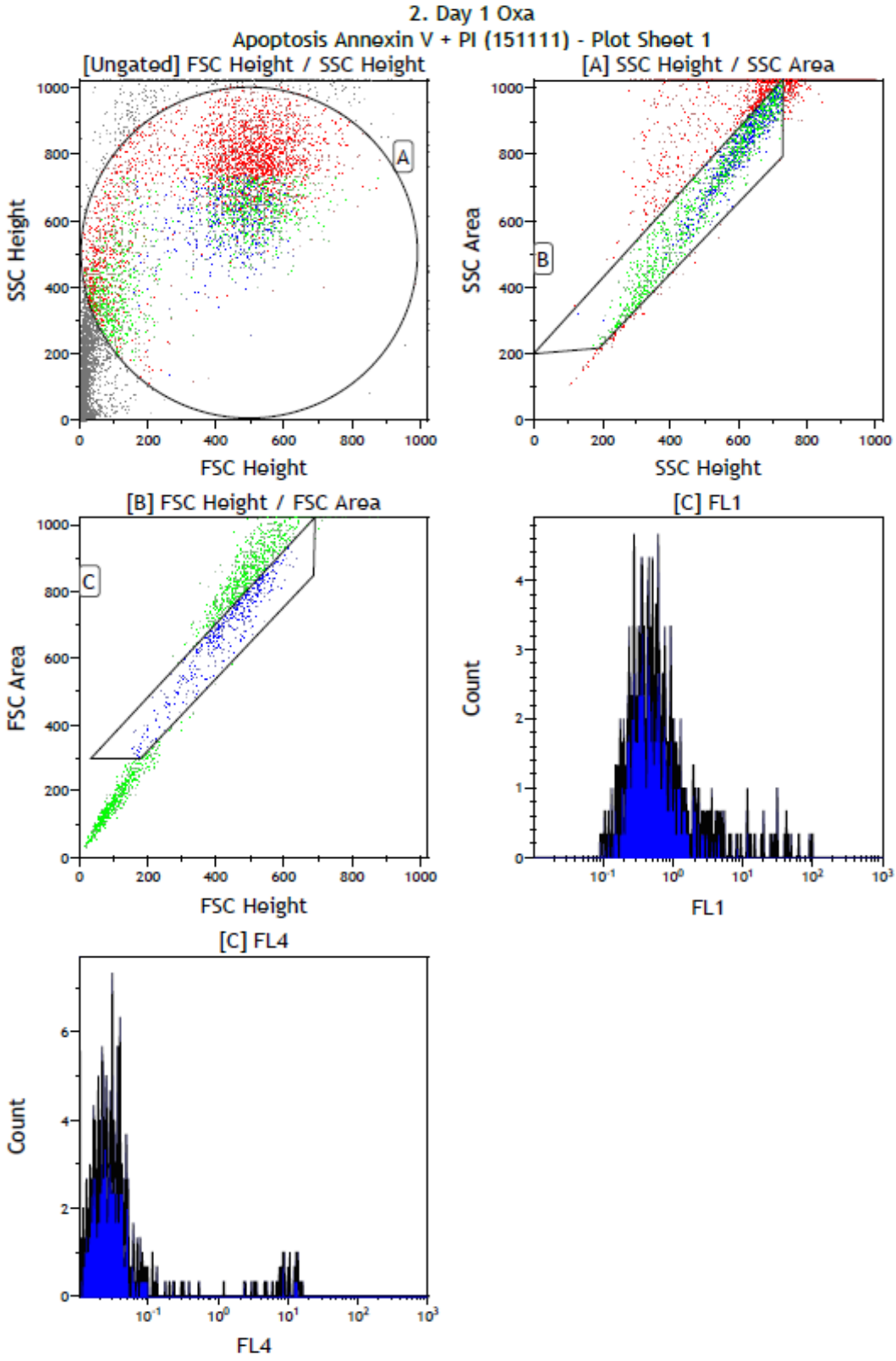


Figure 28: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 24 hours (Set 1).

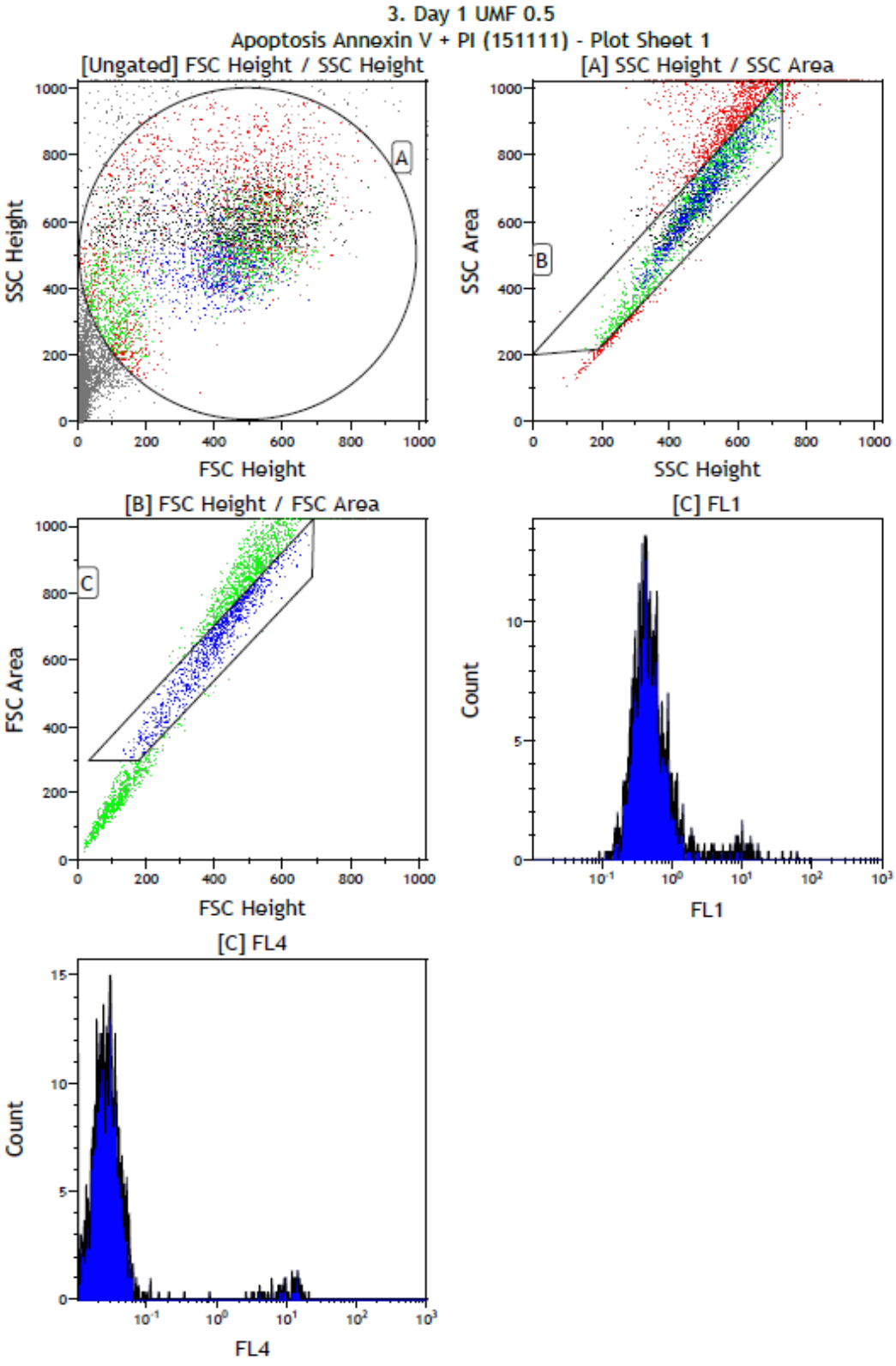


Figure 29: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 24 hours (Set 1).

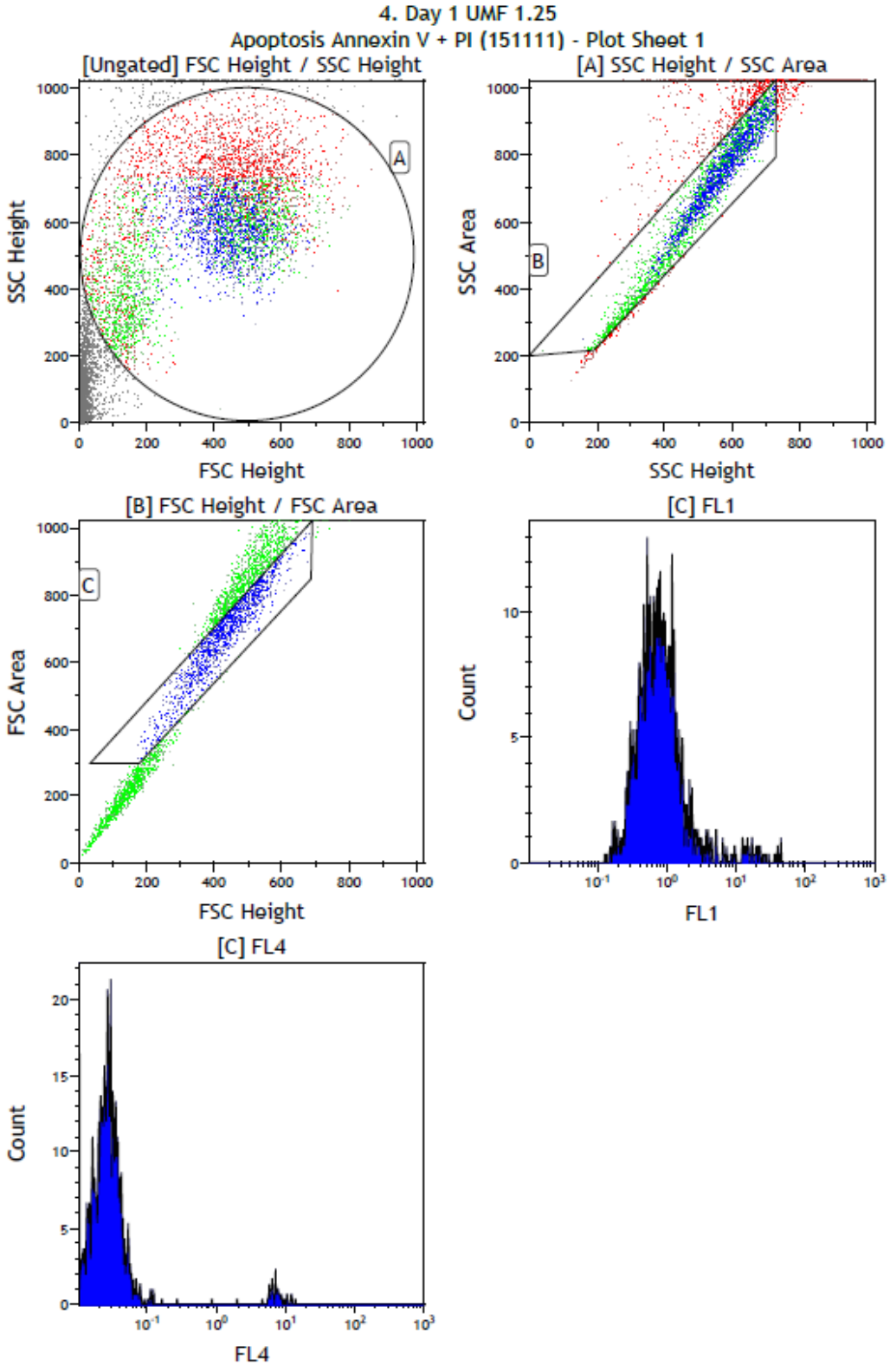


Figure 30: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 24 hours (Set 1).

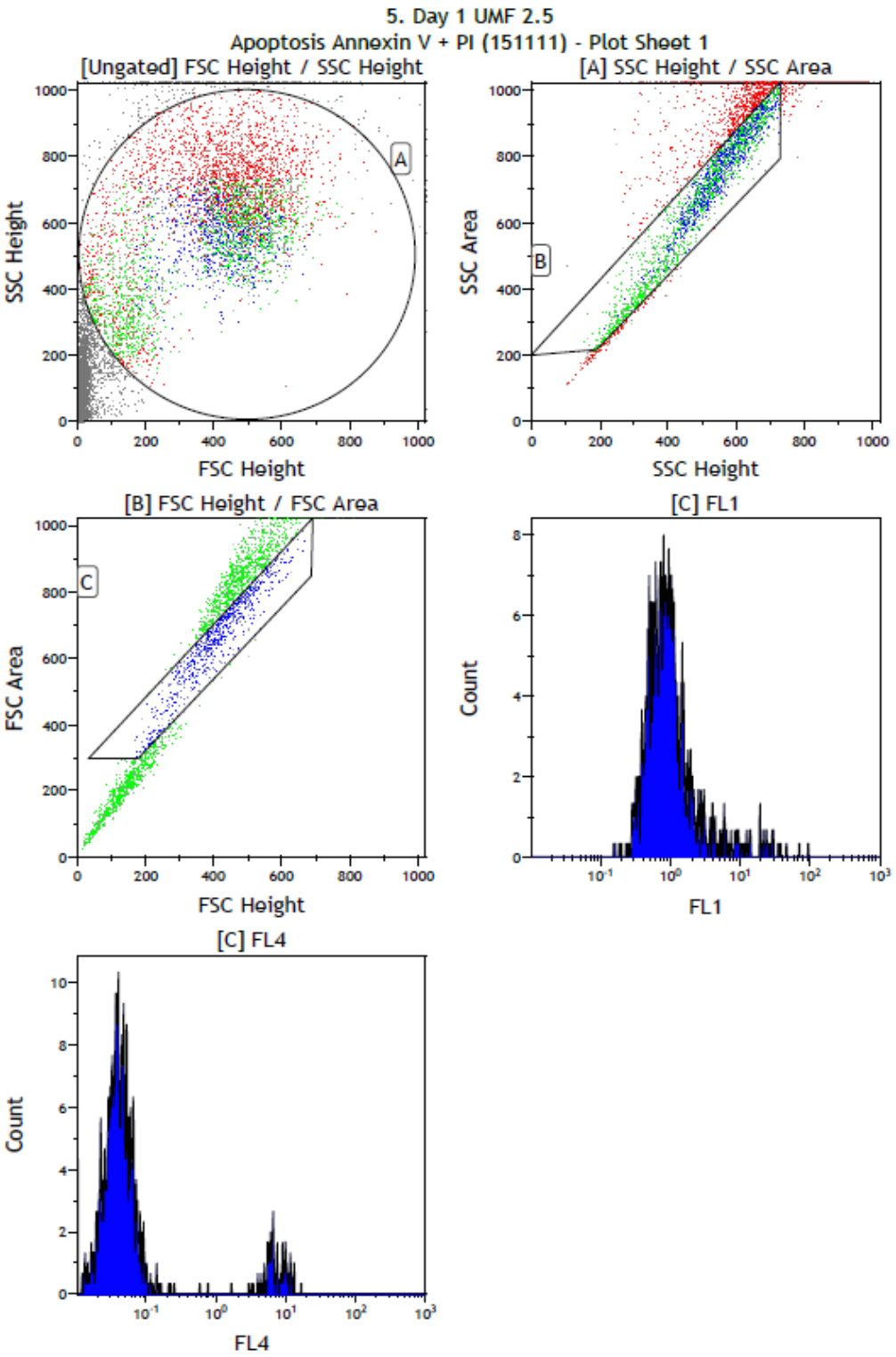


Figure 31: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 24 hours (Set 1).

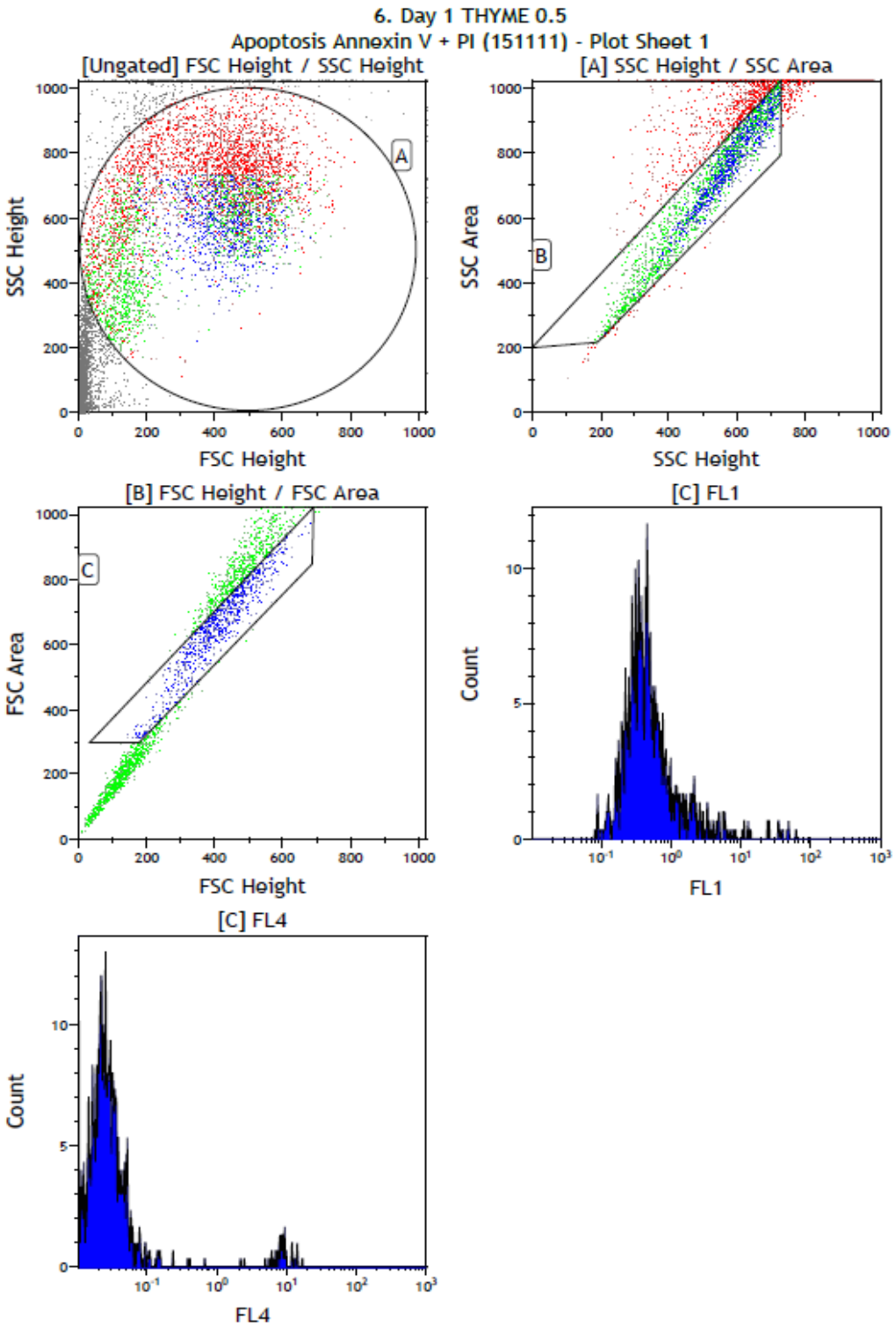


Figure 32: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 24 hours (Set 1).

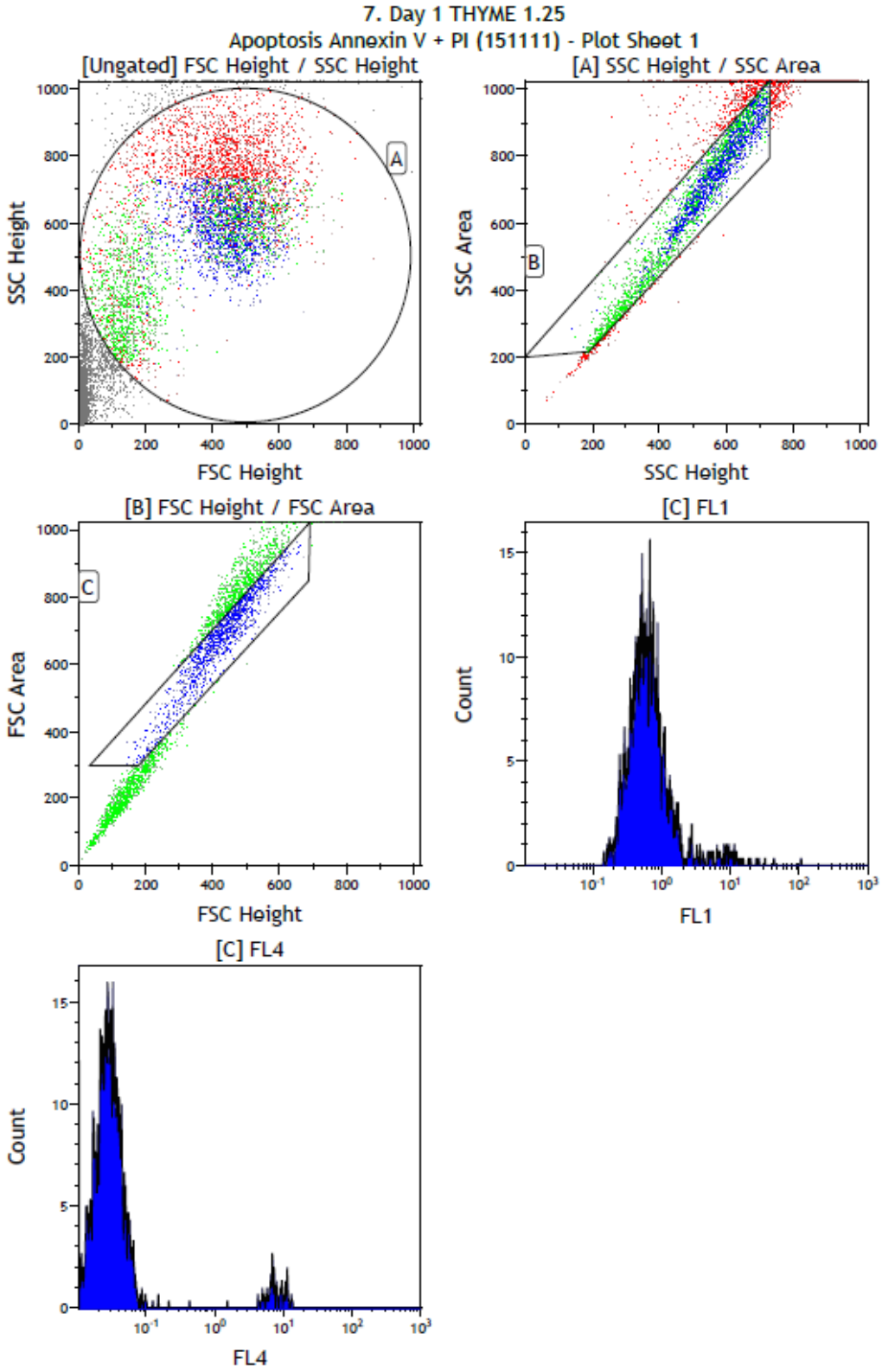


Figure 33: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 24 hours (Set 1).

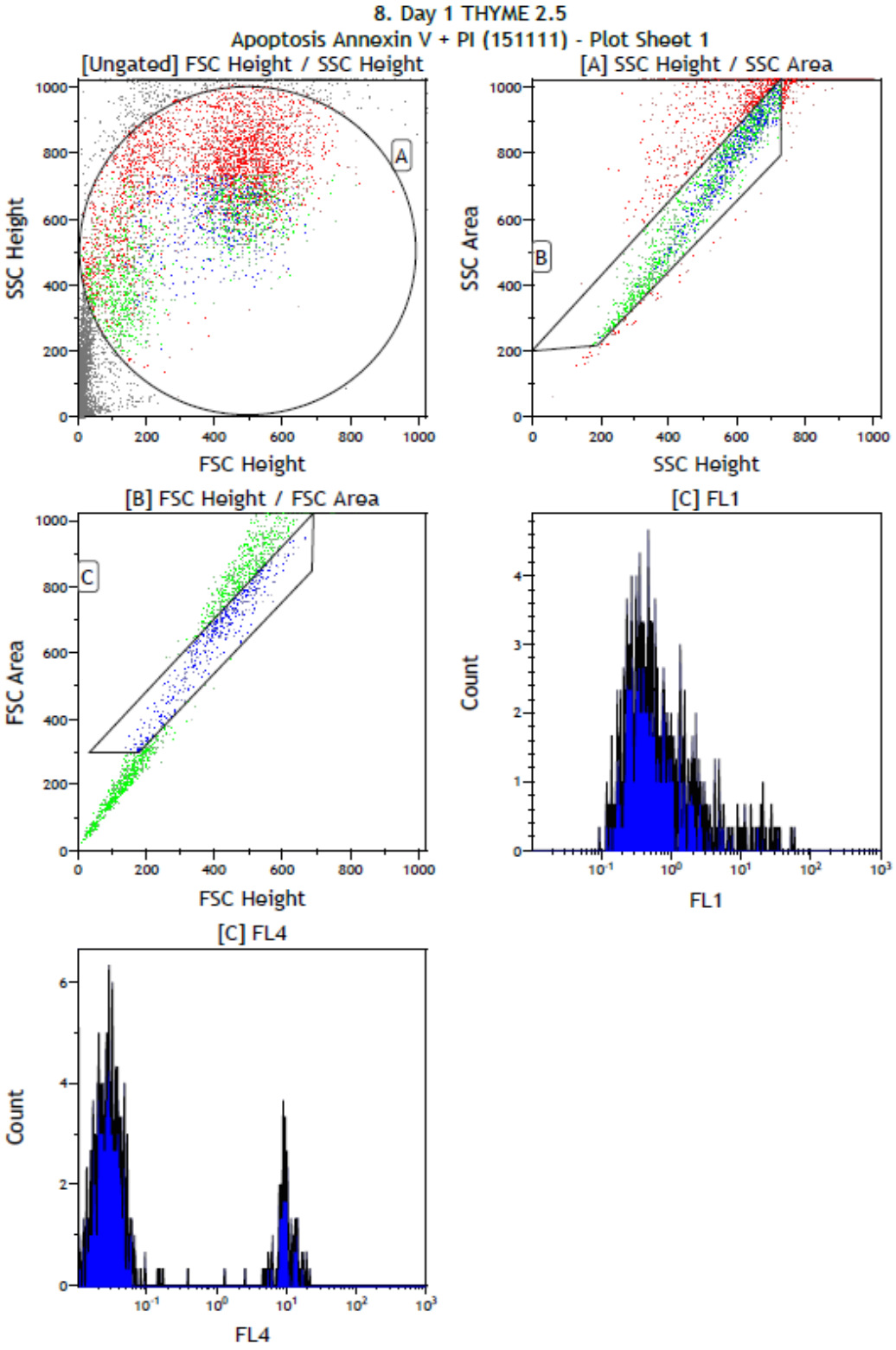


Figure 34: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 24 hours (Set 1).

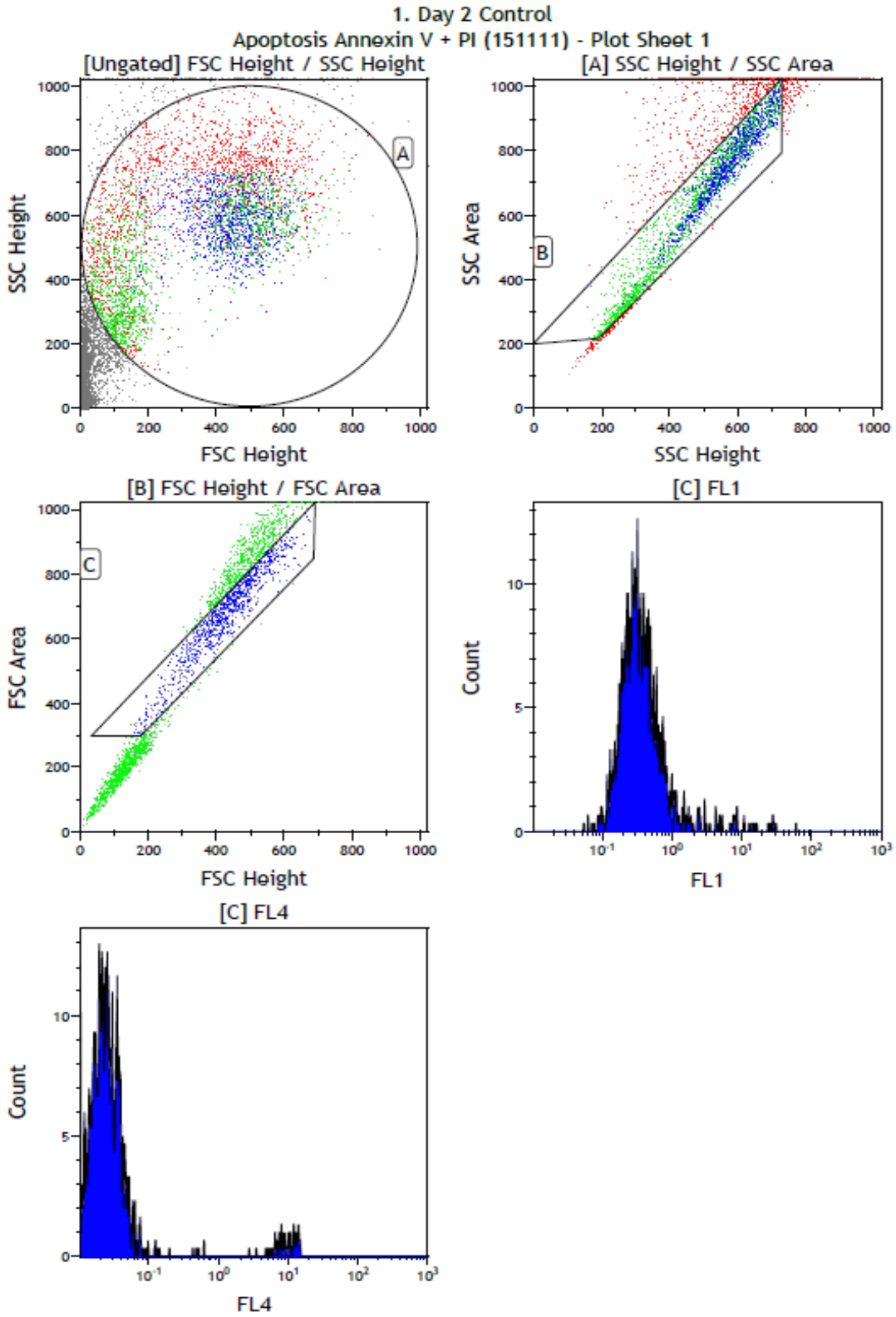


Figure 35: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 48 hours (Set 1).

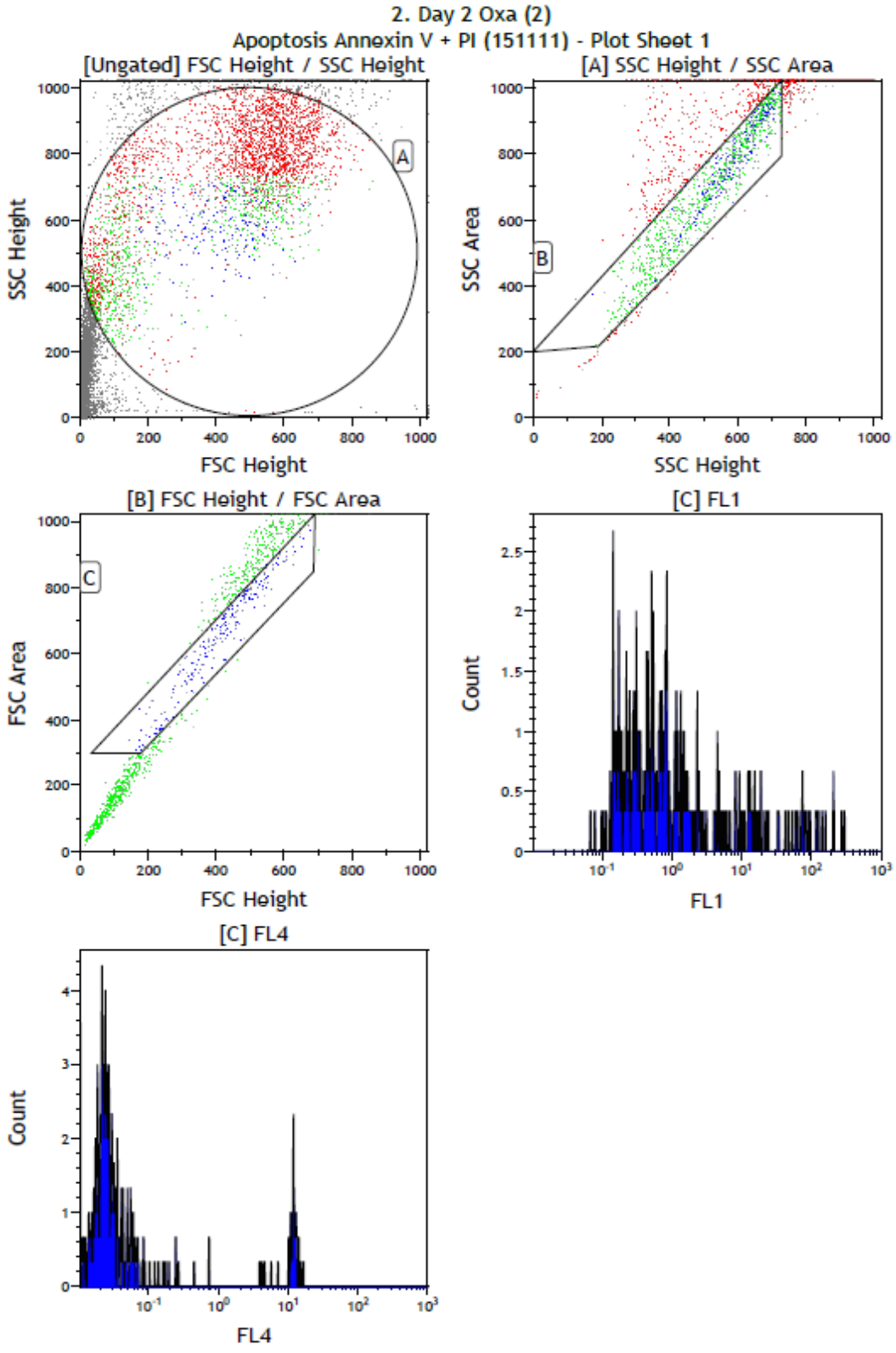


Figure 36: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 48 hours (Set 1).

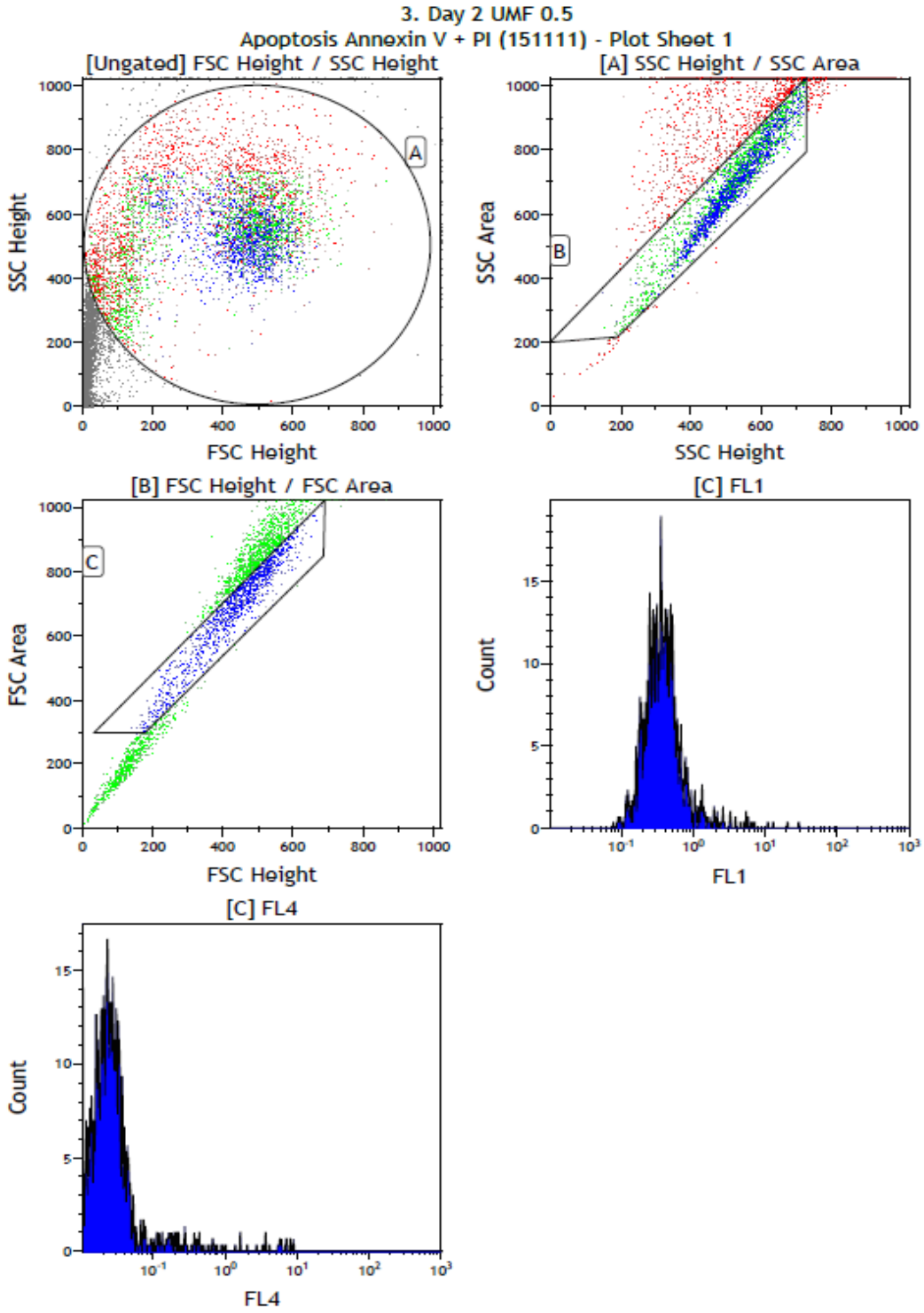


Figure 37: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 48 hours (Set 1).

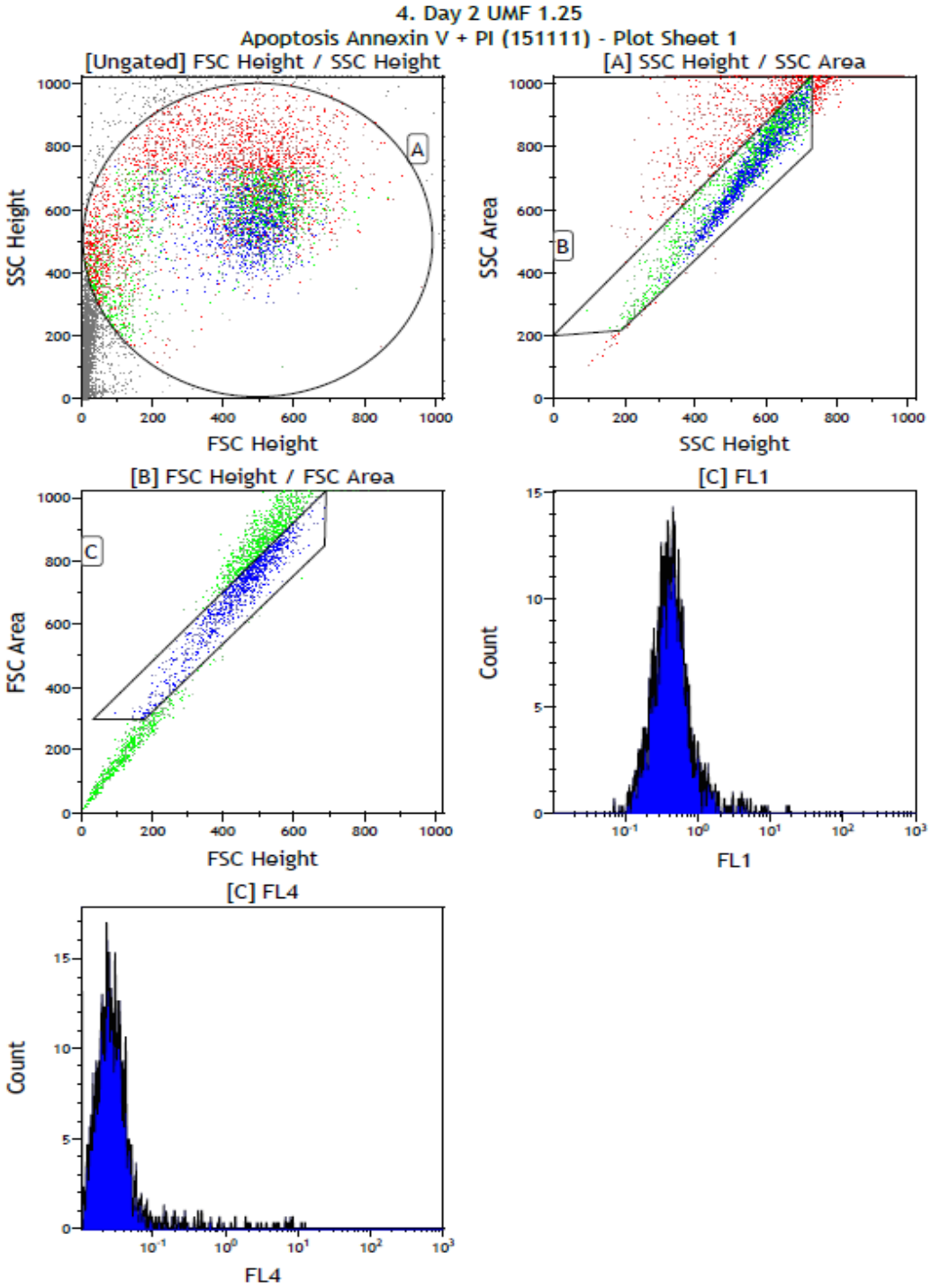


Figure 38: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 48 hours (Set 1).

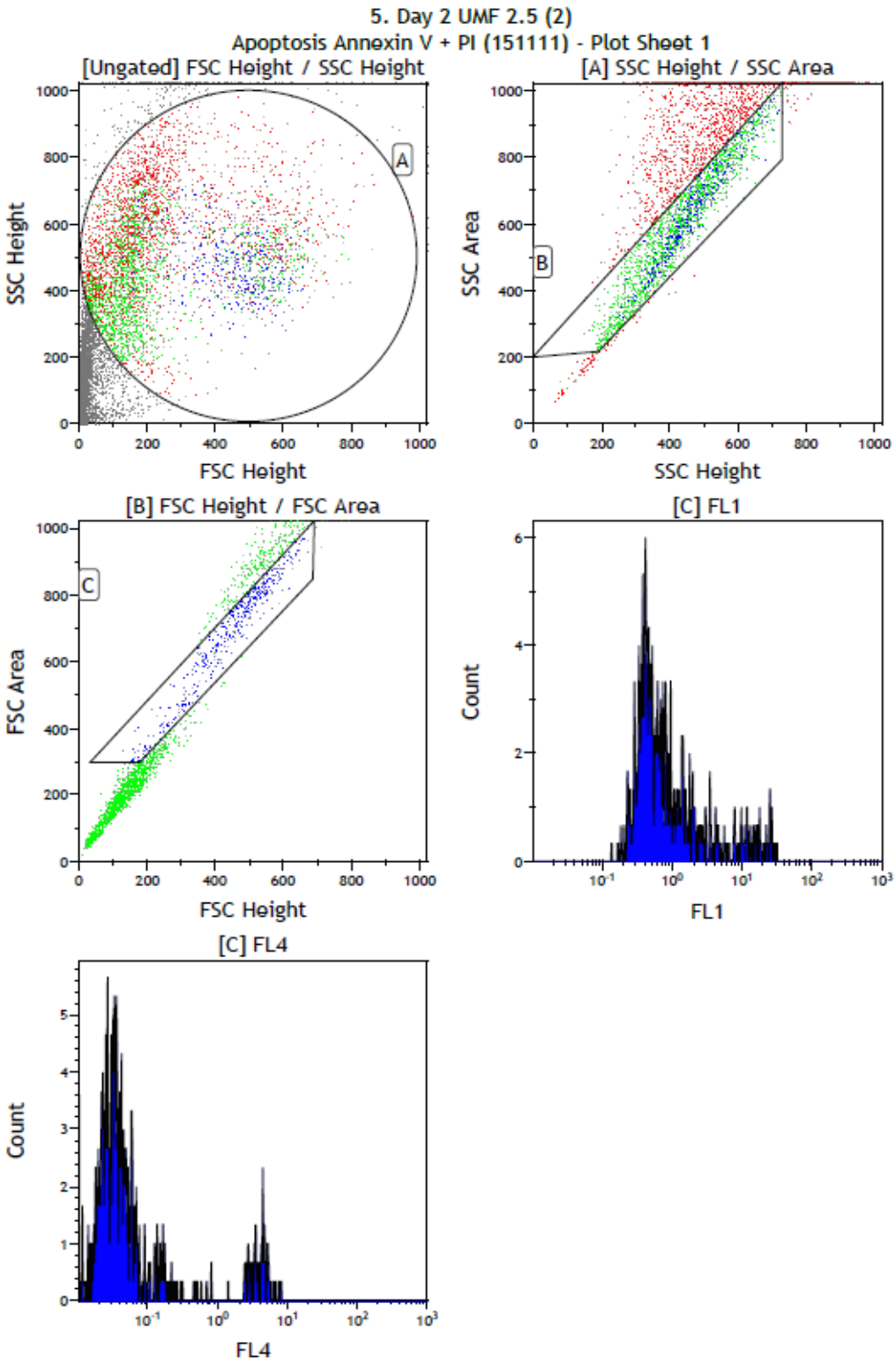


Figure 39: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 48 hours (Set 1).

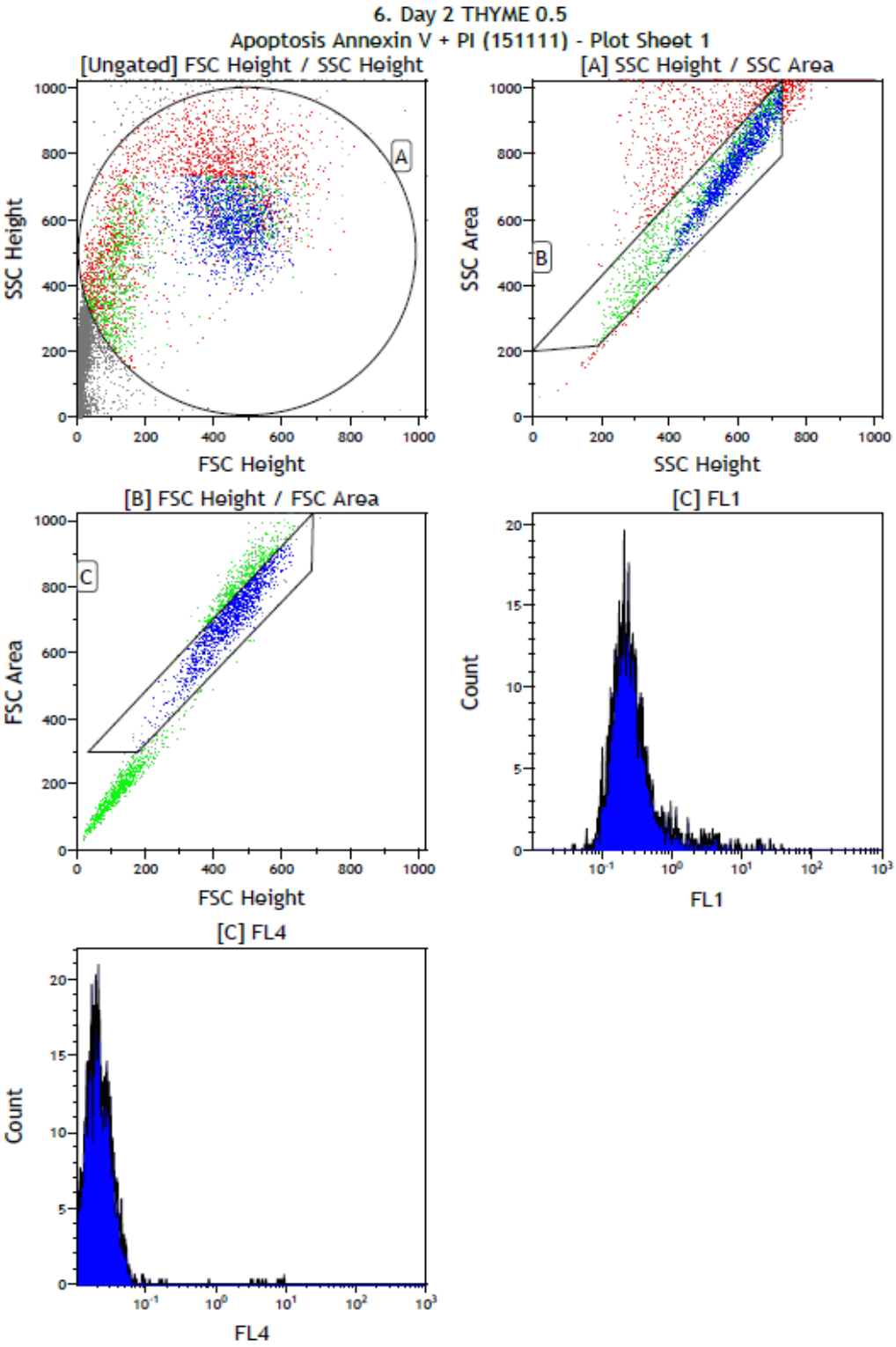


Figure 40: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 48 hours (Set 1).

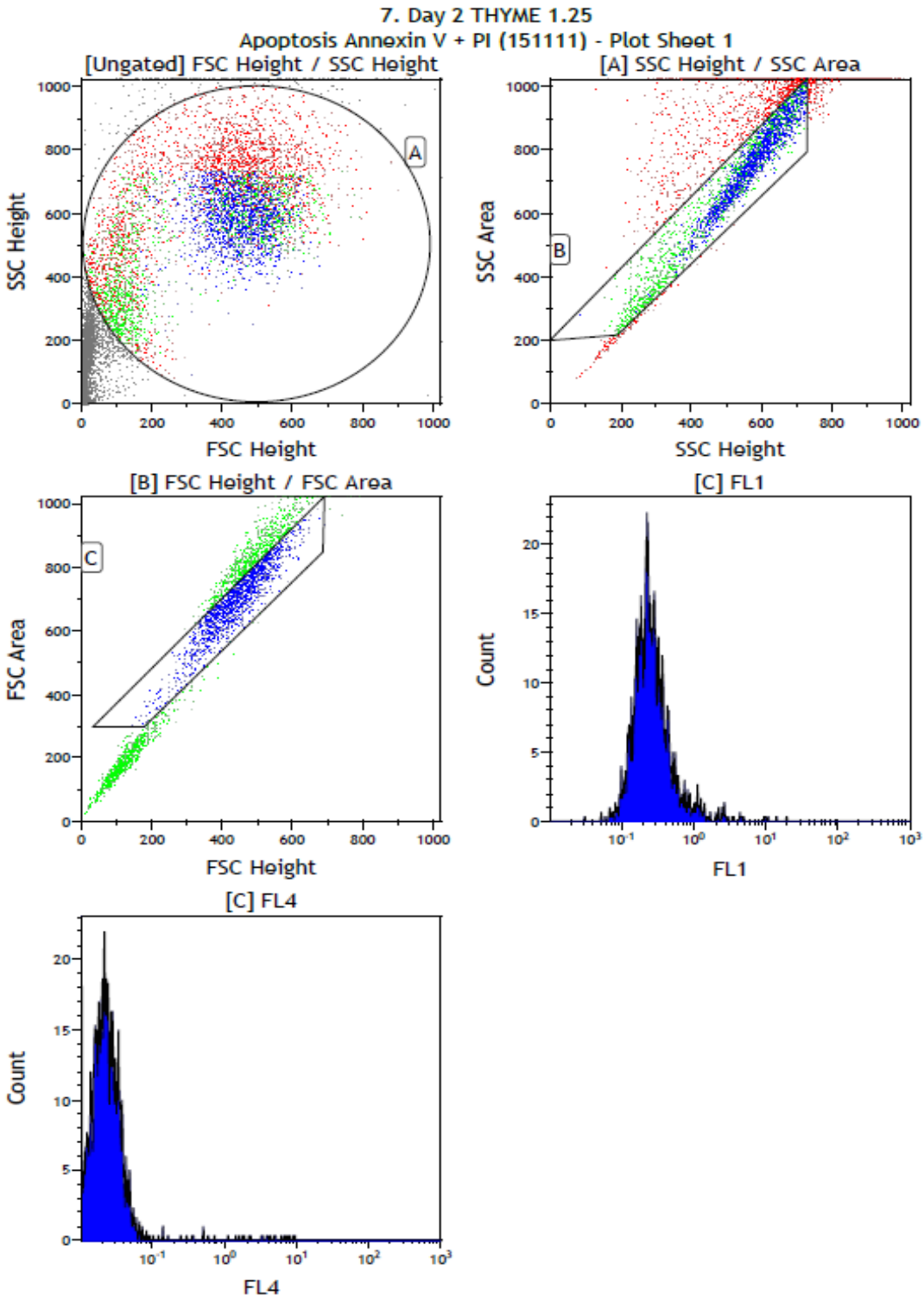


Figure 41: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 48 hours (Set 1).

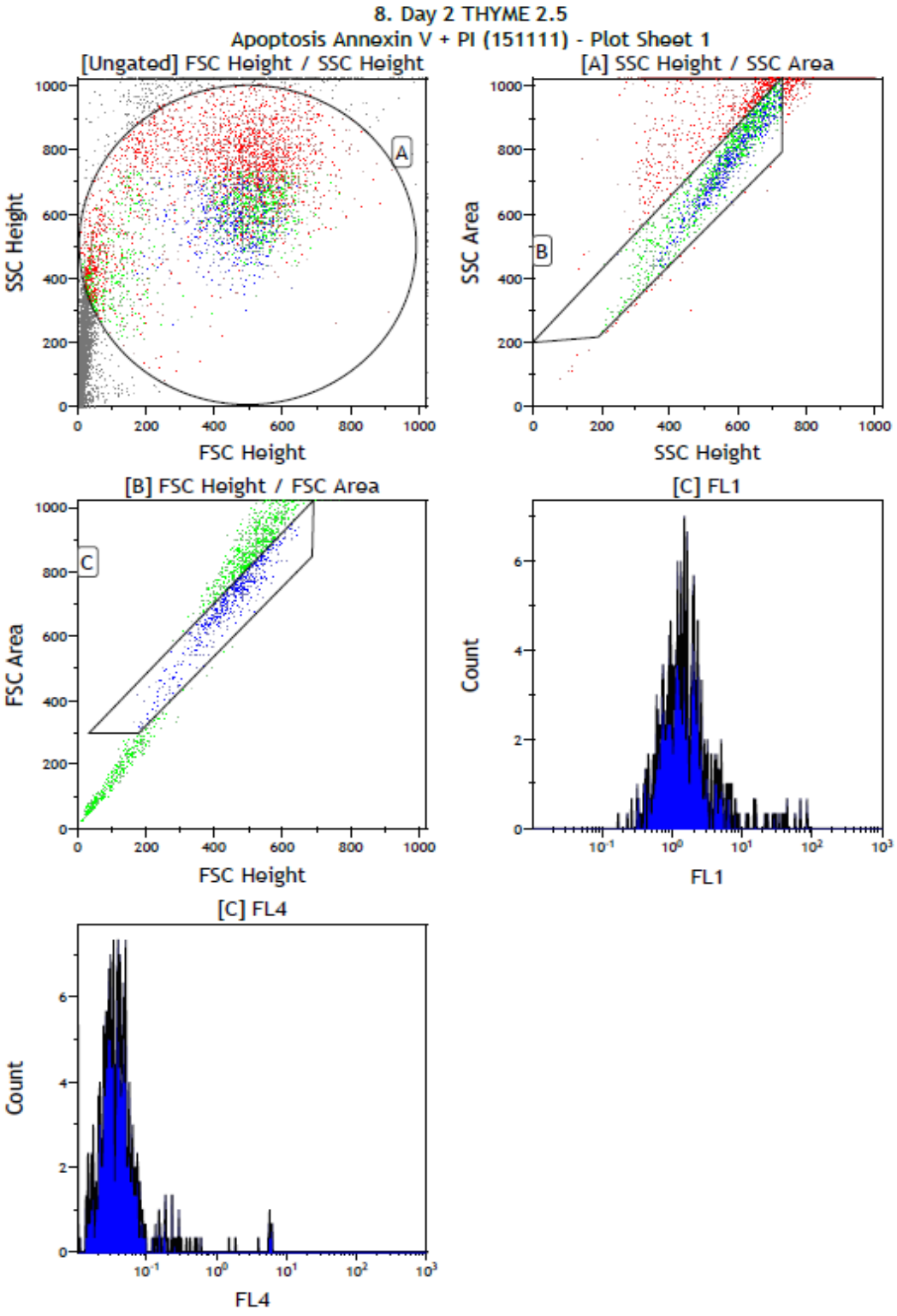


Figure 42: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 48 hours (Set 1).

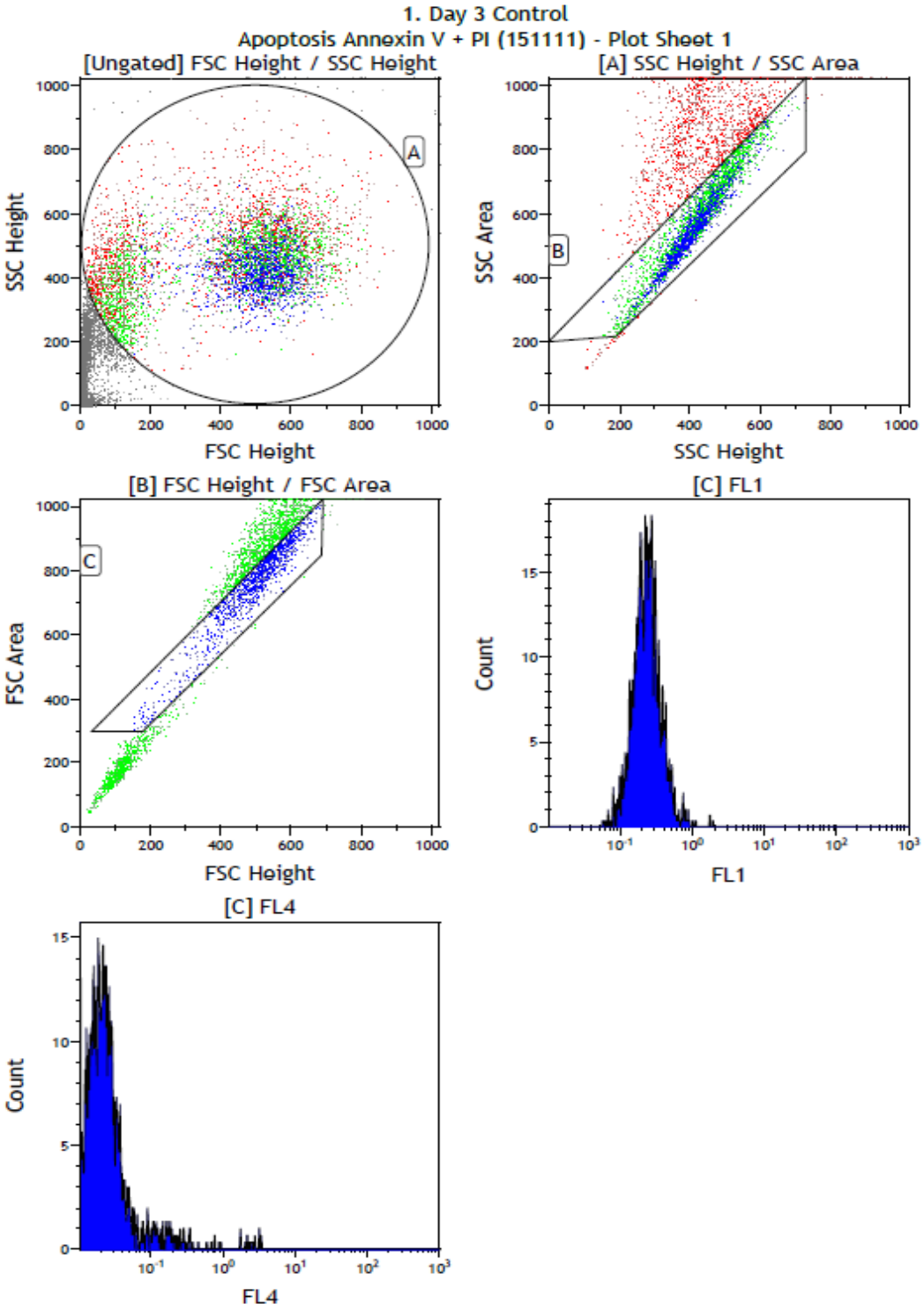


Figure 43: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 72 hours (Set 1).

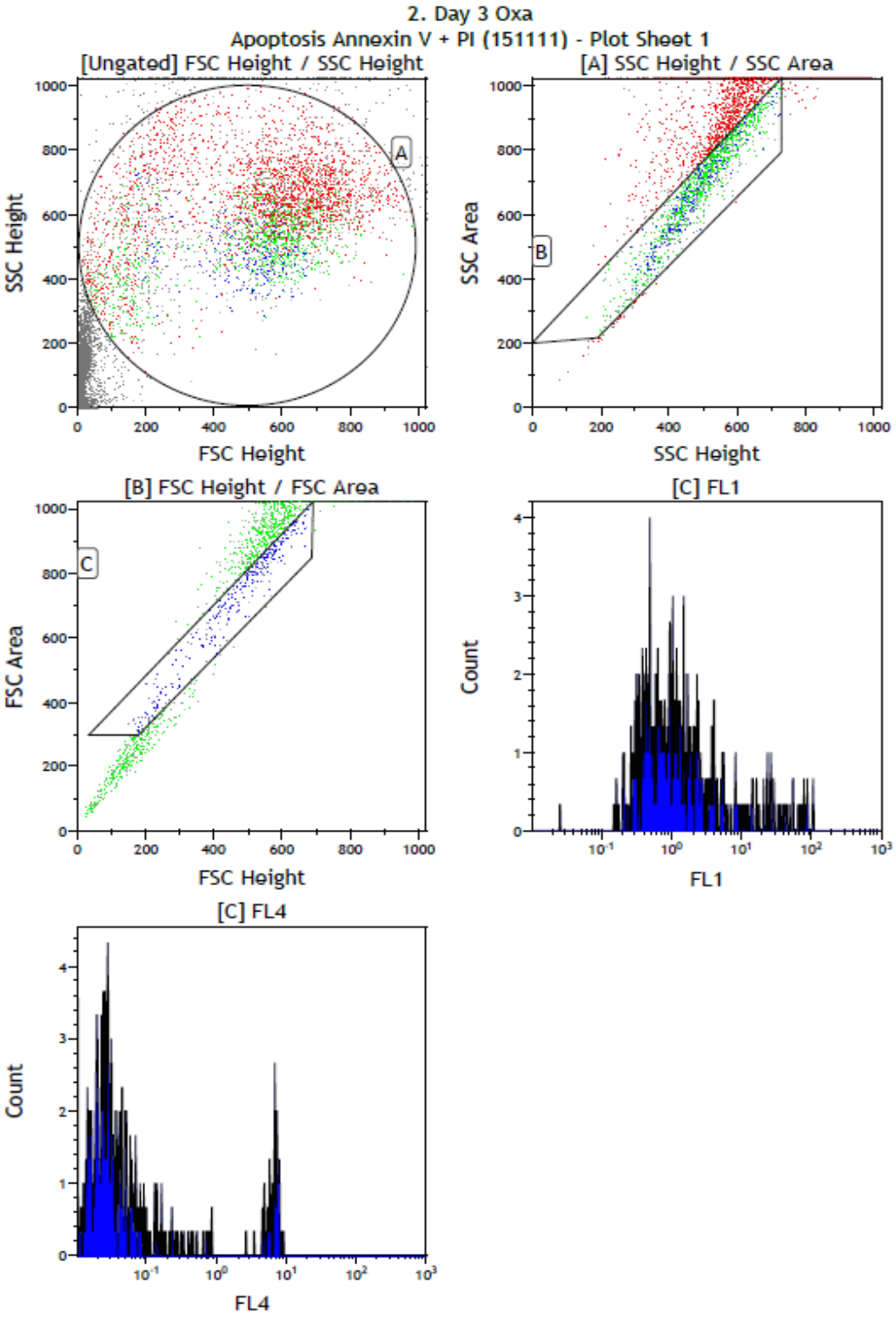


Figure 44: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 72 hours (Set 1).

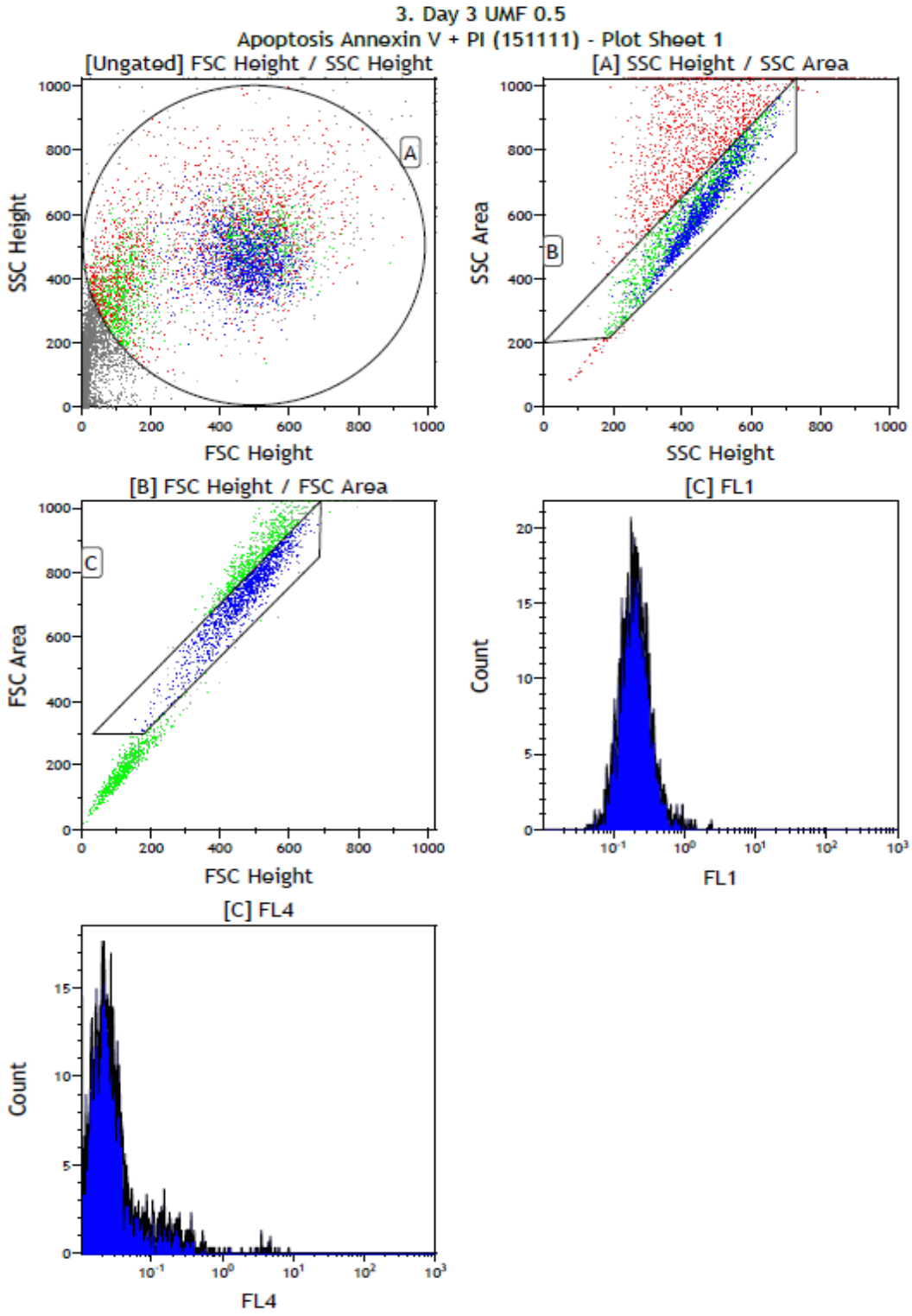


Figure 45: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 72 hours (Set 1).

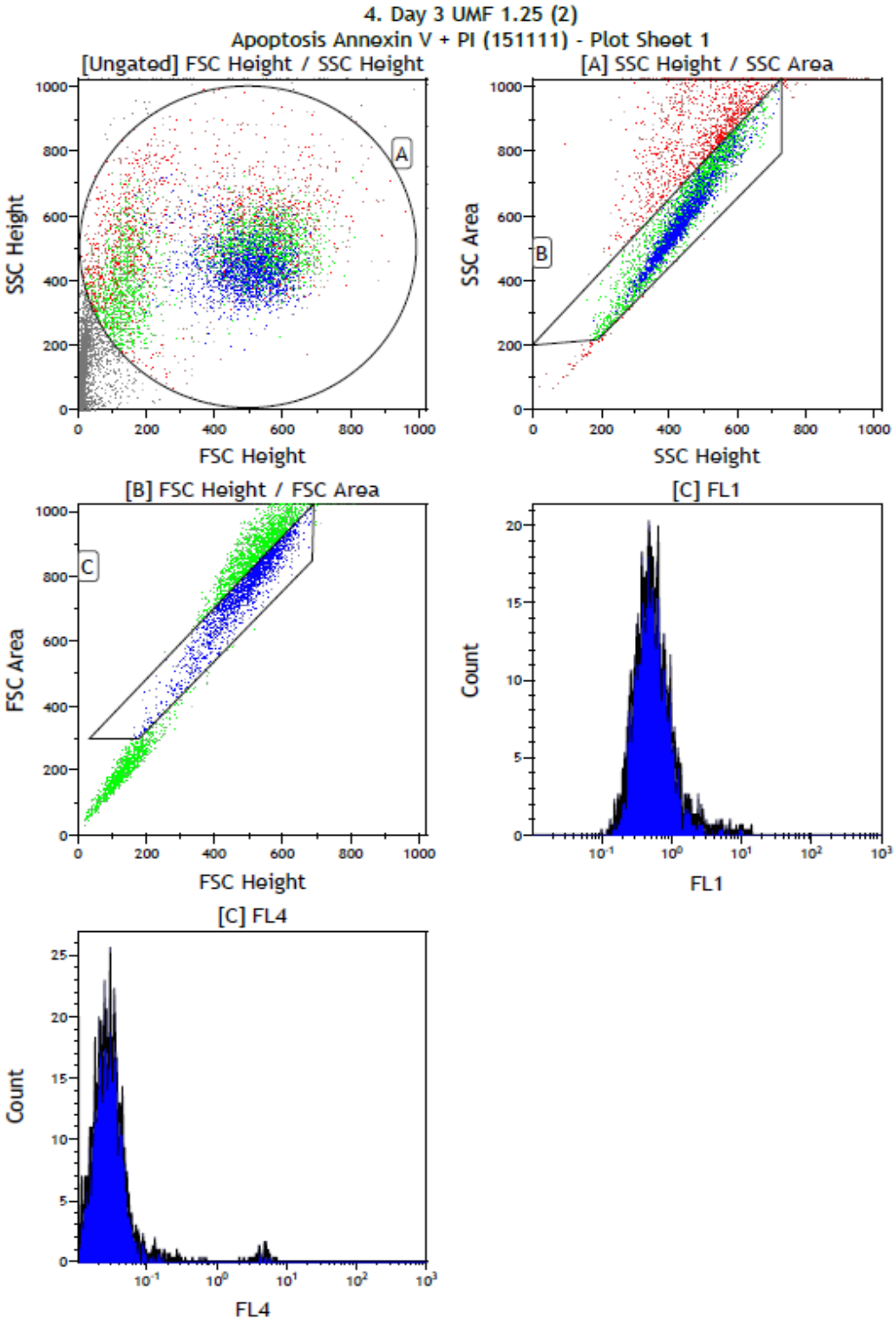


Figure 46: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 72 hours (Set 1).

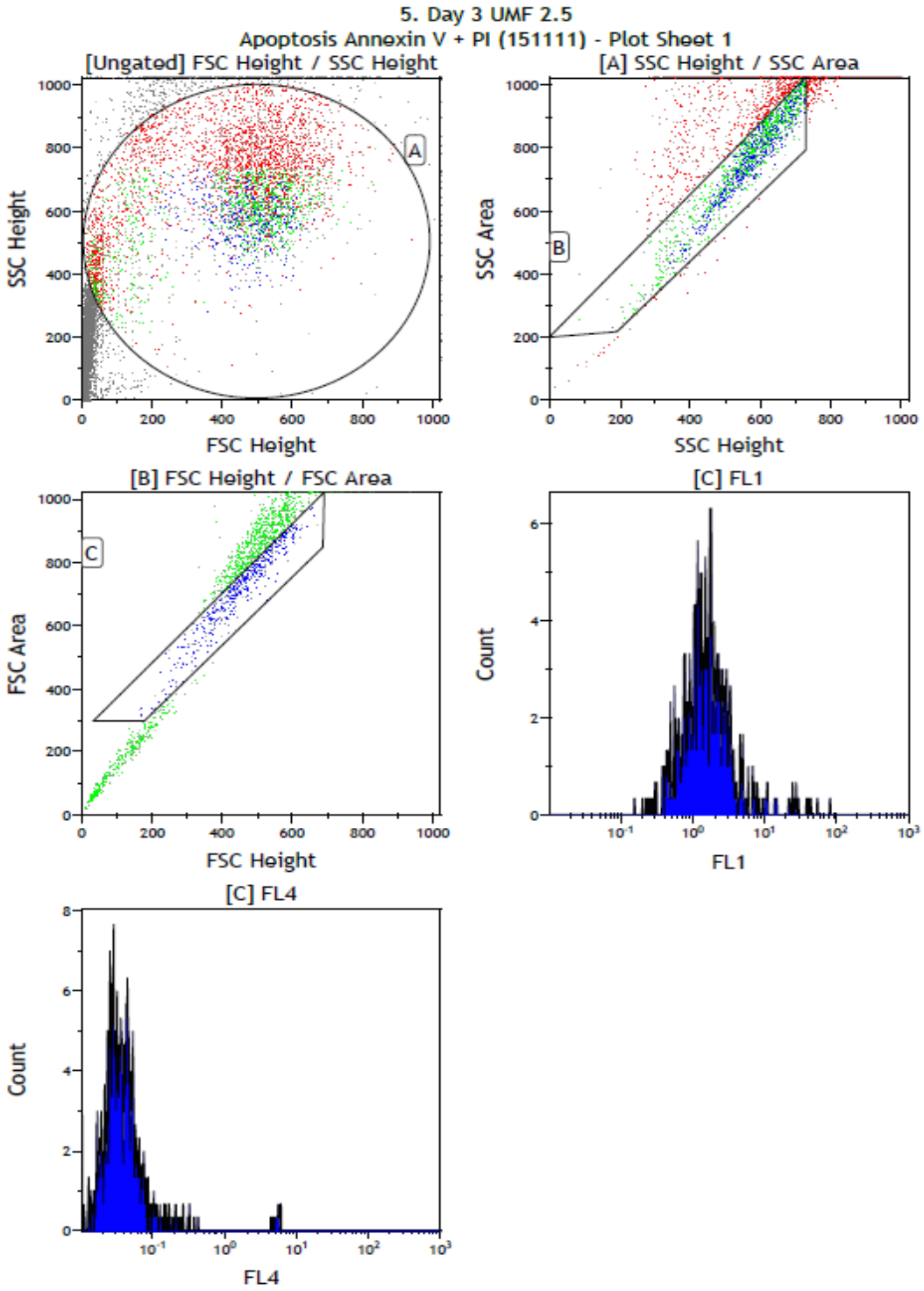


Figure 47: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 72 hours (Set 1).

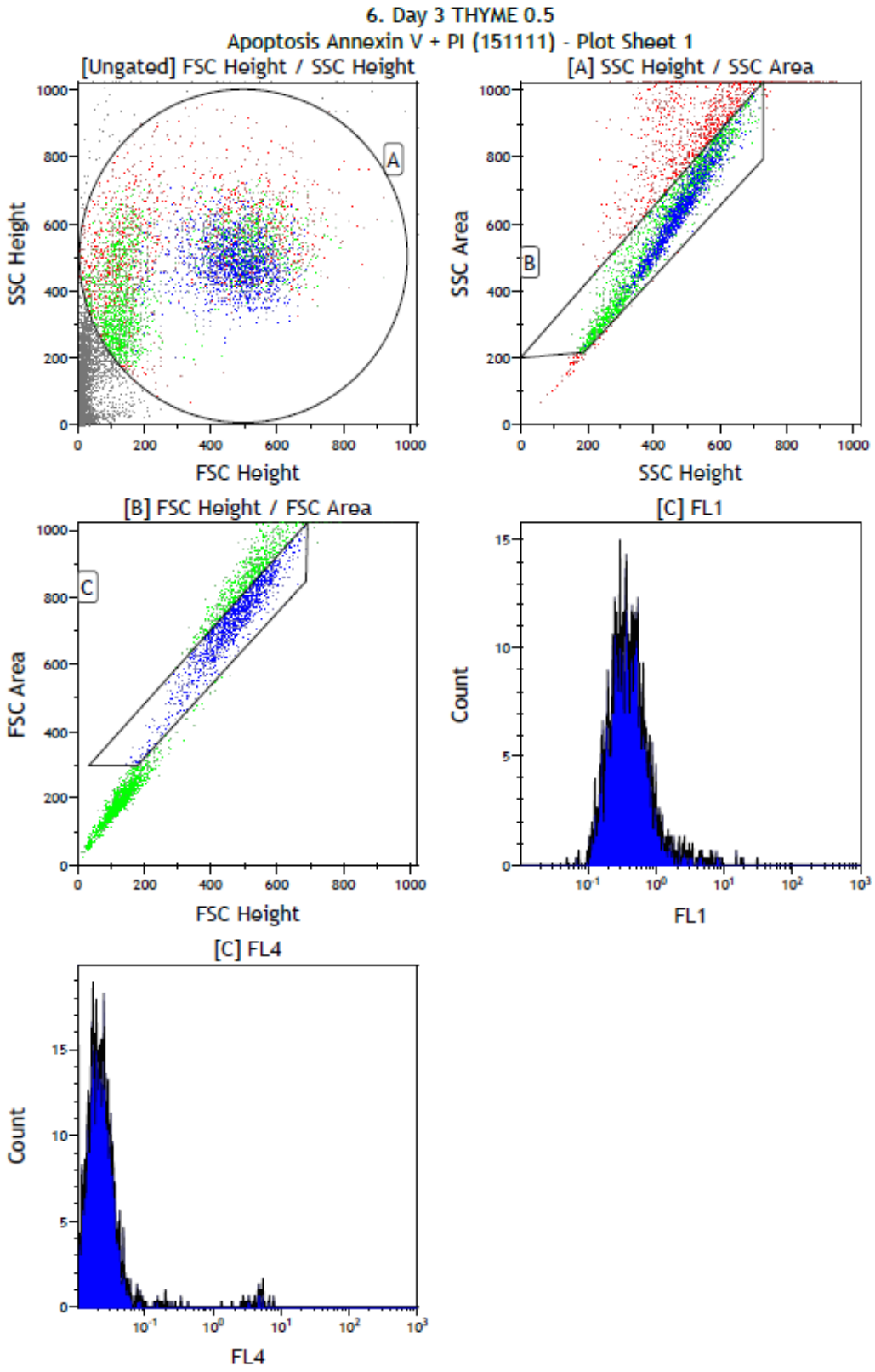


Figure 48: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 72 hours (Set 1).

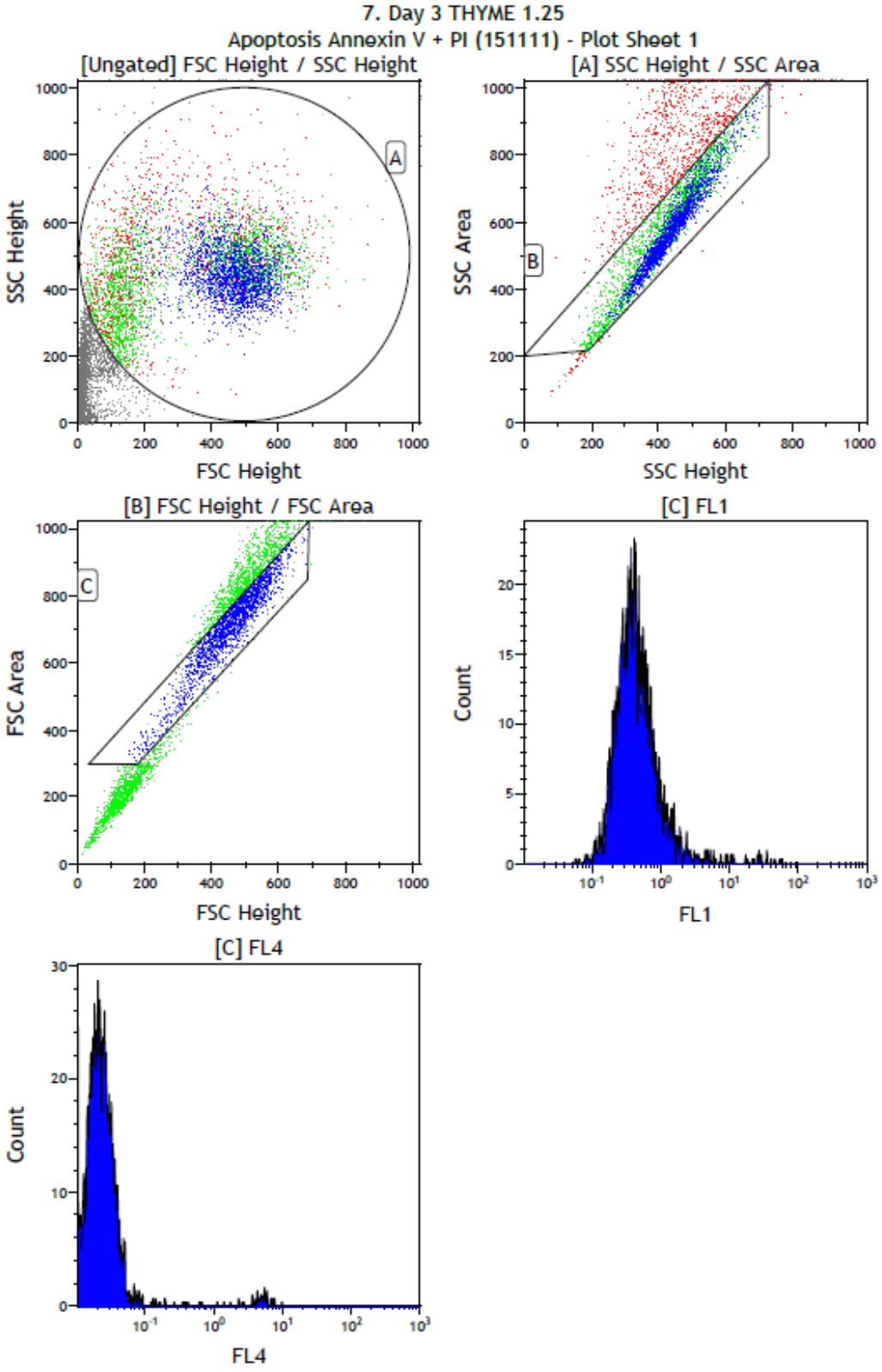


Figure 49: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 72 hours (Set 1).

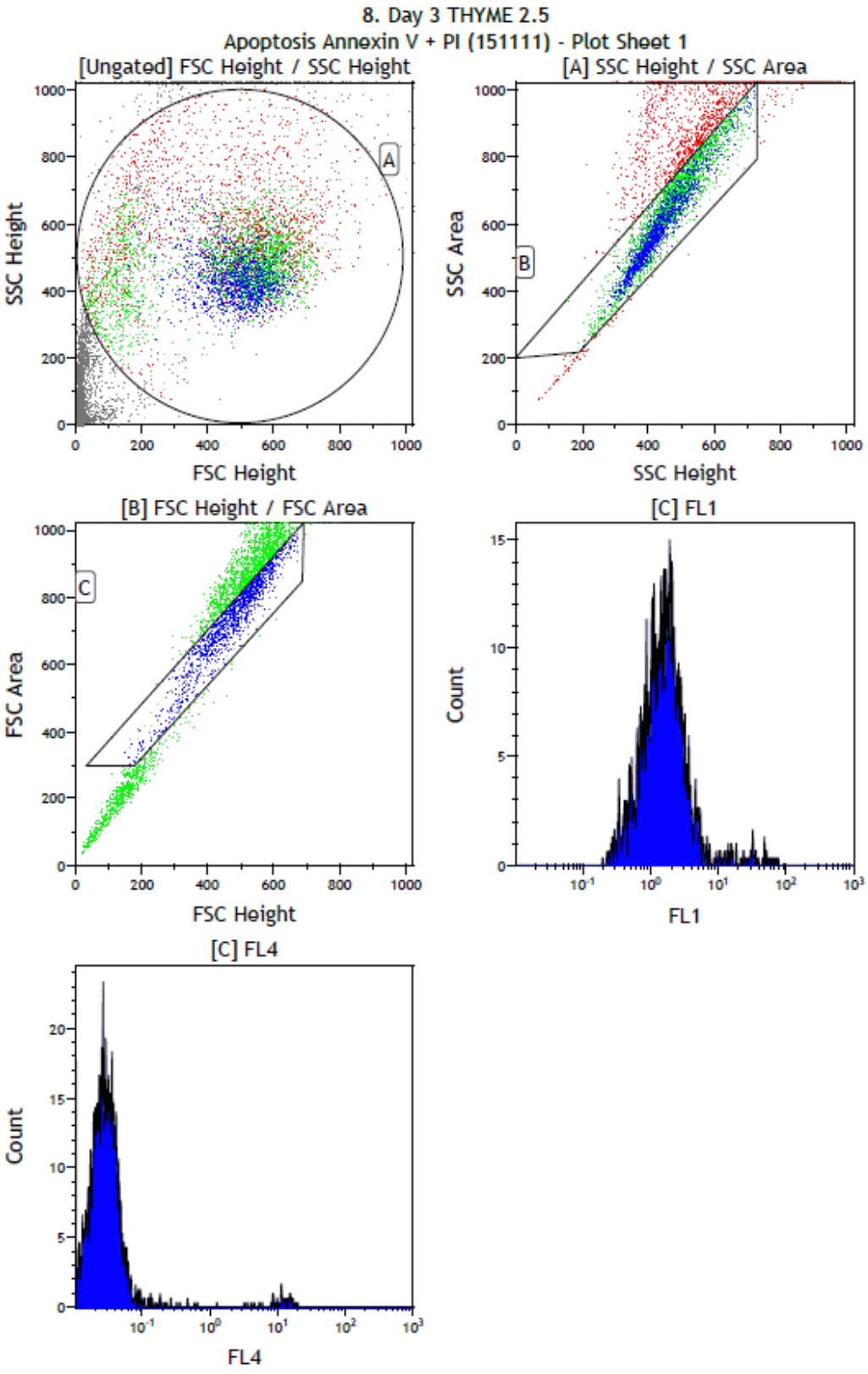


Figure 50: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 72 hours (Set 1).

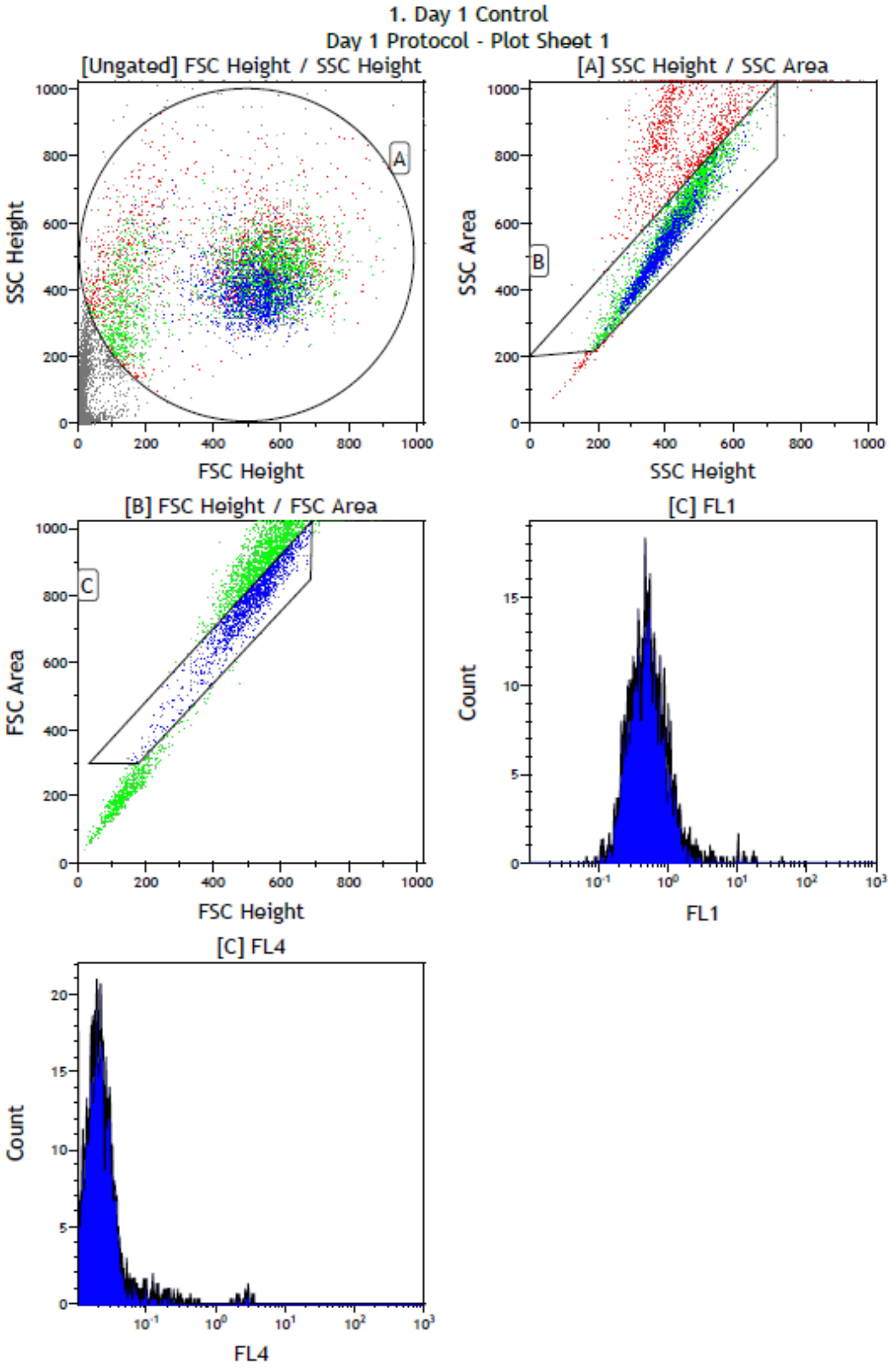


Figure 51: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 24 hours (Set 2).

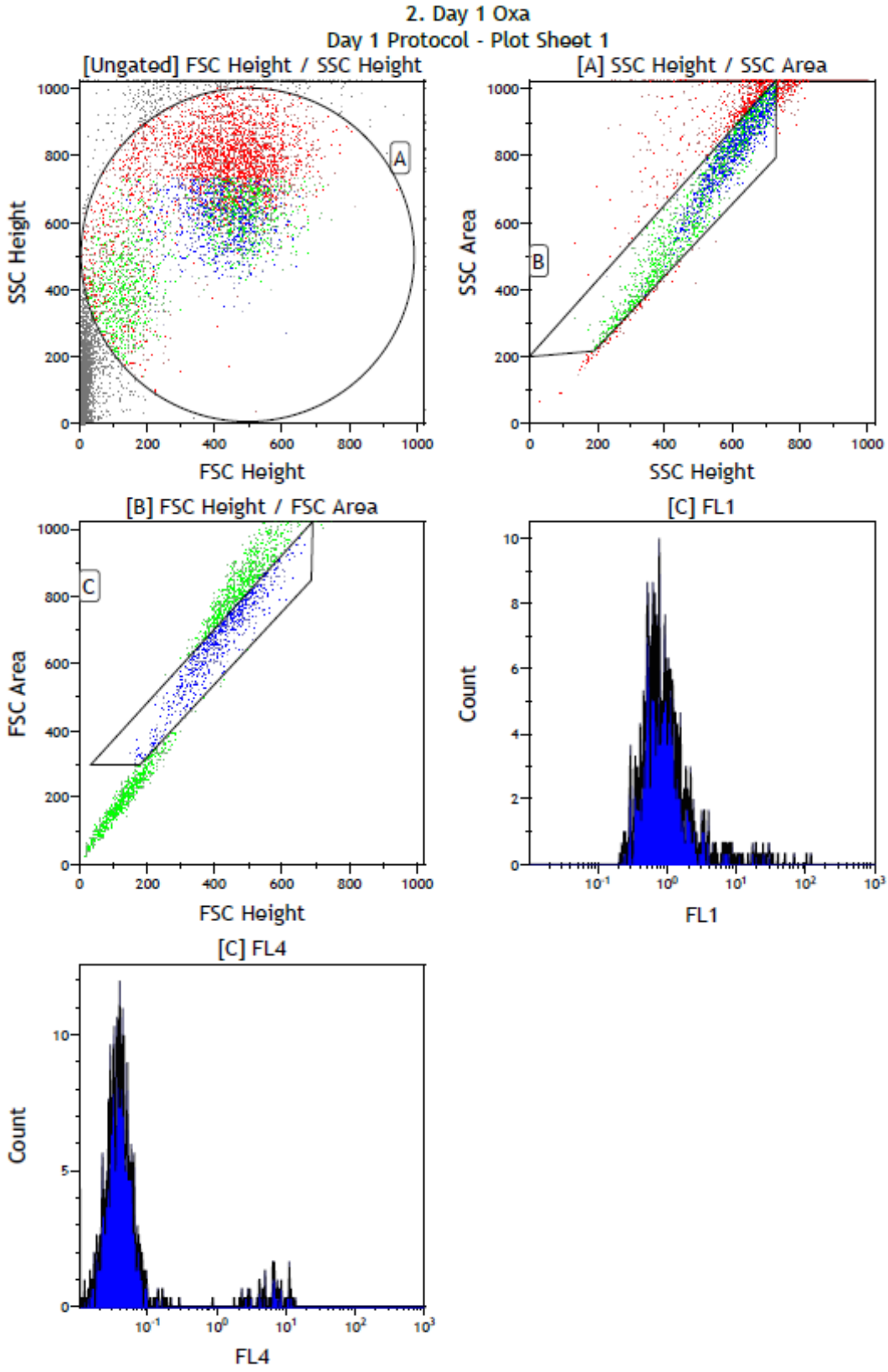


Figure 52: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 24 hours (Set 2).

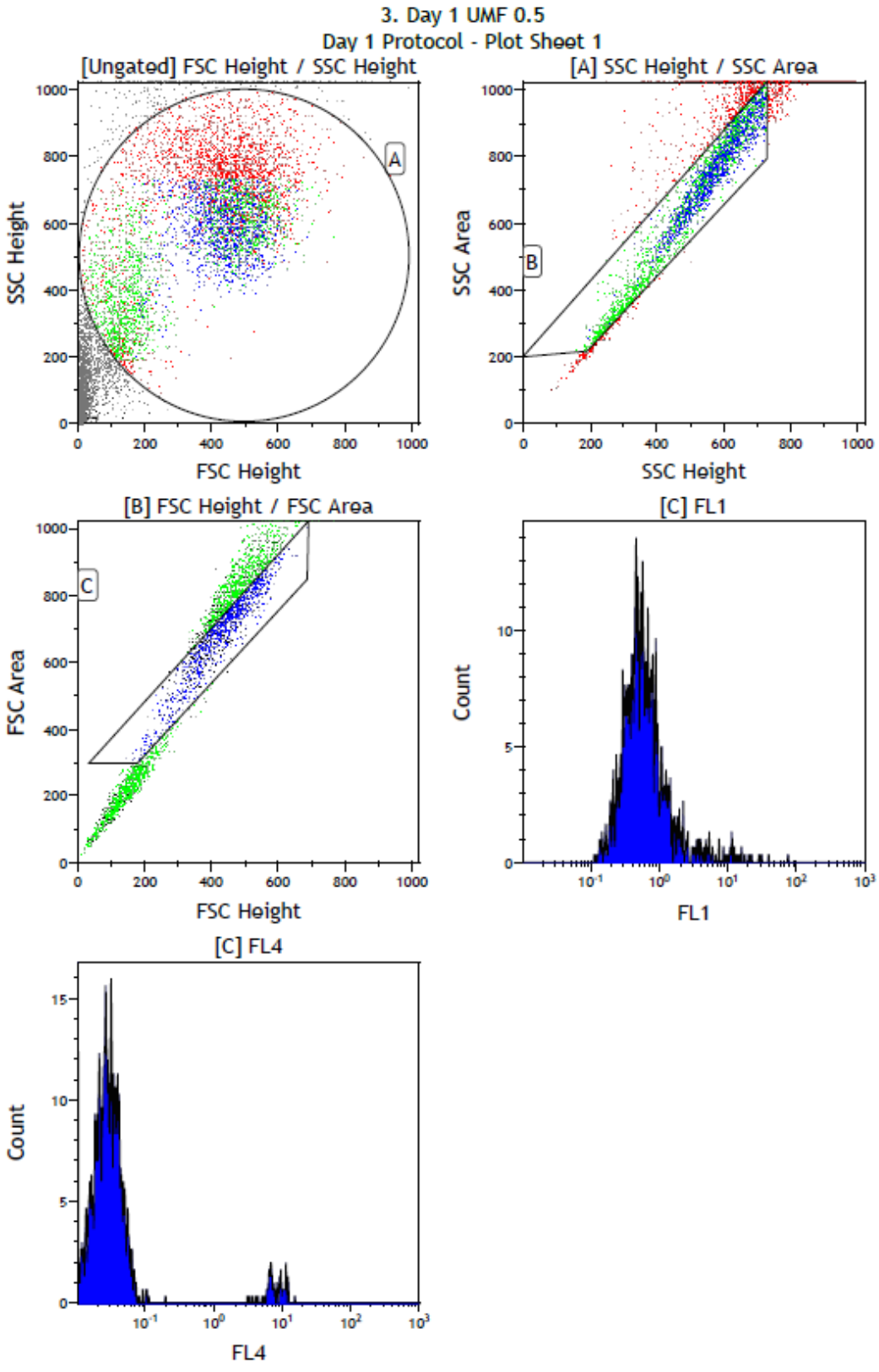


Figure 53: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 24 hours (Set 2).

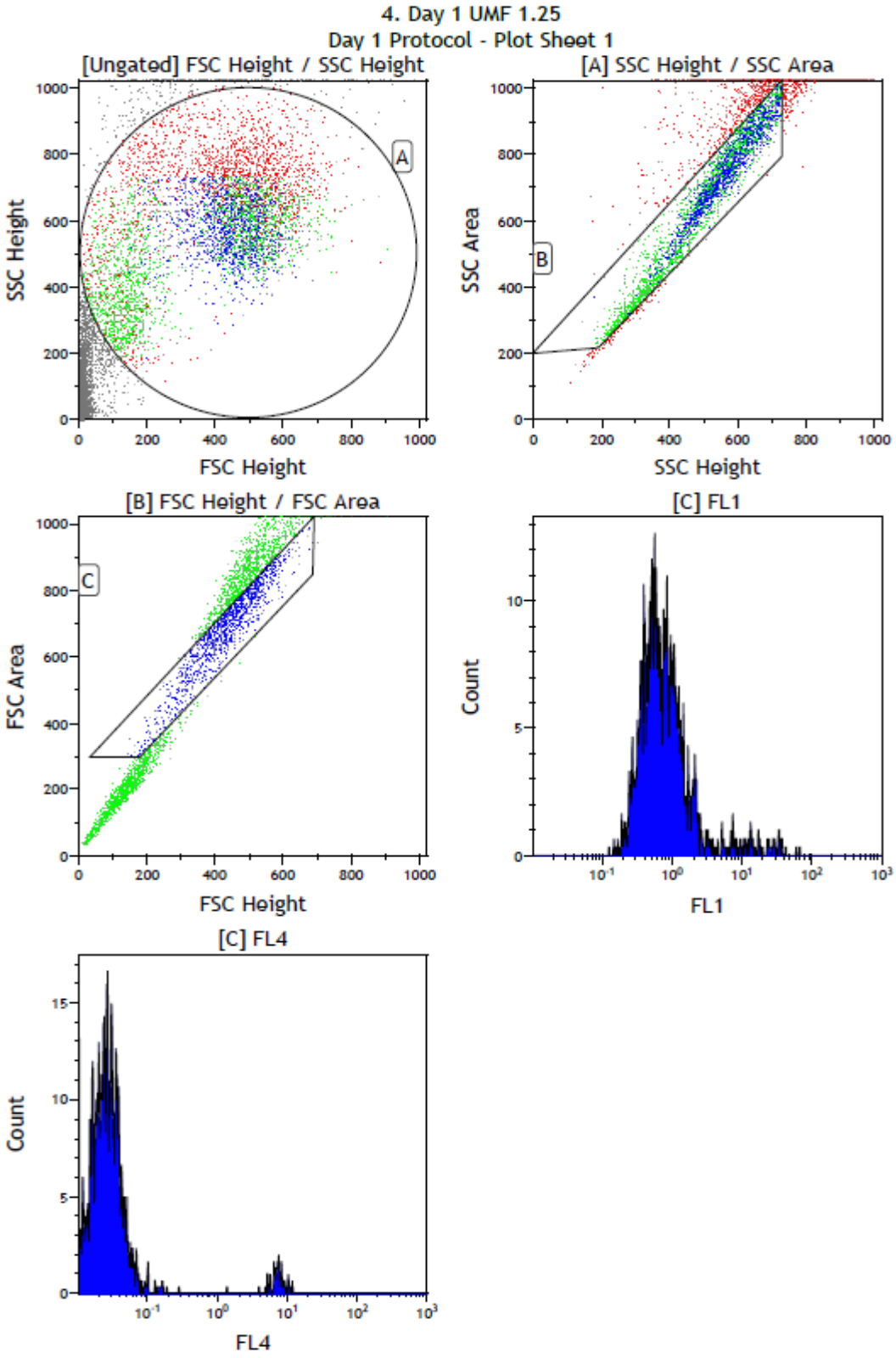


Figure 54: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 24 hours (Set 2).

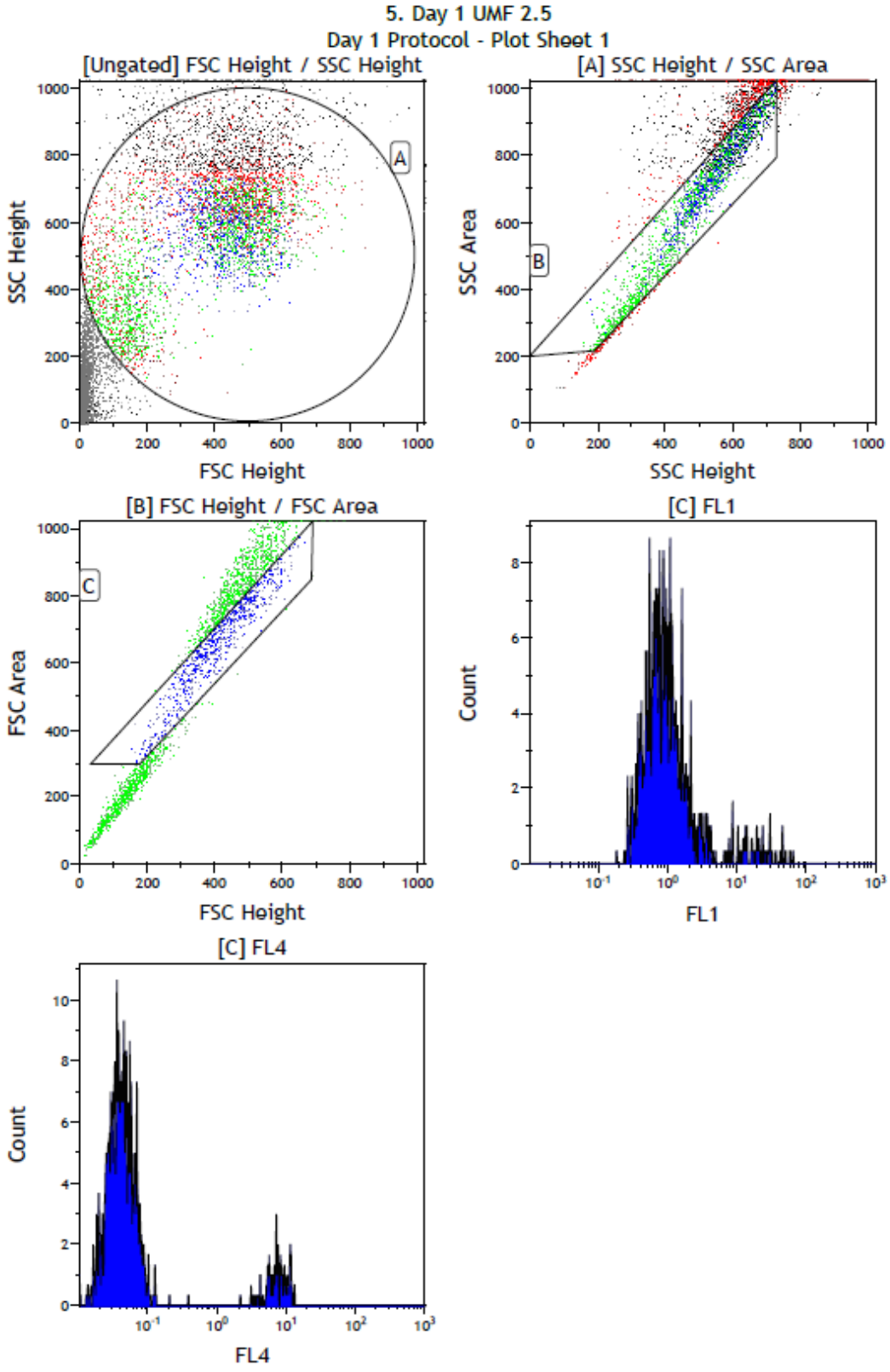


Figure 55: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 24 hours (Set 2).

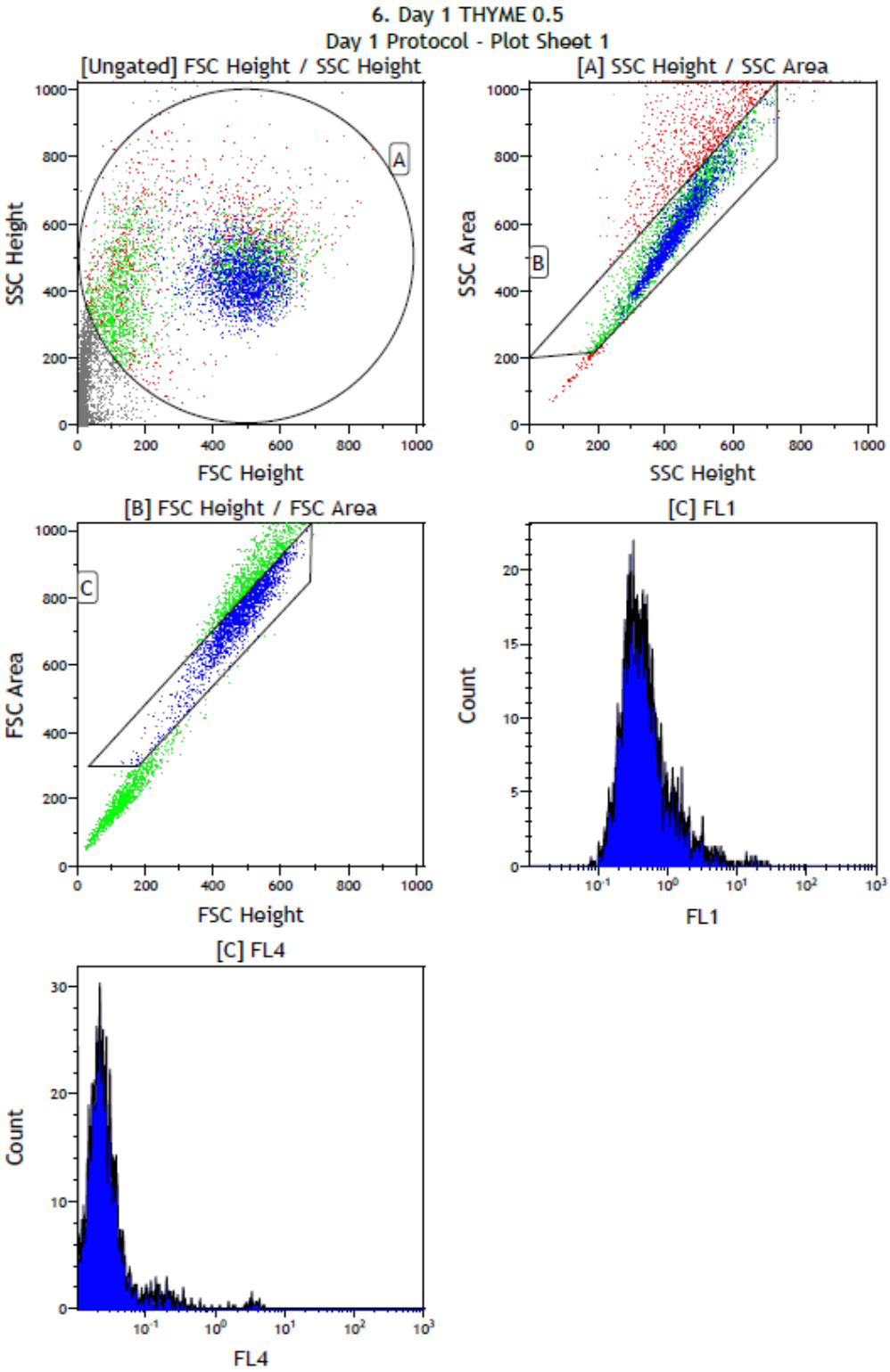


Figure 56: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 24 hours (Set 2).

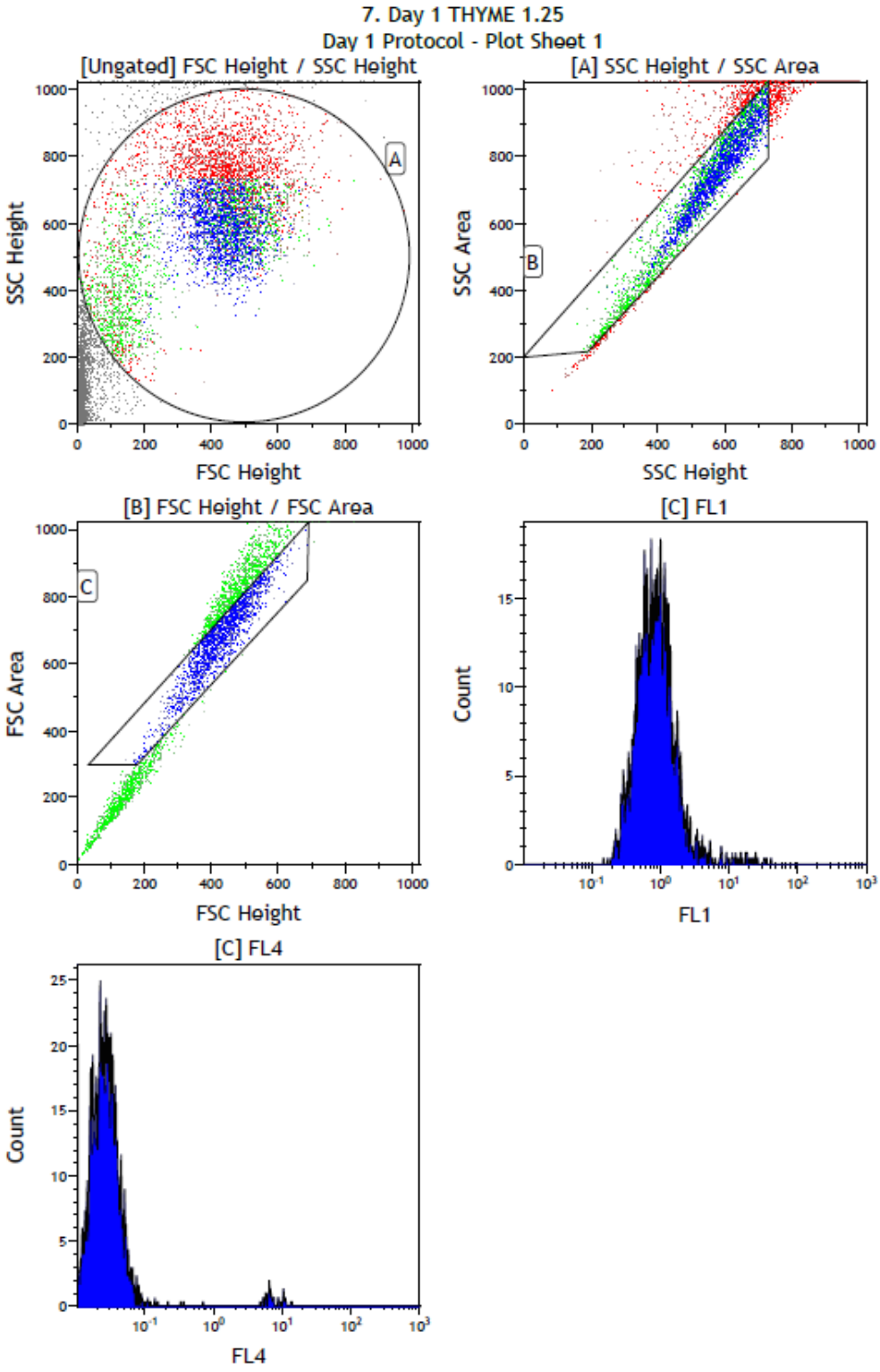


Figure 57: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 24 hours (Set 2).

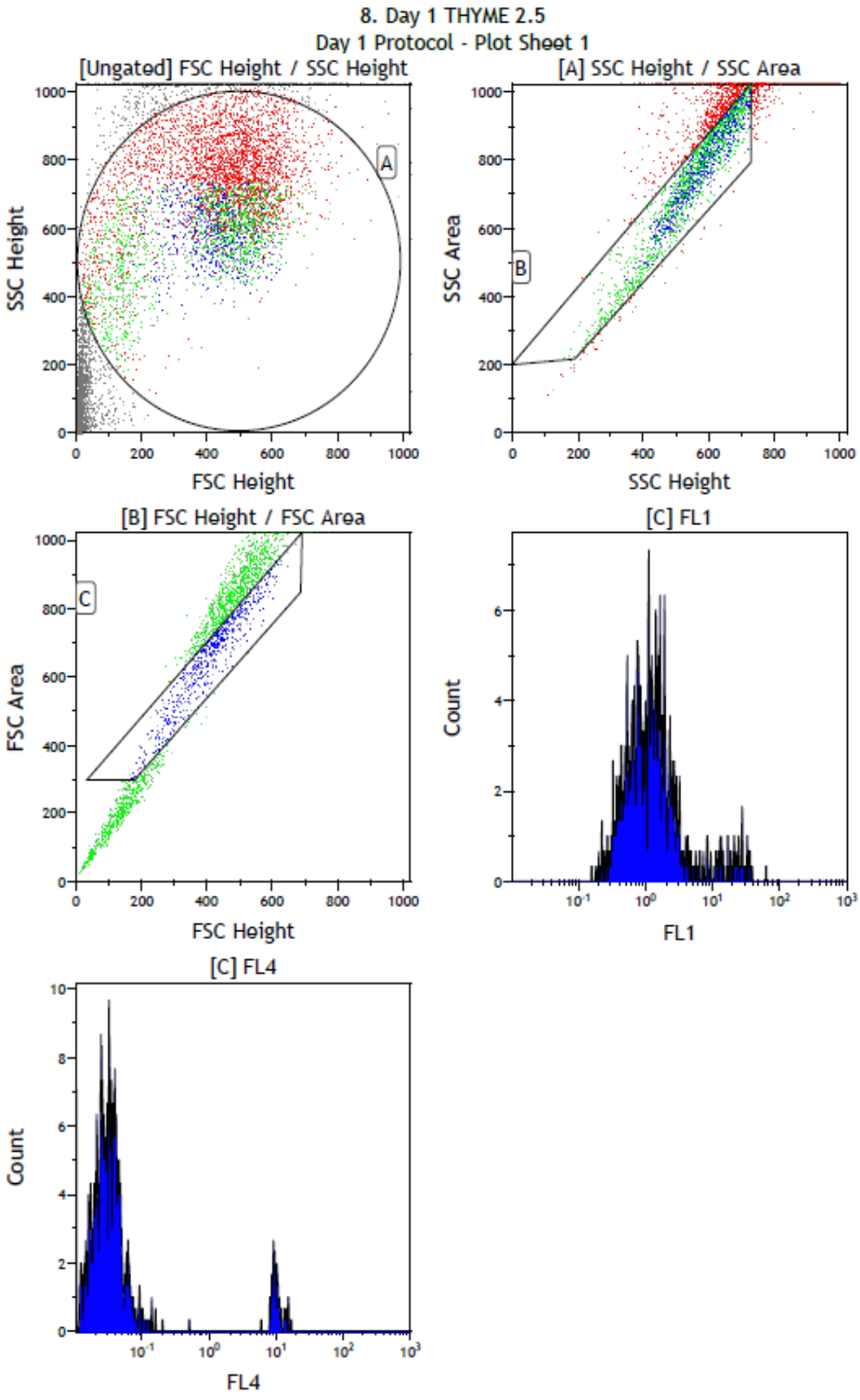


Figure 58: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 24 hours (Set 2).

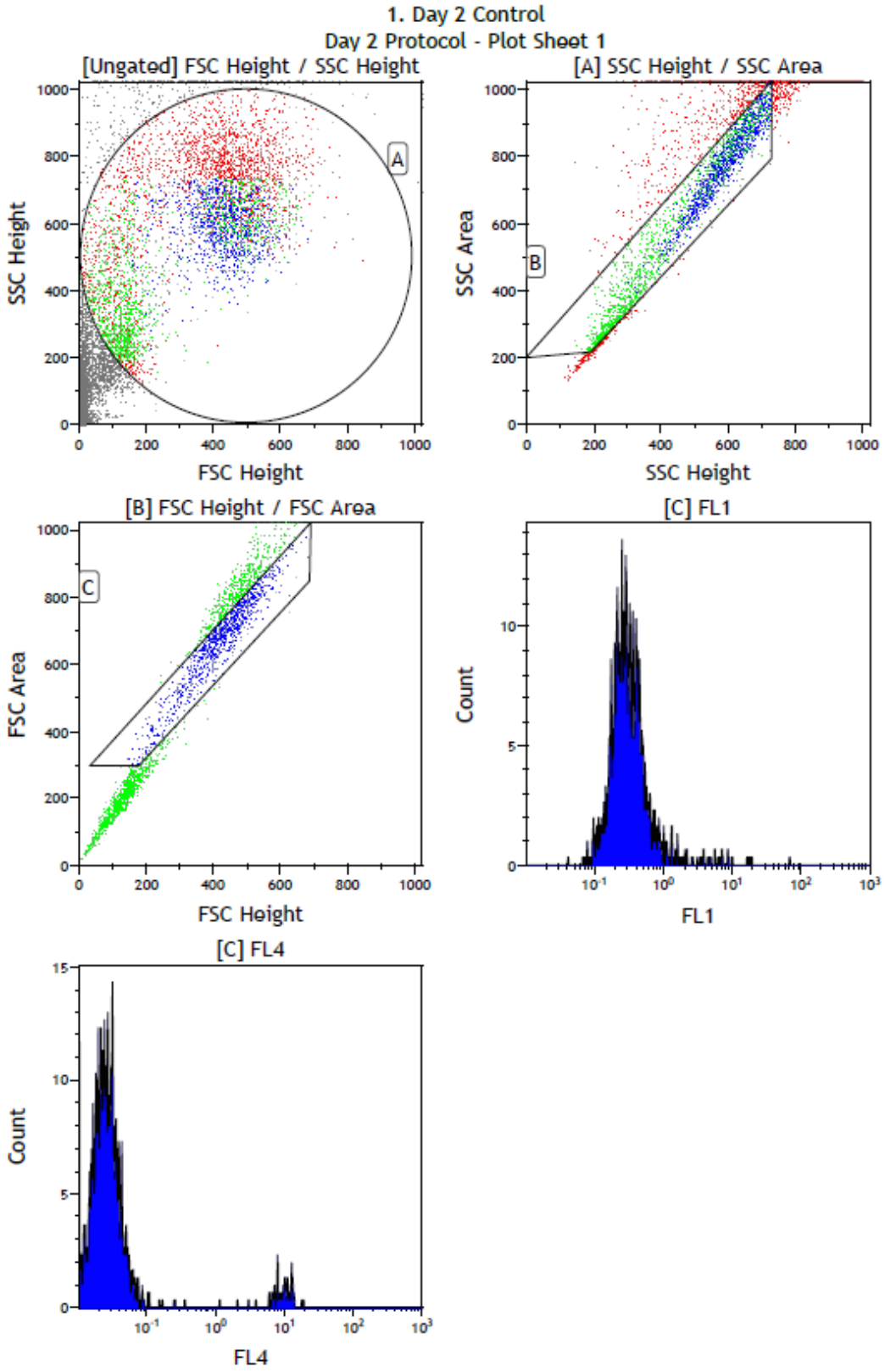


Figure 59: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 48 hours (Set 2).

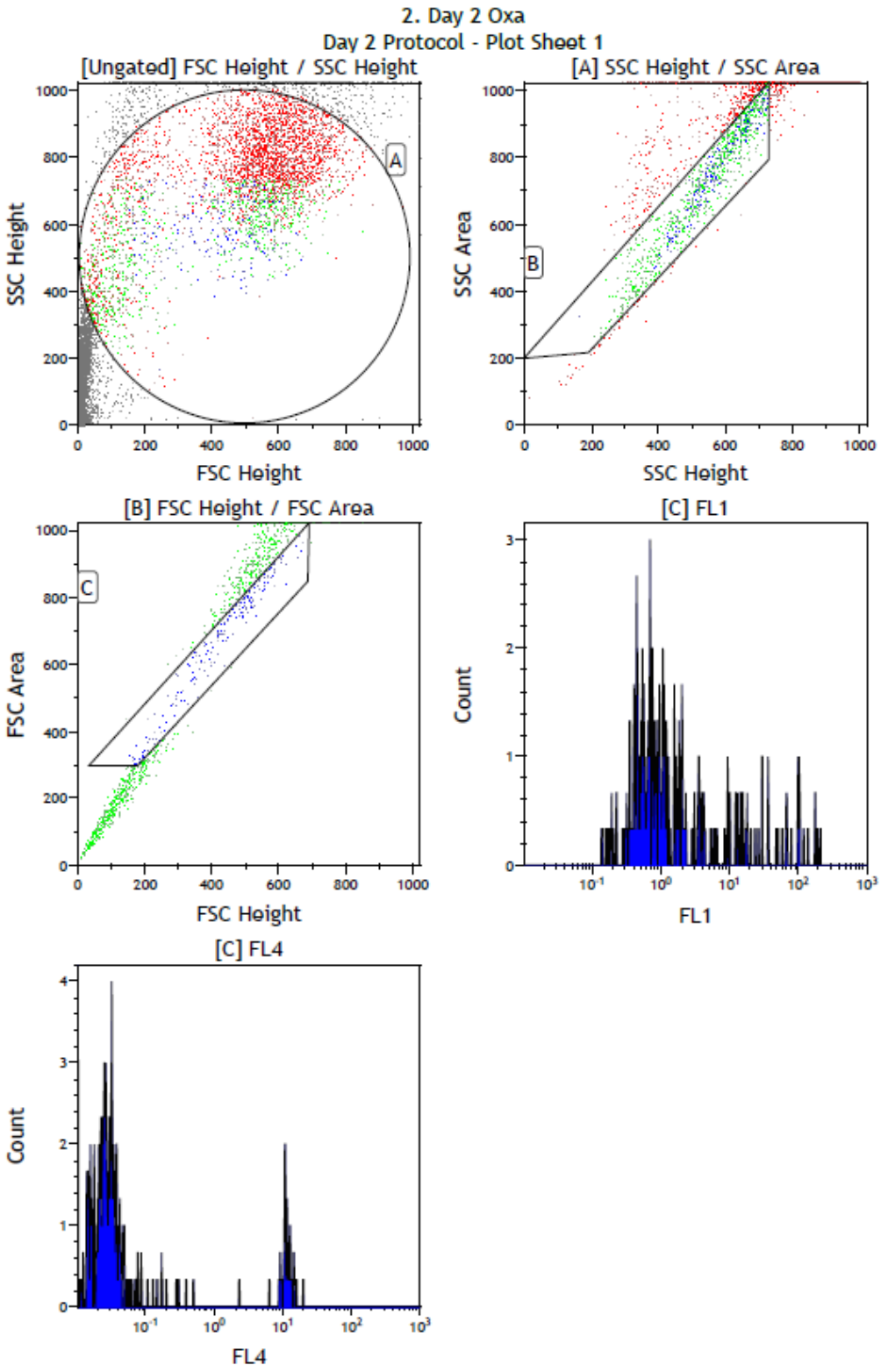


Figure 60: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 48 hours (Set 2).

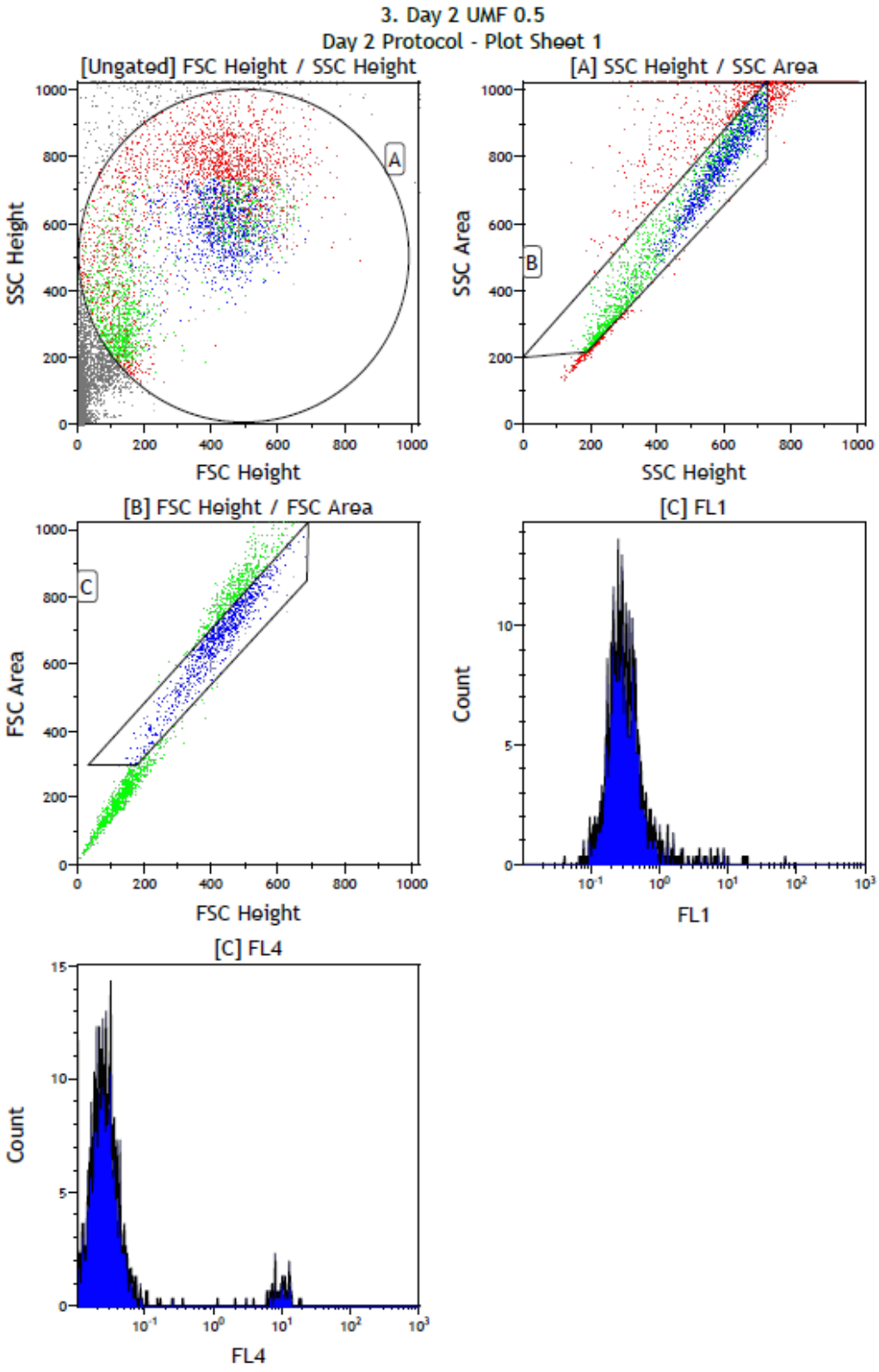


Figure 61: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 48 hours (Set 2).

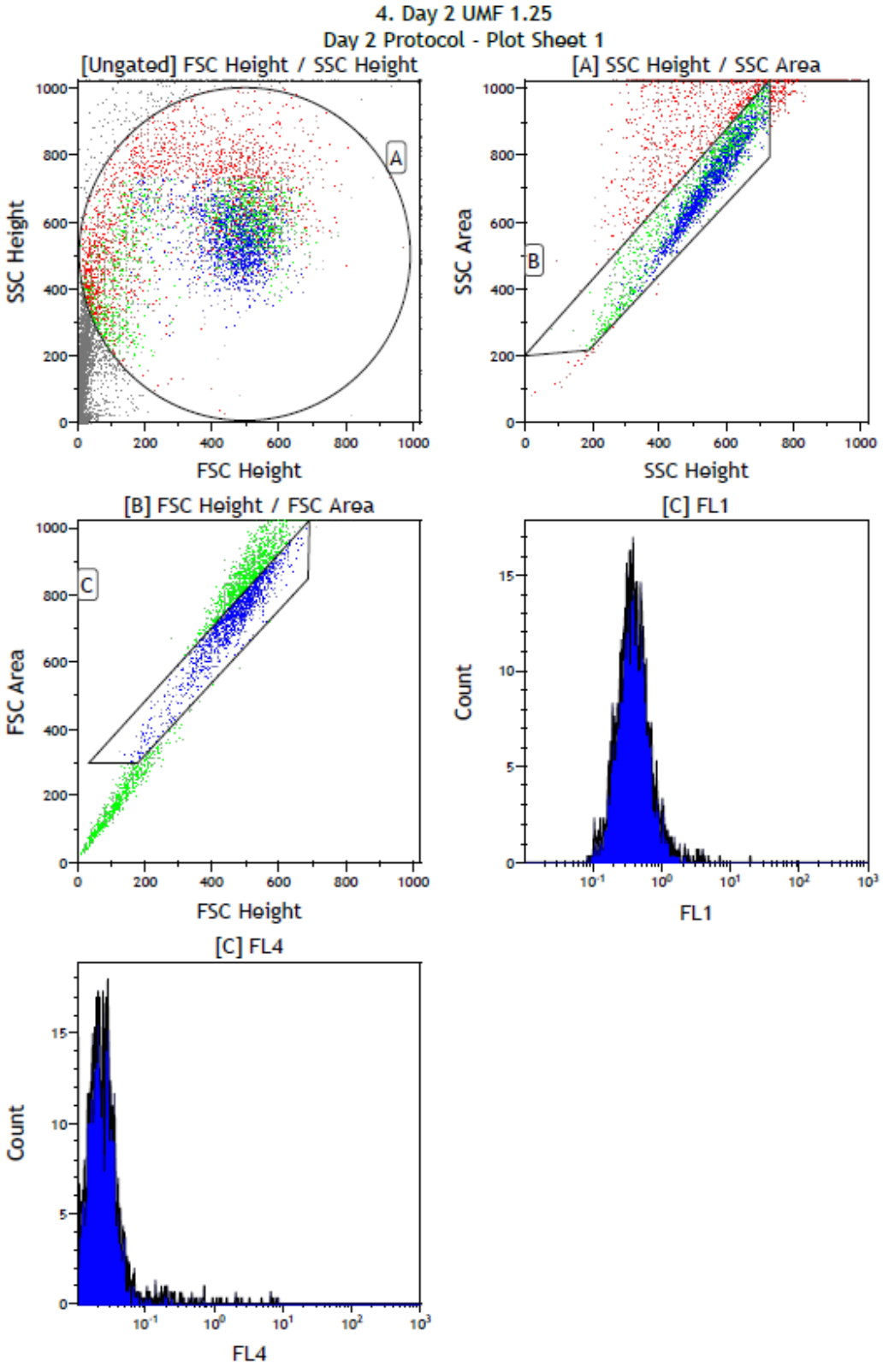


Figure 62: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 48 hours (Set 2).

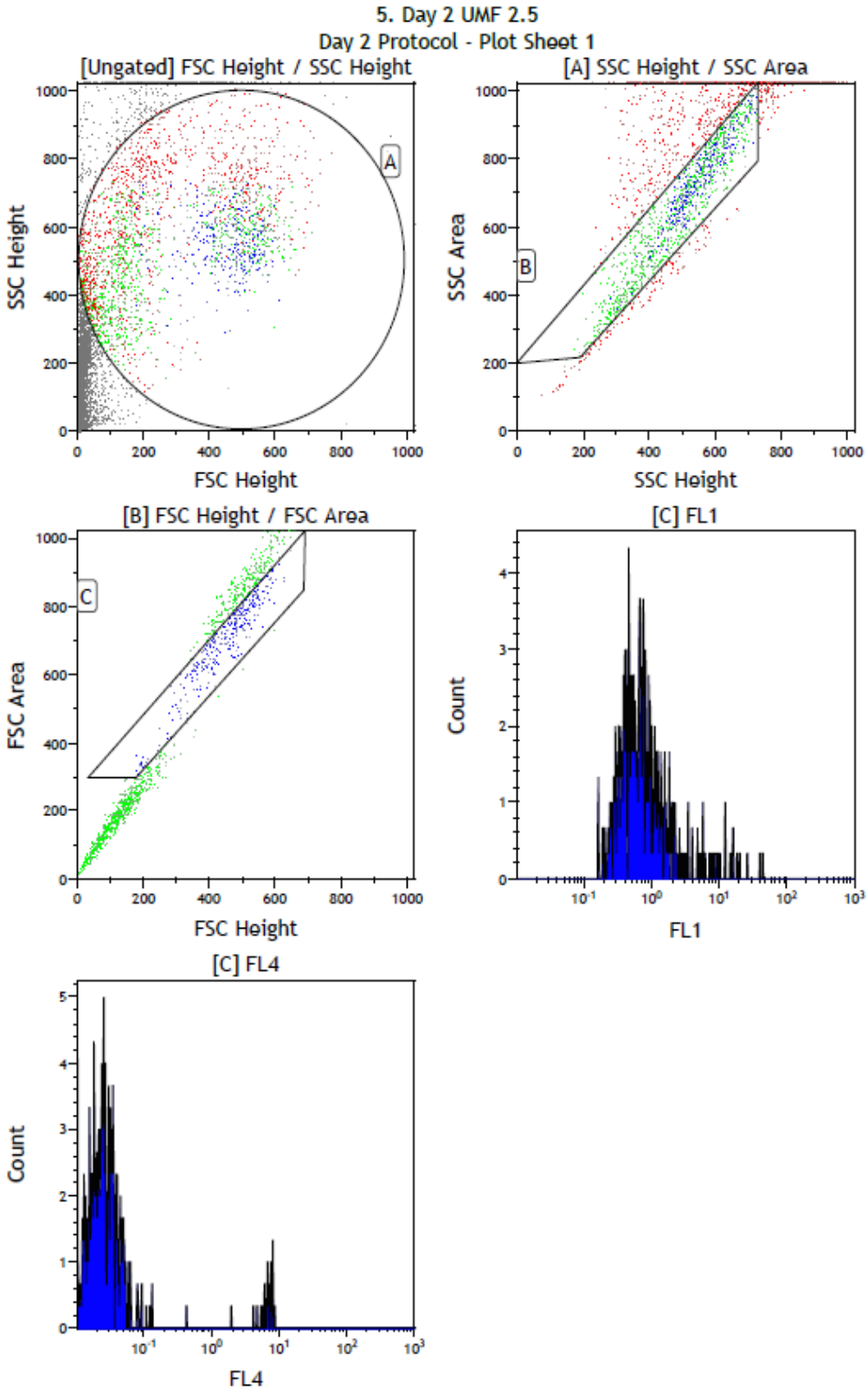


Figure 63: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 48 hours (Set 2).

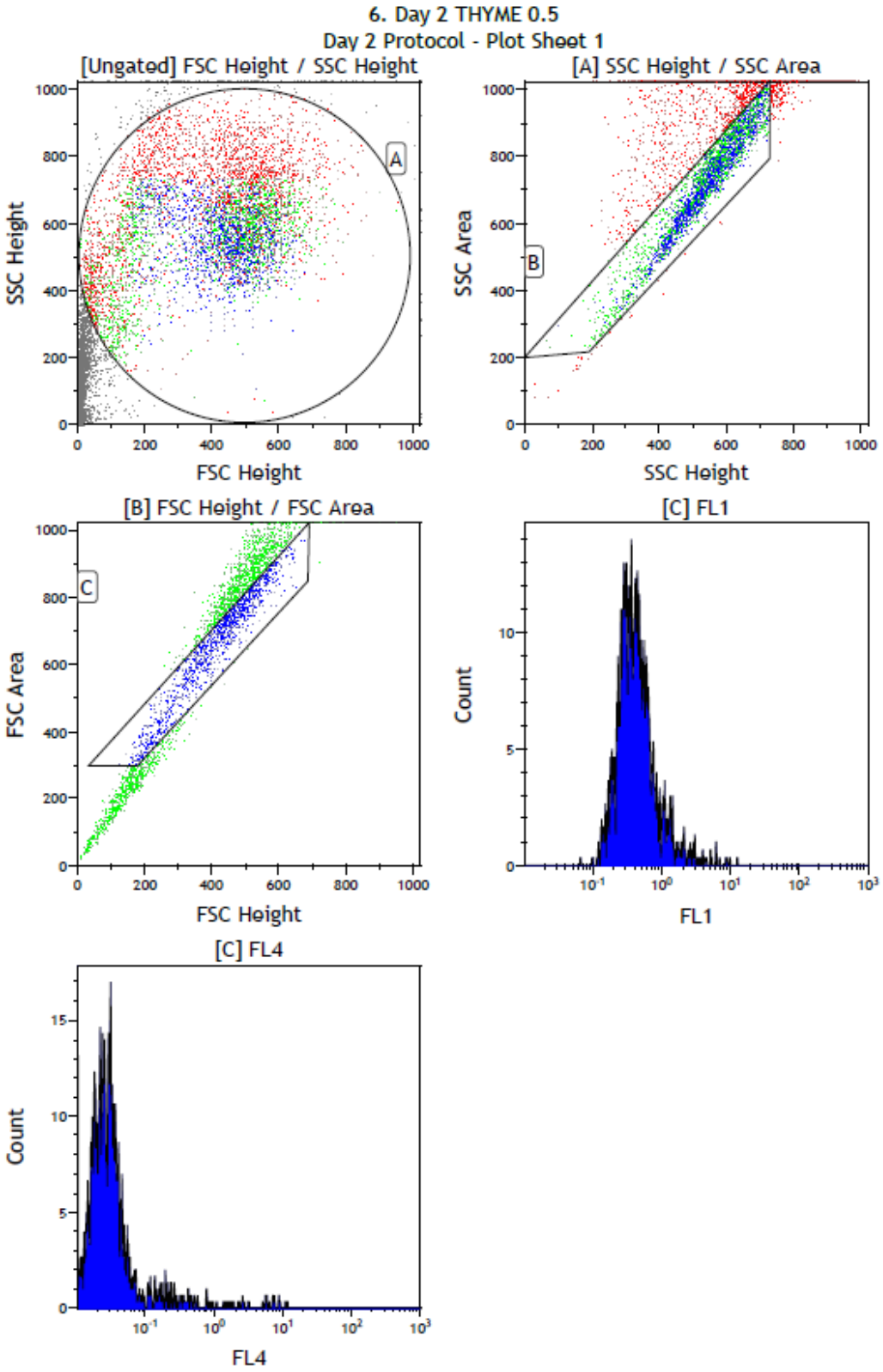


Figure 64: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 48 hours (Set 2).

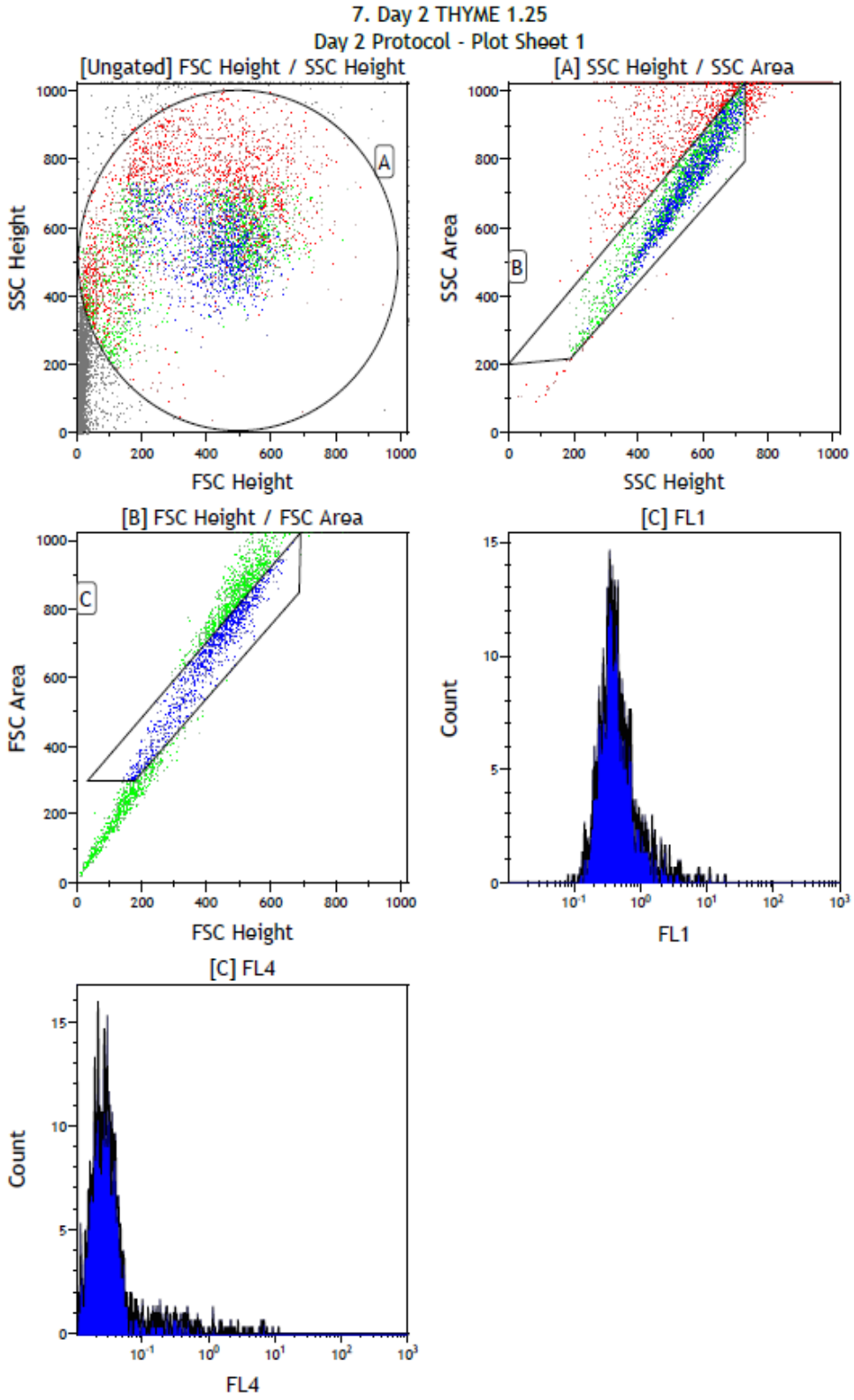


Figure 65: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 48 hours (Set 2).

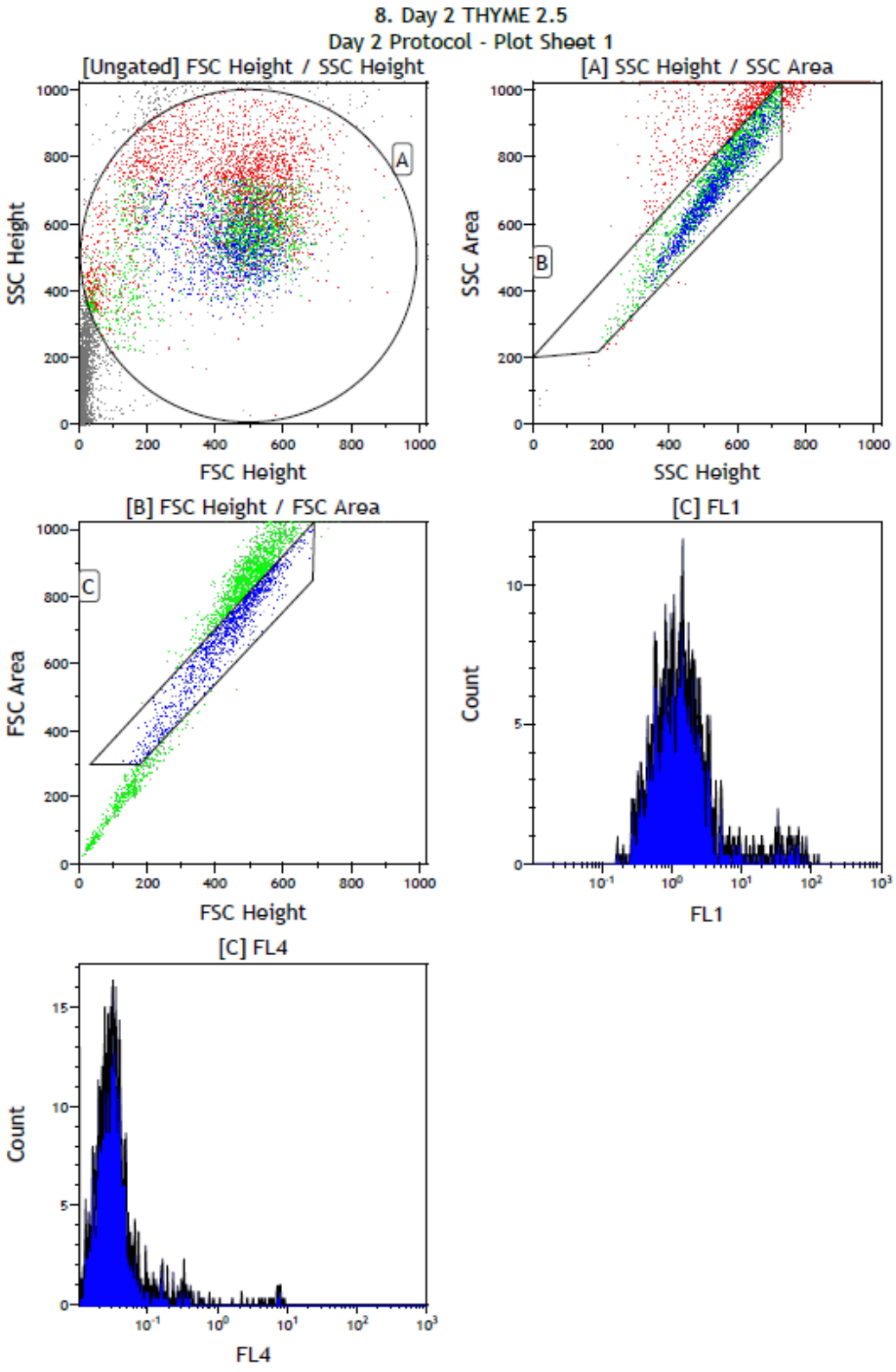


Figure 66: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 48 hours (Set 2).

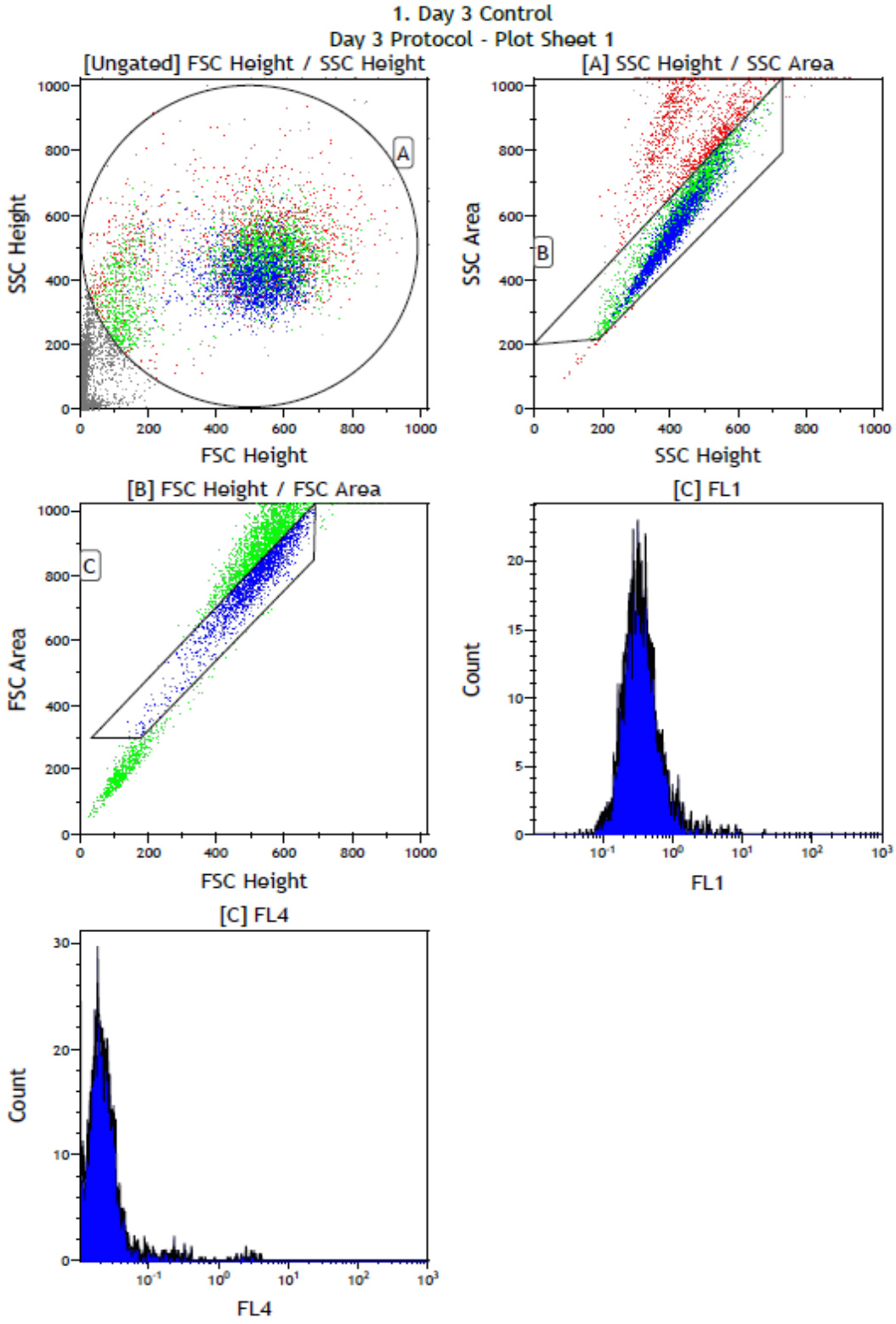


Figure 67: Apoptosis annexin V + PI data plot. Untreated LoVo cells for 72 hours (Set 2).

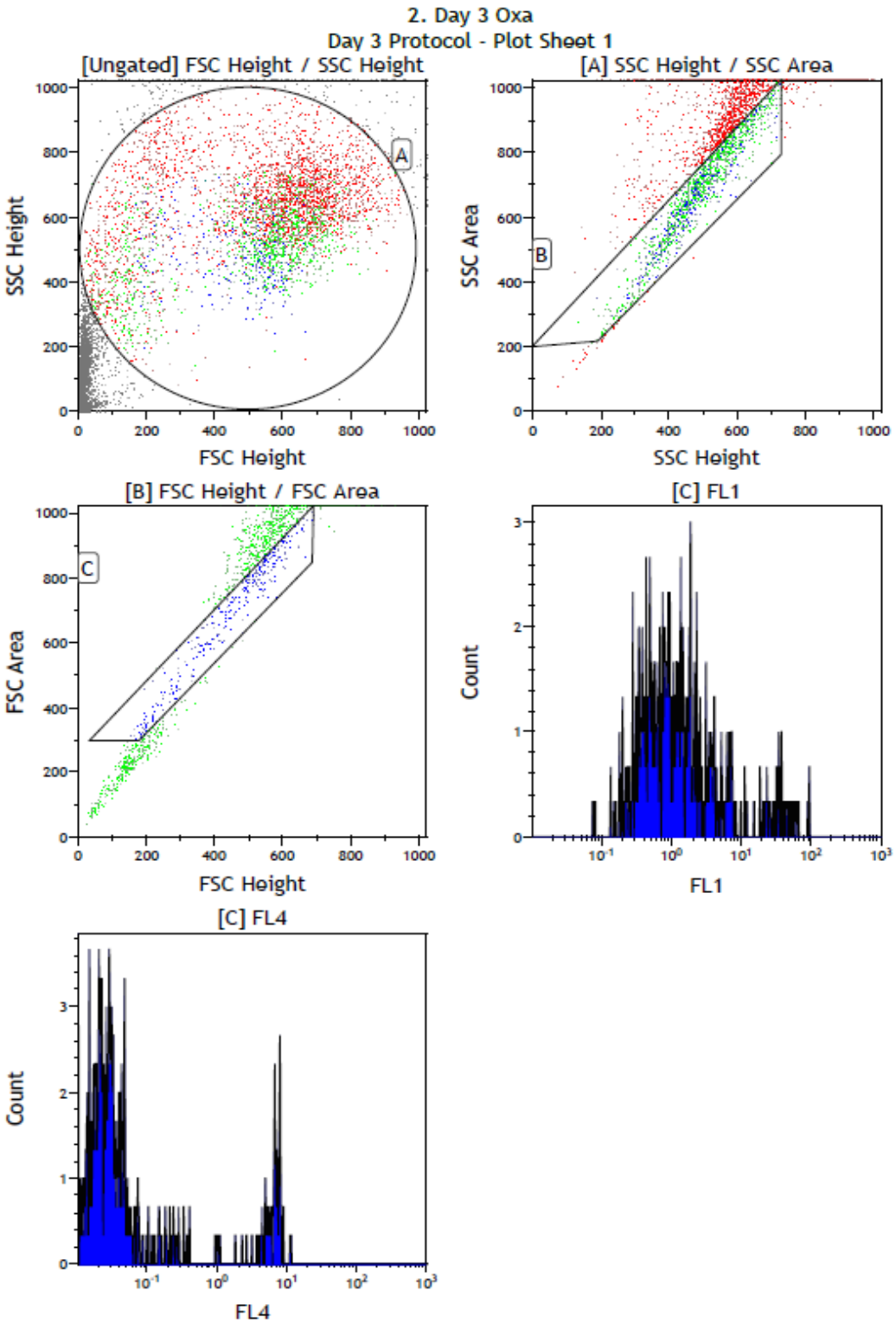


Figure 68: Apoptosis annexin V + PI data plot. LoVo cells treated with oxaliplatin for 72 hours (Set 2).

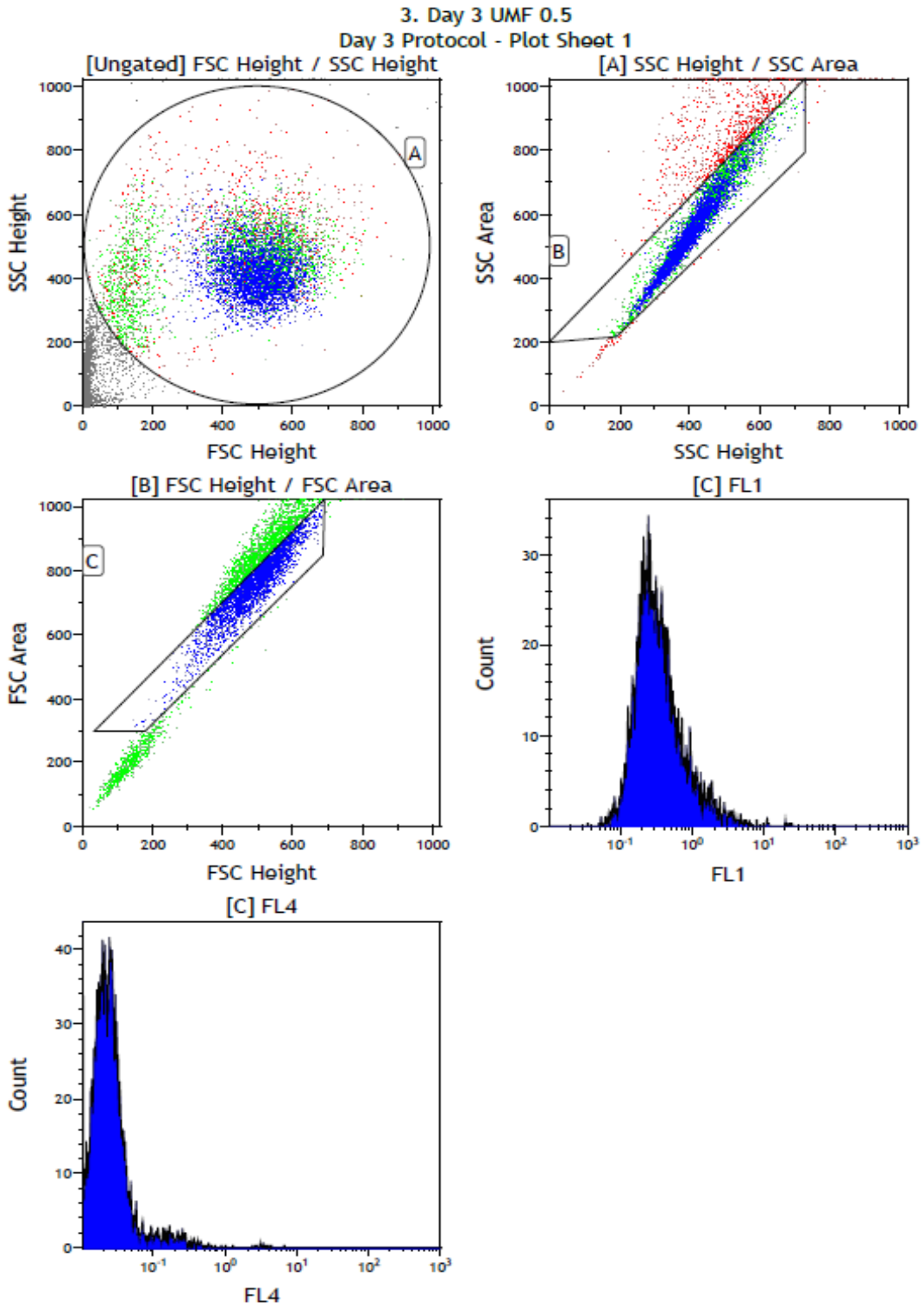


Figure 69: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 0.5% for 72 hours (Set 2).

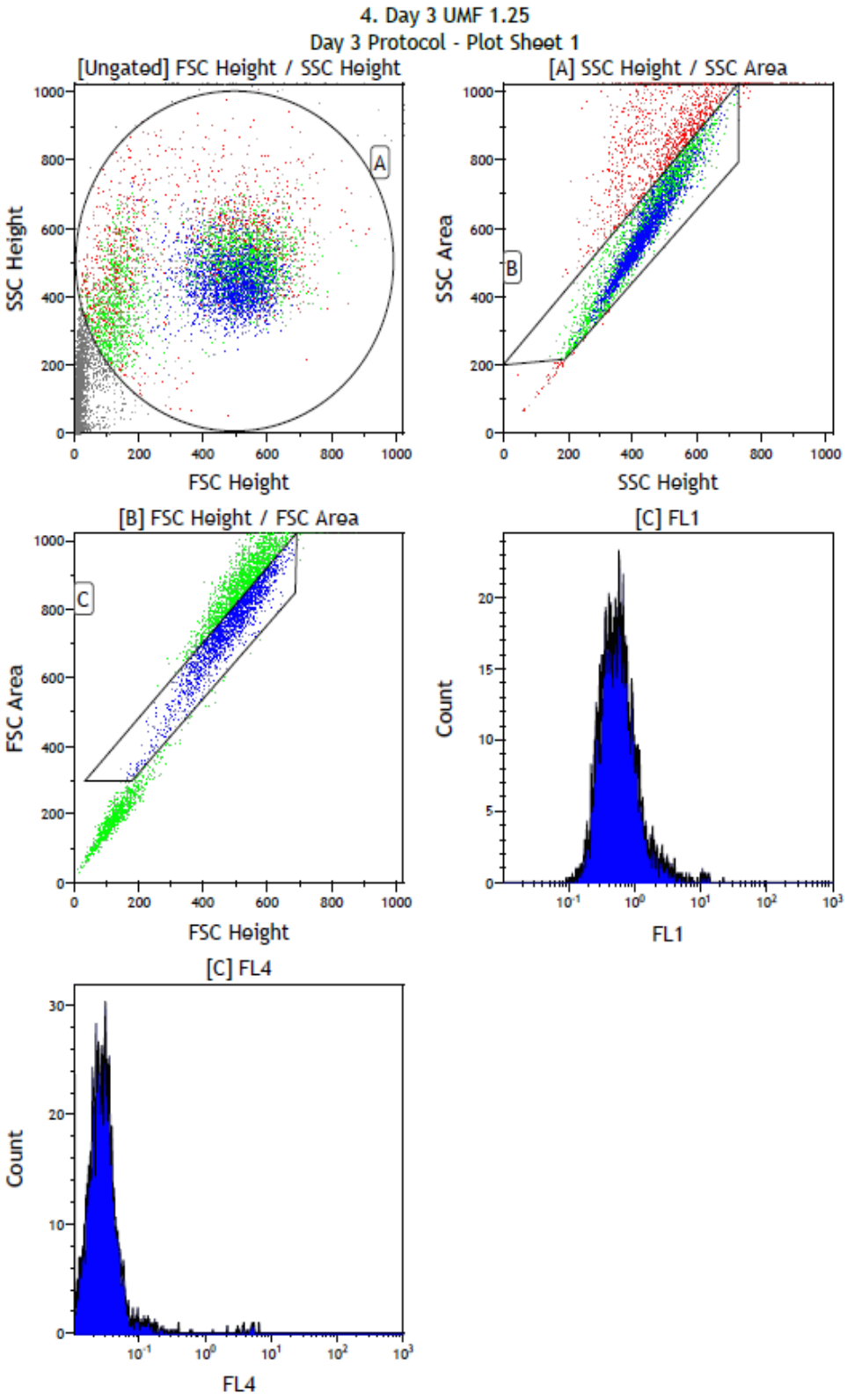


Figure 70: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 1.25% for 72 hours (Set 2).

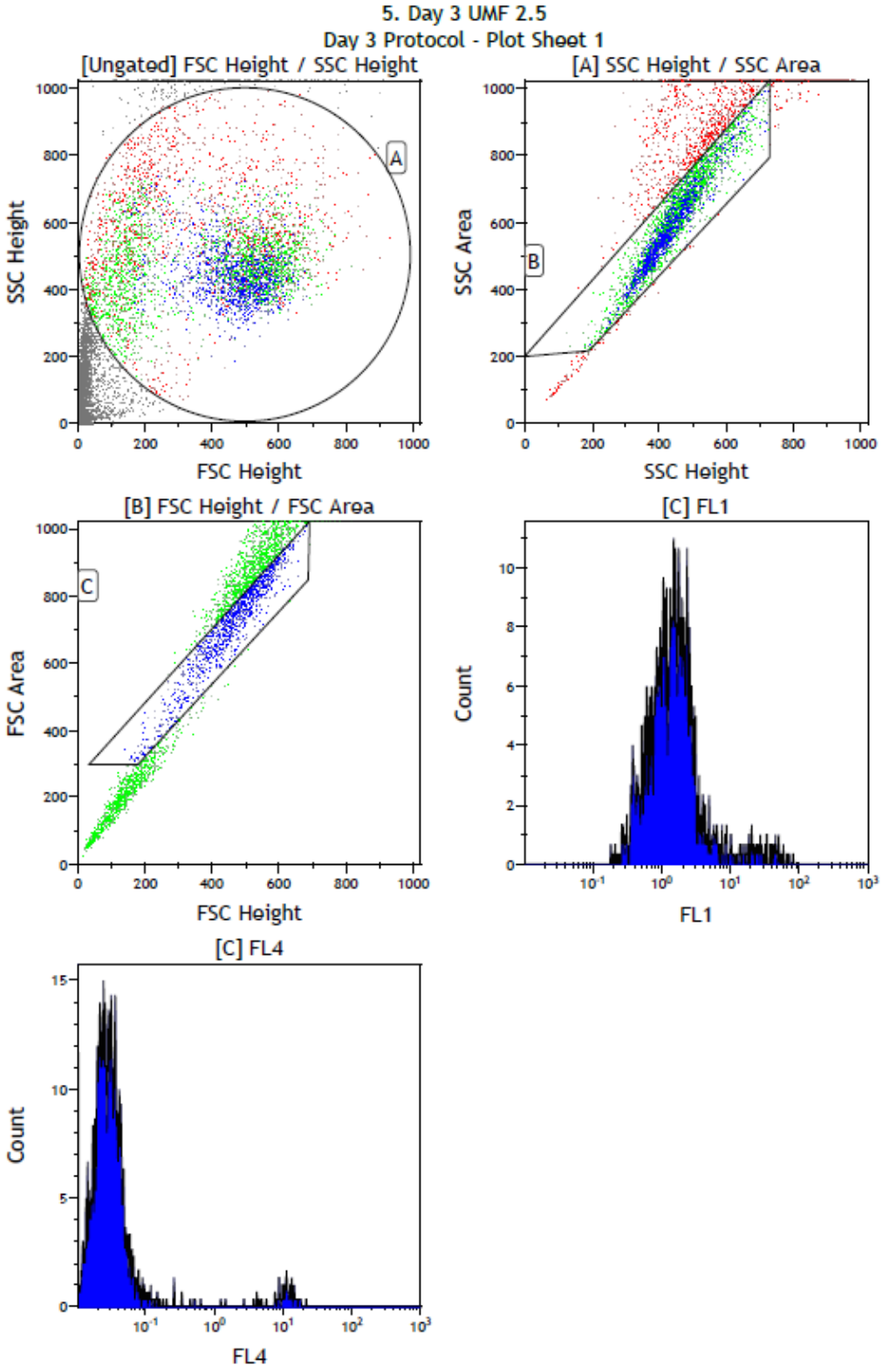


Figure 71: Apoptosis annexin V + PI data plot. LoVo cells treated with UMF+15 2.5% for 72 hours (Set 2).

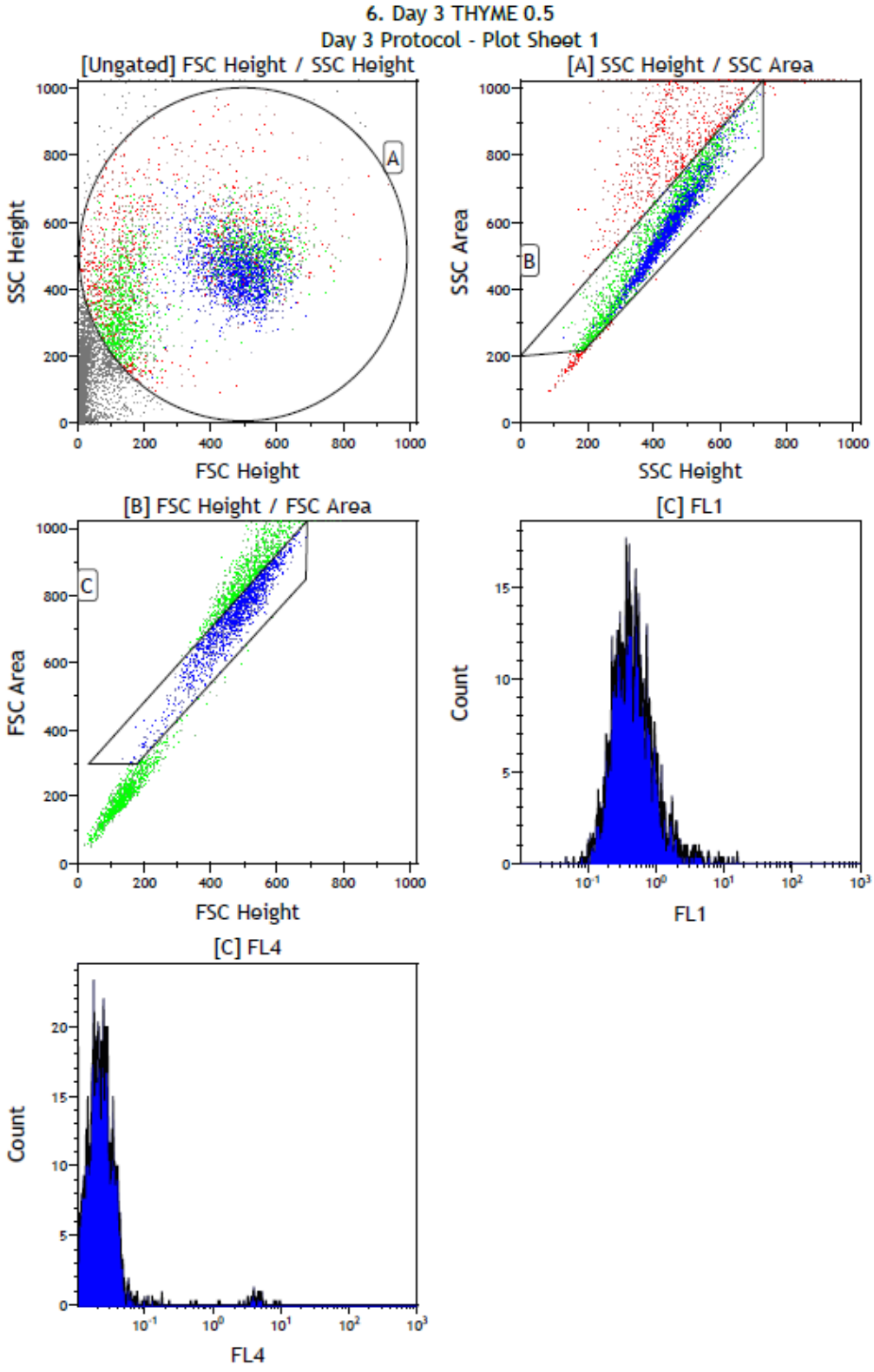


Figure 72: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 0.5% for 72 hours (Set 2).

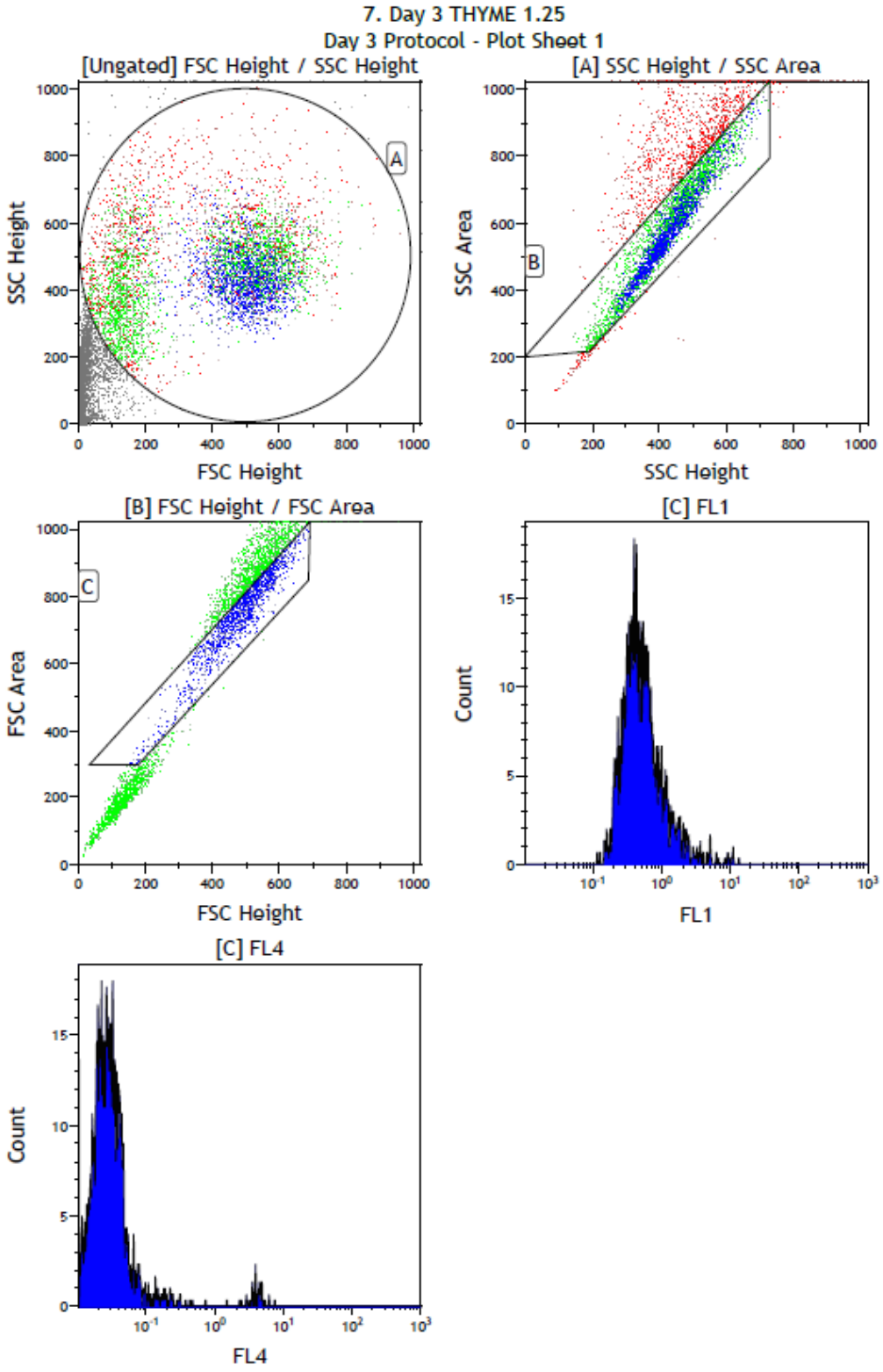


Figure 73: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 1.25% for 72 hours (Set 2).

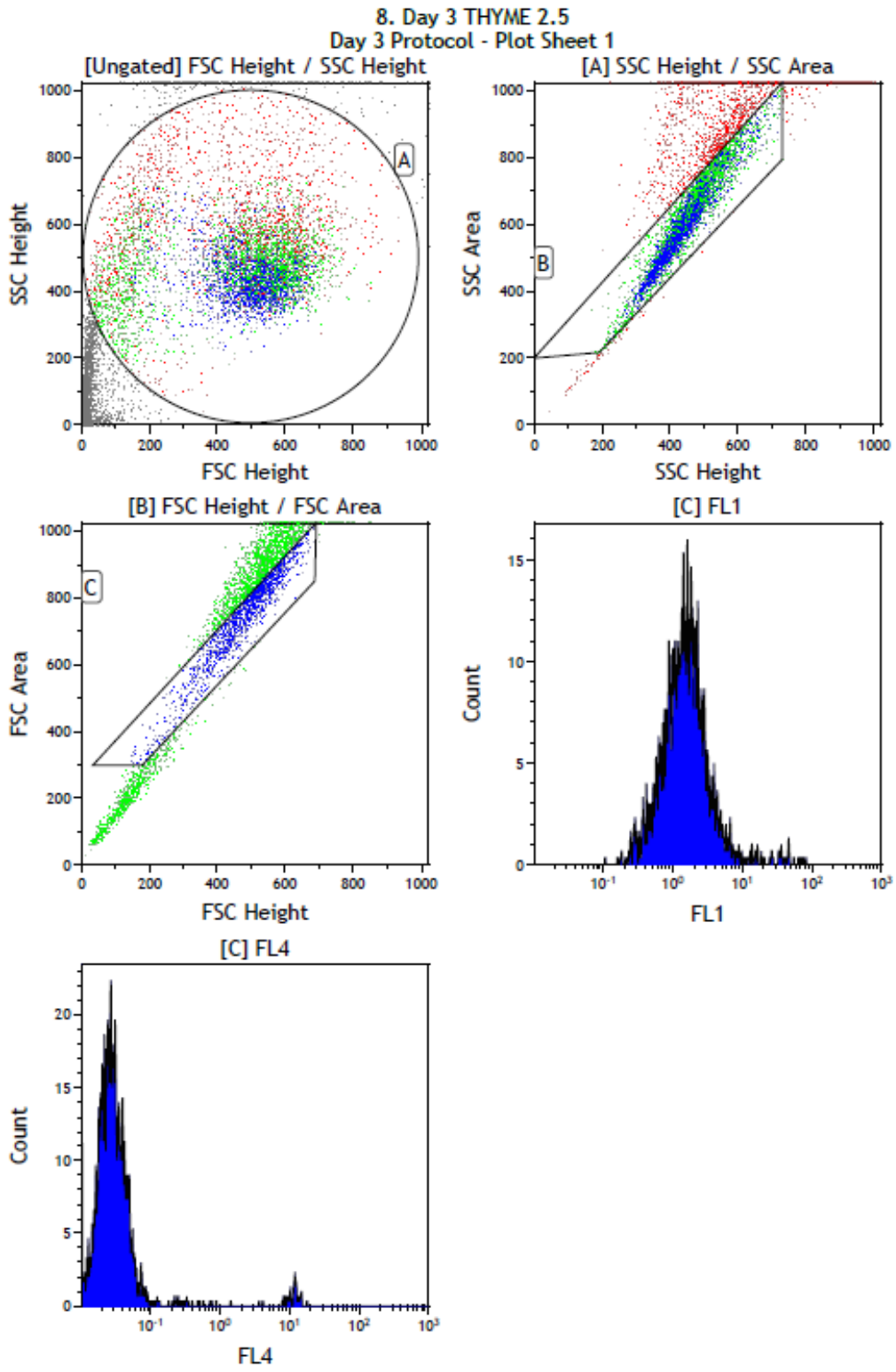
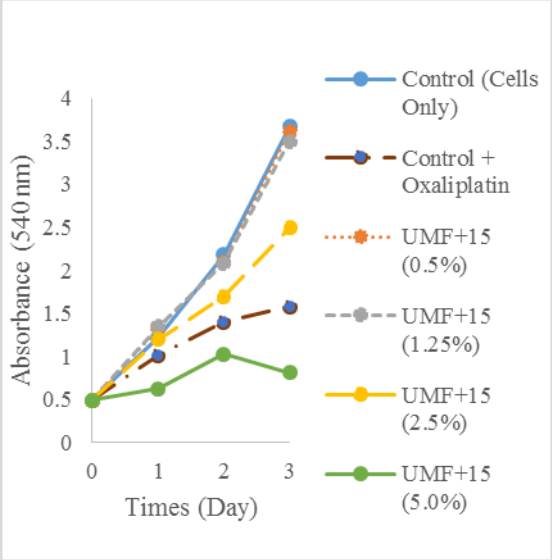


Figure 74: Apoptosis annexin V + PI data plot. LoVo cells treated with Thyme 2.5% for 72 hours (Set 2).

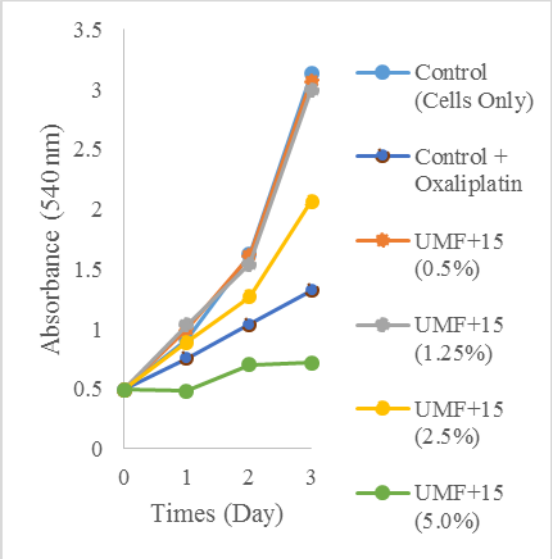
WiDr raw data section

Anti-proliferative effect of honey



Cells were treated with UMF+15 from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

Figure 75: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on WiDr cells (Set 1).



Cells were treated with UMF+15 from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

Figure 76: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on WiDr cells (Set 2).

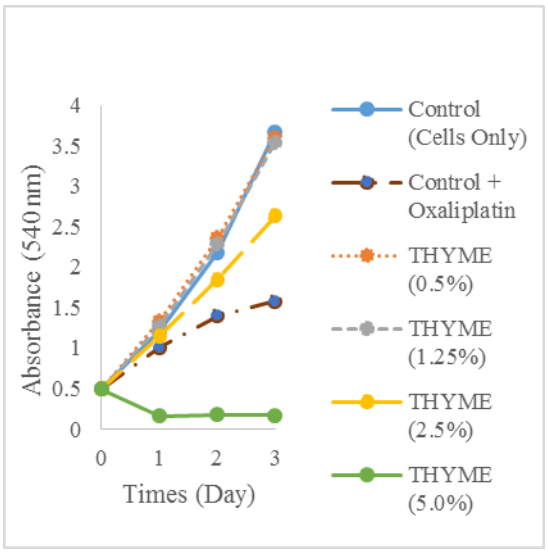


Figure 77: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on WiDr cells (Set 1).

Cells were treated with Thyme from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

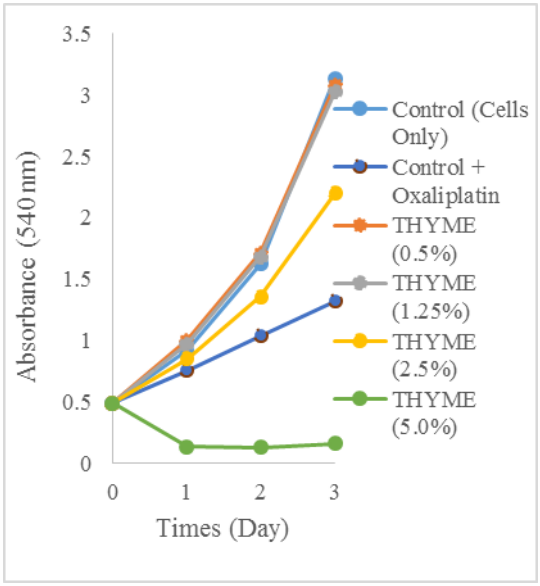


Figure 78: Absorbance at 540nm vs time (24 hours, 48 hours and 72 hours) on WiDr cells (Set 2).

Cells were treated with Thyme from 0.5% to 5% for 24, 48 and 72 hours. Data were obtained as means ± standard errors (n=6).

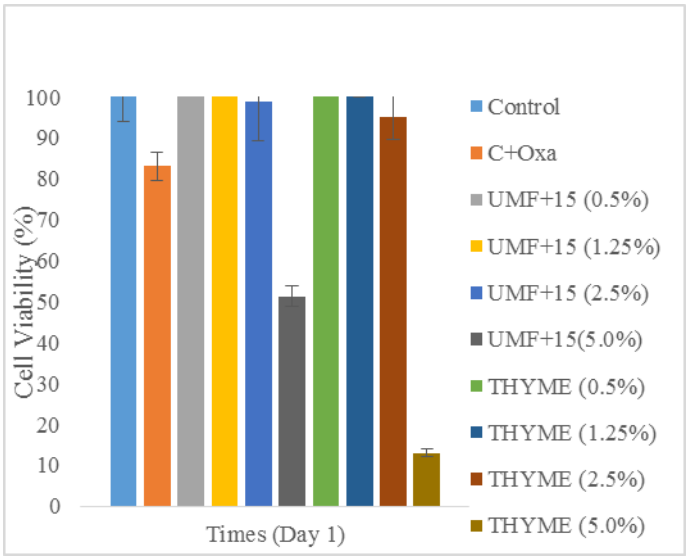


Figure 79: Cell viability (%) vs time (24 hours) on WiDr cells (Set 1).

Data were obtained as means ± standard errors (n=6).

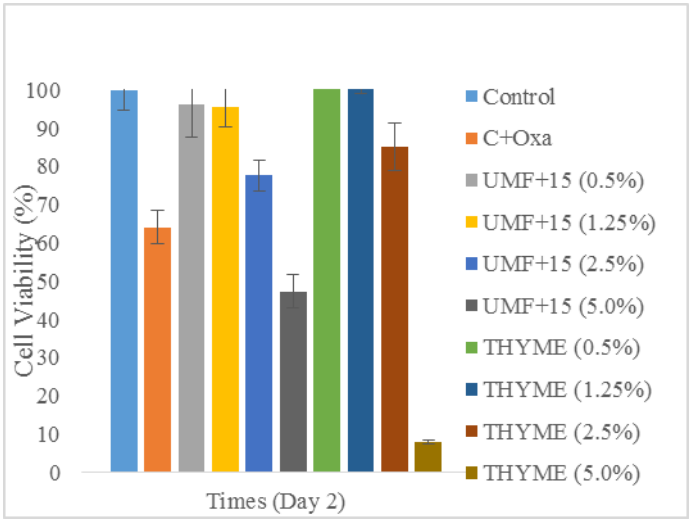


Figure 80: Cell viability (%) vs time (48 hours) on WiDr cells (Set 1).

Data were obtained as means ± standard errors (n=6).

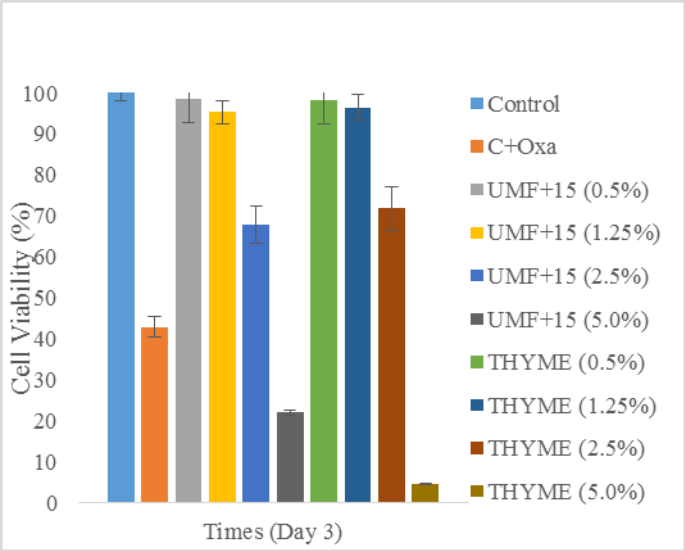


Figure 81: Cell viability (%) vs time (72 hours) on WiDr cells (Set 1).

Data were obtained as means ± standard errors (n=6).

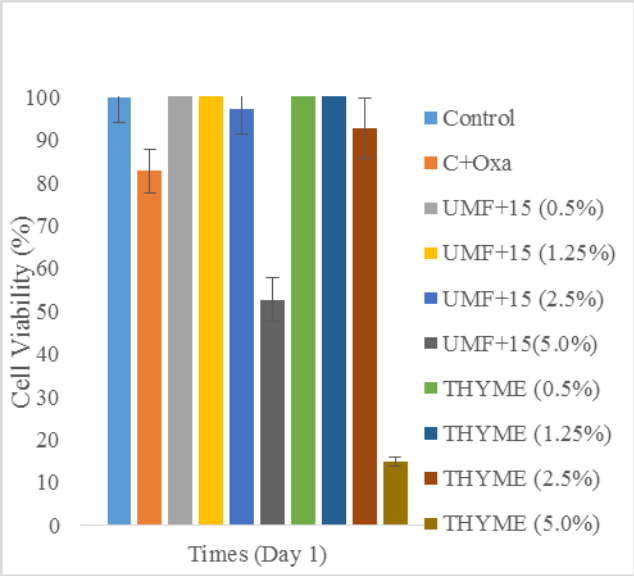


Figure 82: Cell viability (%) vs time (24 hours) on WiDr cells (Set 2).

Data were obtained as means ± standard errors (n=6).

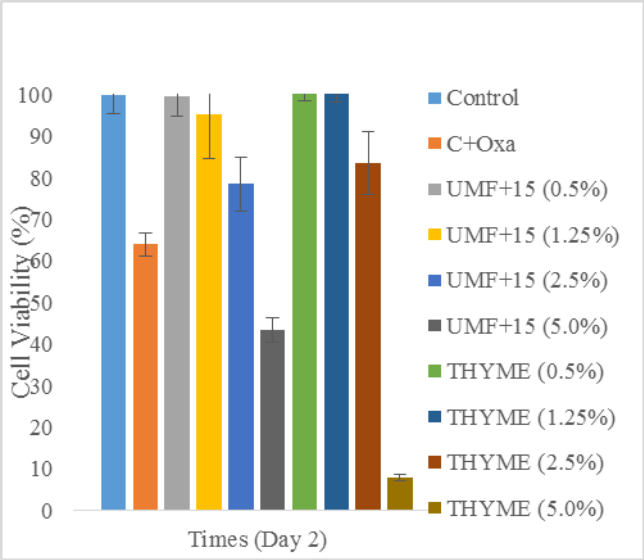


Figure 83: Cell viability (%) vs time (48 hours) on WiDr cells (Set 2).

Data were obtained as means ± standard errors (n=6).

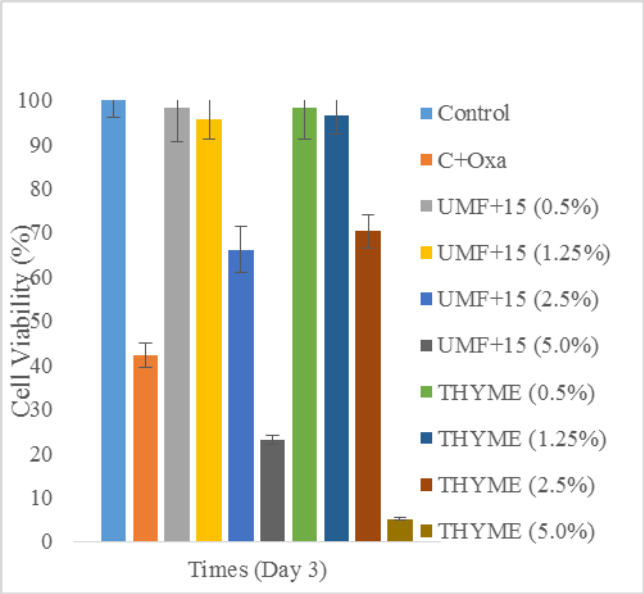


Figure 84: Cell viability (%) vs time (72 hours) on WiDr cells (Set 2).

Data were obtained as means ± standard errors (n=6).

Statistical data analysis by Prism

Table 32: Control Comparison (1way ANOVA)

Table Analyzed	Control Comparison				
ANOVA summary					
F	239.9				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9356				
Brown-Forsythe test					
F (DFn, DFd)	8.764 (2, 33)				
P value	0.0009				
P value summary	***				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	4.06				
P value	0.1313				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	33.66	2	16.83	F (2, 33) = 239.9	P < 0.0001
Residual (within columns)	2.315	33	0.07016		
Total	35.98	35			
Data summary					
Number of treatments (columns)	3				
Number of values (total)	36				

Table 33: Control Comparison (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	2
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control (Day 1) vs. Control (Day 2)	-0.8367	-1.087 to -0.5869	Yes	****
Control (Day 1) vs. Control (Day 3)	-2.337	-2.587 to -2.088	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Control (Day 1) vs. Control (Day 2)	1.067	1.904	-0.8367	0.1081	12	12	7.737	33
Control (Day 1) vs. Control (Day 3)	1.067	3.405	-2.337	0.1081	12	12	21.61	33

Table 34: UMF+15 vs Control Day 1 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 1				
ANOVA summary					
F	171.9				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9259				
Brown-Forsythe test					
F (DFn, DFd)	1.196 (4, 55)				
P value	0.3228				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	5.238				
P value	0.2638				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	28370	4	7092	F (4, 55) = 171.9	P < 0.0001
Residual (within columns)	2270	55	41.27		
Total	30640	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 35: UMF+15 vs Control Day 1 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	-9.097	-15.87 to -2.324	Yes	**
Control vs. UMF+15 1.25%	-11.89	-18.66 to -5.113	Yes	***
Control vs. UMF+15 2.5%	1.884	-4.889 to 8.657	No	ns
Control vs. UMF+15 5%	48	41.22 to 54.77	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	109.1	-9.097	2.623	12	12	3.469	55
Control vs. UMF+15 1.25%	100	111.9	-11.89	2.623	12	12	4.532	55
Control vs. UMF+15 2.5%	100	98.12	1.884	2.623	12	12	0.7183	55
Control vs. UMF+15 5%	100	52	48	2.623	12	12	18.3	55

Table 36: UMF+15 vs Control Day 2 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 2				
ANOVA summary					
F	177.9				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9283				
Brown-Forsythe test					
F (DFn, DFd)	1.928 (4, 55)				
P value	0.1188				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
Bartlett's test					
Bartlett's statistic (corrected)	6.654				
P value	0.1553				
P value summary	ns				
Significantly different standard deviations? (P < 0.05)	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	25280	4	6320	F (4, 55) = 177.9	P < 0.0001
Residual (within columns)	1954	55	35.53		
Total	27234	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 37: UMF+15 vs Control Day 2 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	2.092	-4.193 to 8.376	No	ns
Control vs. UMF+15 1.25%	4.519	-1.765 to 10.80	No	ns
Control vs. UMF+15 2.5%	21.96	15.68 to 28.25	Yes	****
Control vs. UMF+15 5%	54.63	48.34 to 60.91	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	97.91	2.092	2.433	12	12	0.8596	55
Control vs. UMF+15 1.25%	100	95.48	4.519	2.433	12	12	1.857	55
Control vs. UMF+15 2.5%	100	78.04	21.96	2.433	12	12	9.026	55
Control vs. UMF+15 5%	100	45.37	54.63	2.433	12	12	22.45	55

Table 38: UMF+15 vs Control Day 3 (1way ANOVA)

Table Analyzed	UMF+15 vs Control Day 3				
ANOVA summary					
F	761.7				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9823				
Brown-Forsythe test					
F (DFn, DFd)	4.771 (4, 55)				
P value	0.0022				
P value summary	**				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	28.22				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	52826	4	13207	F (4, 55) = 761.7	P < 0.0001
Residual (within columns)	953.6	55	17.34		
Total	53780	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 39: UMF+15 vs Control Day 3 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. UMF+15 0.5%	1.627	-2.763 to 6.017	No	ns
Control vs. UMF+15 1.25%	4.459	0.06907 to 8.849	Yes	*
Control vs. UMF+15 2.5%	32.97	28.58 to 37.36	Yes	****
Control vs. UMF+15 5%	77.53	73.14 to 81.92	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. UMF+15 0.5%	100	98.37	1.627	1.7	12	12	0.9571	55
Control vs. UMF+15 1.25%	100	95.54	4.459	1.7	12	12	2.623	55
Control vs. UMF+15 2.5%	100	67.03	32.97	1.7	12	12	19.4	55
Control vs. UMF+15 5%	100	22.47	77.53	1.7	12	12	45.61	55

Table 40: Thyme vs Control Day 1 (1way ANOVA)

Table Analyzed	THYME vs Control Day 1				
ANOVA summary					
F	775.9				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9826				
Brown-Forsythe test					
F (DFn, DFd)	3.291 (4, 55)				
P value	0.0173				
P value summary	*				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	19.4				
P value	0.0007				
P value summary	***				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	77095	4	19274	F (4, 55) = 775.9	P < 0.0001
Residual (within columns)	1366	55	24.84		
Total	78462	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 41: Thyme vs Control Day 1 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. THYME 0.5%	-10.04	-15.30 to -4.790	Yes	****
Control vs. THYME 1.25%	-5.847	-11.10 to -0.5926	Yes	*
Control vs. THYME 2.5%	6.045	0.7906 to 11.30	Yes	*
Control vs. THYME 5%	86.12	80.86 to 91.37	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	110	-10.04	2.035	12	12	4.937	55
Control vs. THYME 1.25%	100	105.8	-5.847	2.035	12	12	2.874	55
Control vs. THYME 2.5%	100	93.95	6.045	2.035	12	12	2.971	55
Control vs. THYME 5%	100	13.88	86.12	2.035	12	12	42.33	55

Table 42: Thyme vs Control Day 2 (1way ANOVA)

Table Analyzed	THYME vs Control Day 2				
ANOVA summary					
F	742.2				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9818				
Brown-Forsythe test					
F (DFn, DFd)	3.498 (4, 55)				
P value	0.0129				
P value summary	*				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	38.05				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	83307	4	20827	F (4, 55) = 742.2	P < 0.0001
Residual (within columns)	1543	55	28.06		
Total	84850	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 43: Thyme vs Control Day 2 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. THYME 0.5%	-7.035	-12.62 to -1.450	Yes	**
Control vs. THYME 1.25%	-4.267	-9.852 to 1.318	No	ns
Control vs. THYME 2.5%	15.68	10.09 to 21.26	Yes	****
Control vs. THYME 5%	92.15	86.57 to 97.74	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	107	-7.035	2.163	12	12	3.253	55
Control vs. THYME 1.25%	100	104.3	-4.267	2.163	12	12	1.973	55
Control vs. THYME 2.5%	100	84.32	15.68	2.163	12	12	7.249	55
Control vs. THYME 5%	100	7.846	92.15	2.163	12	12	42.61	55

Table 44: Thyme vs Control Day 3 (1way ANOVA)

Table Analyzed	THYME vs Control Day 3				
ANOVA summary					
F	1223				
P value	< 0.0001				
P value summary	****				
Are differences among means statistically significant? (P < 0.05)	Yes				
R square	0.9889				
Brown-Forsythe test					
F (DFn, DFd)	4.563 (4, 55)				
P value	0.003				
P value summary	**				
Significantly different standard deviations? (P < 0.05)	Yes				
Bartlett's test					
Bartlett's statistic (corrected)	50.42				
P value	< 0.0001				
P value summary	****				
Significantly different standard deviations? (P < 0.05)	Yes				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	78818	4	19705	F (4, 55) = 1223	P < 0.0001
Residual (within columns)	886.3	55	16.11		
Total	79704	59			
Data summary					
Number of treatments (columns)	5				
Number of values (total)	60				

Table 45: Thyme vs Control Day 3 (1way ANOVA Multiple Comparison)

Number of families	1
Number of comparisons per family	4
Alpha	0.05

Bonferroni's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Control vs. THYME 0.5%	1.623	-2.609 to 5.855	No	ns
Control vs. THYME 1.25%	3.48	-0.7525 to 7.712	No	ns
Control vs. THYME 2.5%	28.95	24.72 to 33.18	Yes	****
Control vs. THYME 5%	95.15	90.92 to 99.39	Yes	****

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
Control vs. THYME 0.5%	100	98.38	1.623	1.639	12	12	0.9904	55
Control vs. THYME 1.25%	100	96.52	3.48	1.639	12	12	2.123	55
Control vs. THYME 2.5%	100	71.05	28.95	1.639	12	12	17.66	55
Control vs. THYME 5%	100	4.846	95.15	1.639	12	12	58.06	55

Table 46: Transform of WiDr IC50 with UMF+15 and Thyme honey.

Transform of UMF+15 IC_{50}	
log(inhibitor) vs. response -- Variable slope (four parameters)	Cell Viability (%)
Best-fit values	
Bottom	-1.159
Top	101.8
LogIC50	0.5175
HillSlope	-2.57
IC50	3.292
Span	103
Std. Error	
Bottom	2.165
Top	0.657
LogIC50	0.01224
HillSlope	0.1464
Span	2.397
95% Confidence Intervals	
Bottom	-7.170 to 4.851
Top	100.0 to 103.7
LogIC50	0.4835 to 0.5515
HillSlope	-2.977 to -2.164
IC50	3.045 to 3.560
Span	96.35 to 109.7
Goodness of Fit	
Degrees of Freedom	4
R square	0.9994
Absolute Sum of Squares	6.333
Sy.x	1.258
Number of points	
Analyzed	8

Transform of THYME IC_{50}	
log(inhibitor) vs. response -- Variable slope (four parameters)	Cell Viability (%)
Best-fit values	
Bottom	3.683
Top	98.31
LogIC50	0.4473
HillSlope	-8.596
IC50	2.801
Span	94.62
Std. Error	
Bottom	1.386
Top	0.6248
LogIC50	0.02577
HillSlope	4.347
Span	1.555
95% Confidence Intervals	
Bottom	-0.1641 to 7.531
Top	96.57 to 100.0
LogIC50	0.3757 to 0.5188
HillSlope	-20.66 to 3.472
IC50	2.375 to 3.302
Span	90.30 to 98.94
Goodness of Fit	
Degrees of Freedom	4
R square	0.9994
Absolute Sum of Squares	7.725
Sy.x	1.39
Number of points	
Analyzed	8

Apoptosis effect of honey

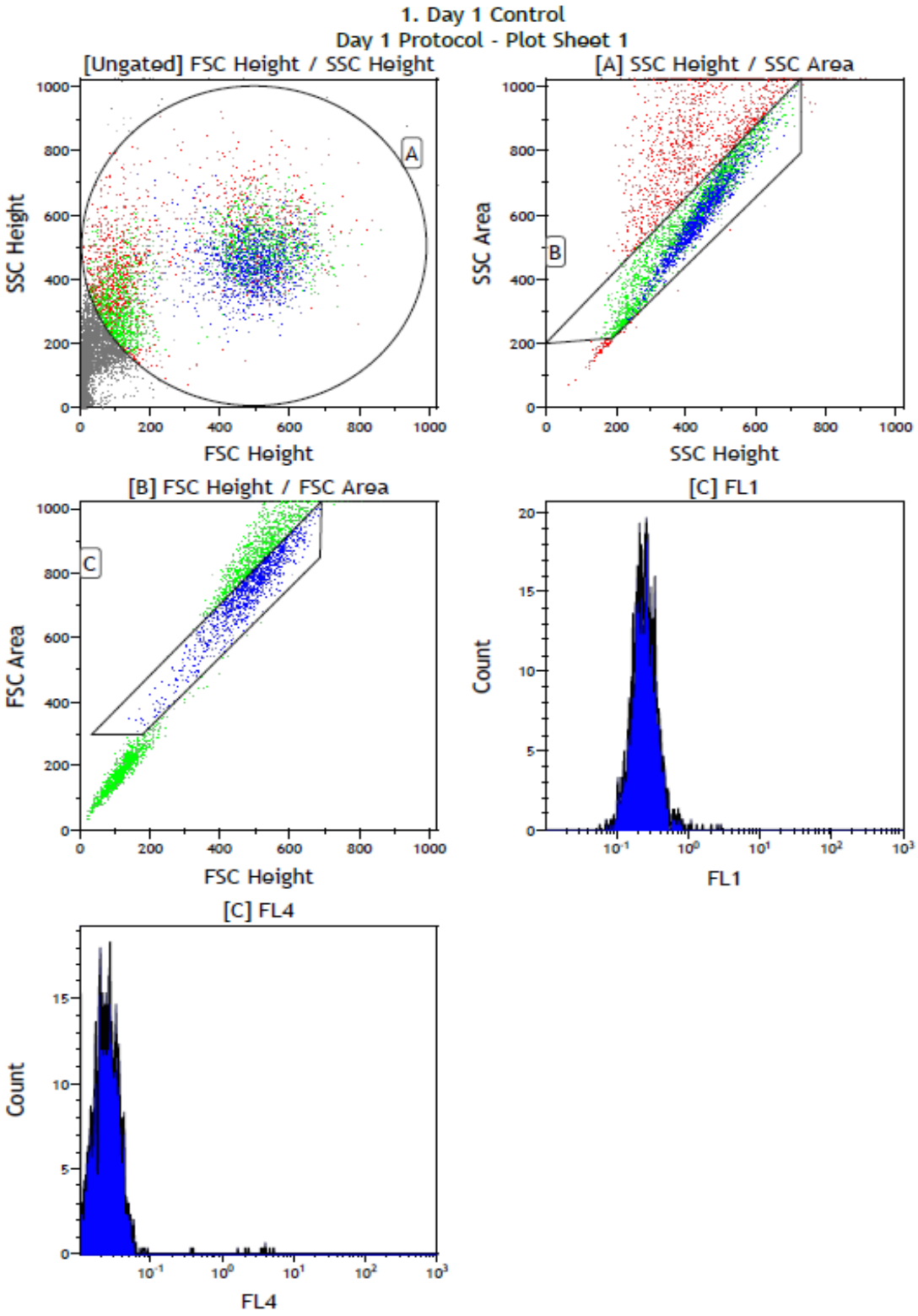


Figure 85: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 24 hours (Set 1).

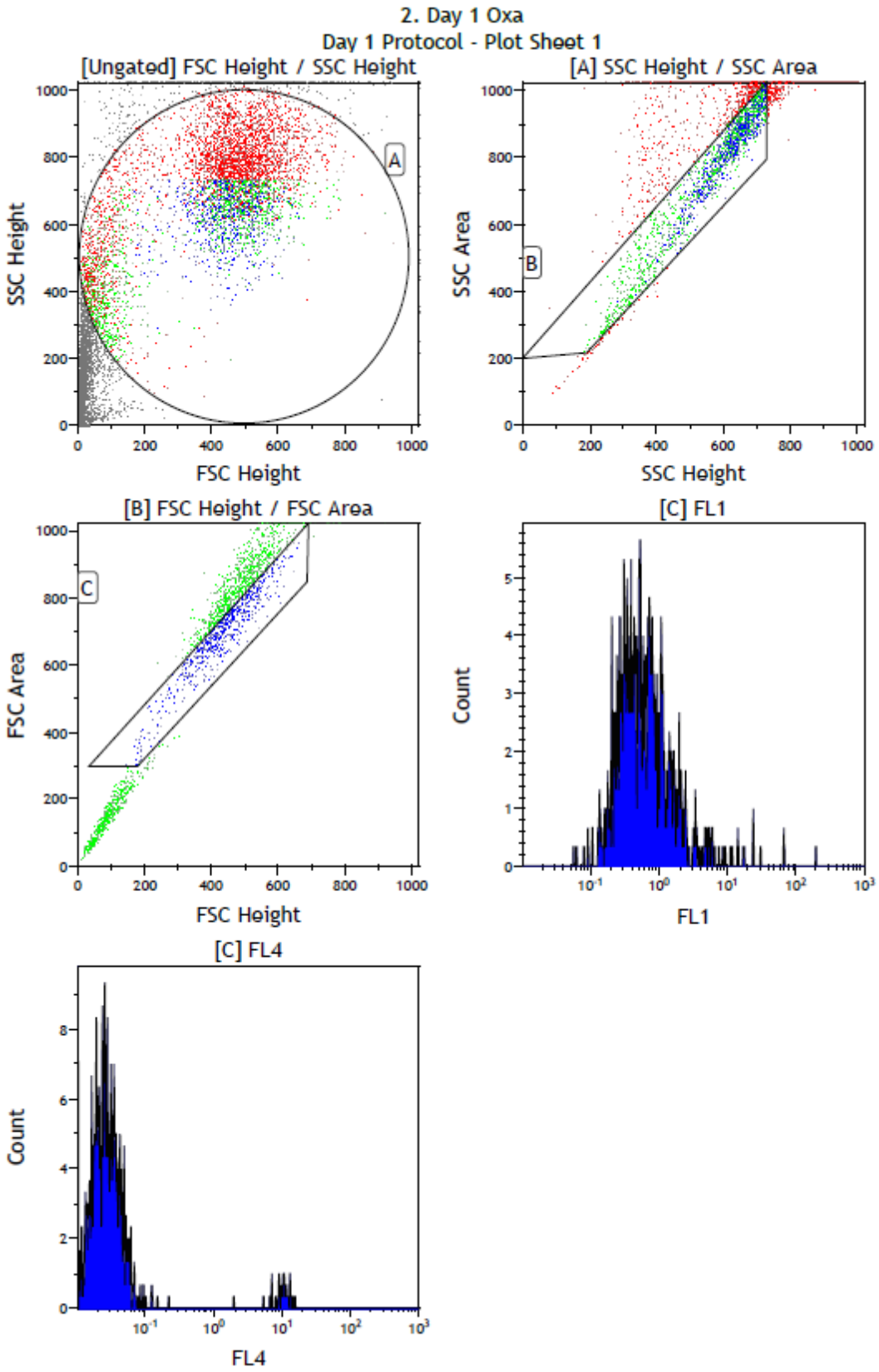


Figure 86: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 24 hours (Set 1).

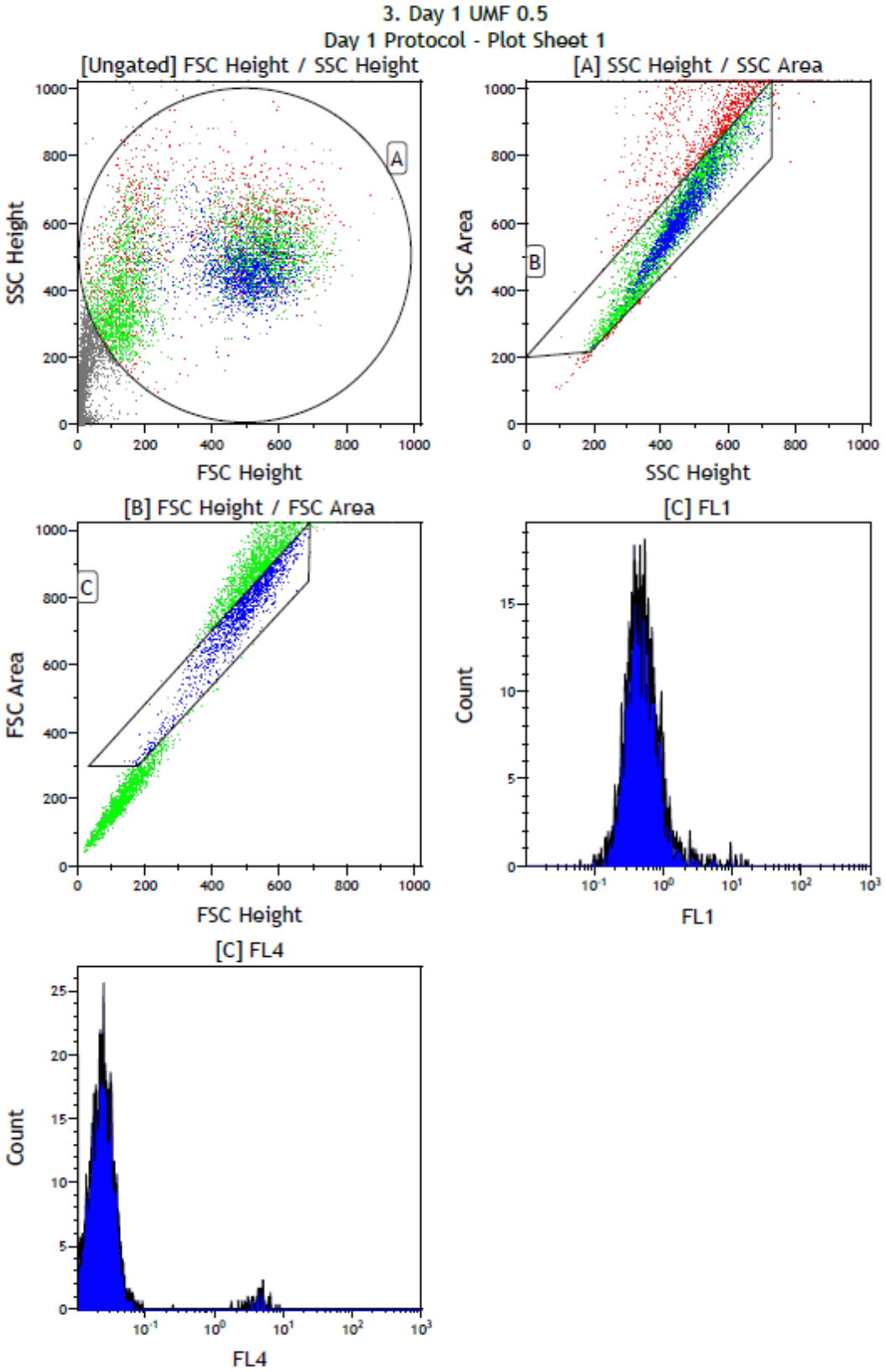


Figure 87: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 24 hours (Set 1).

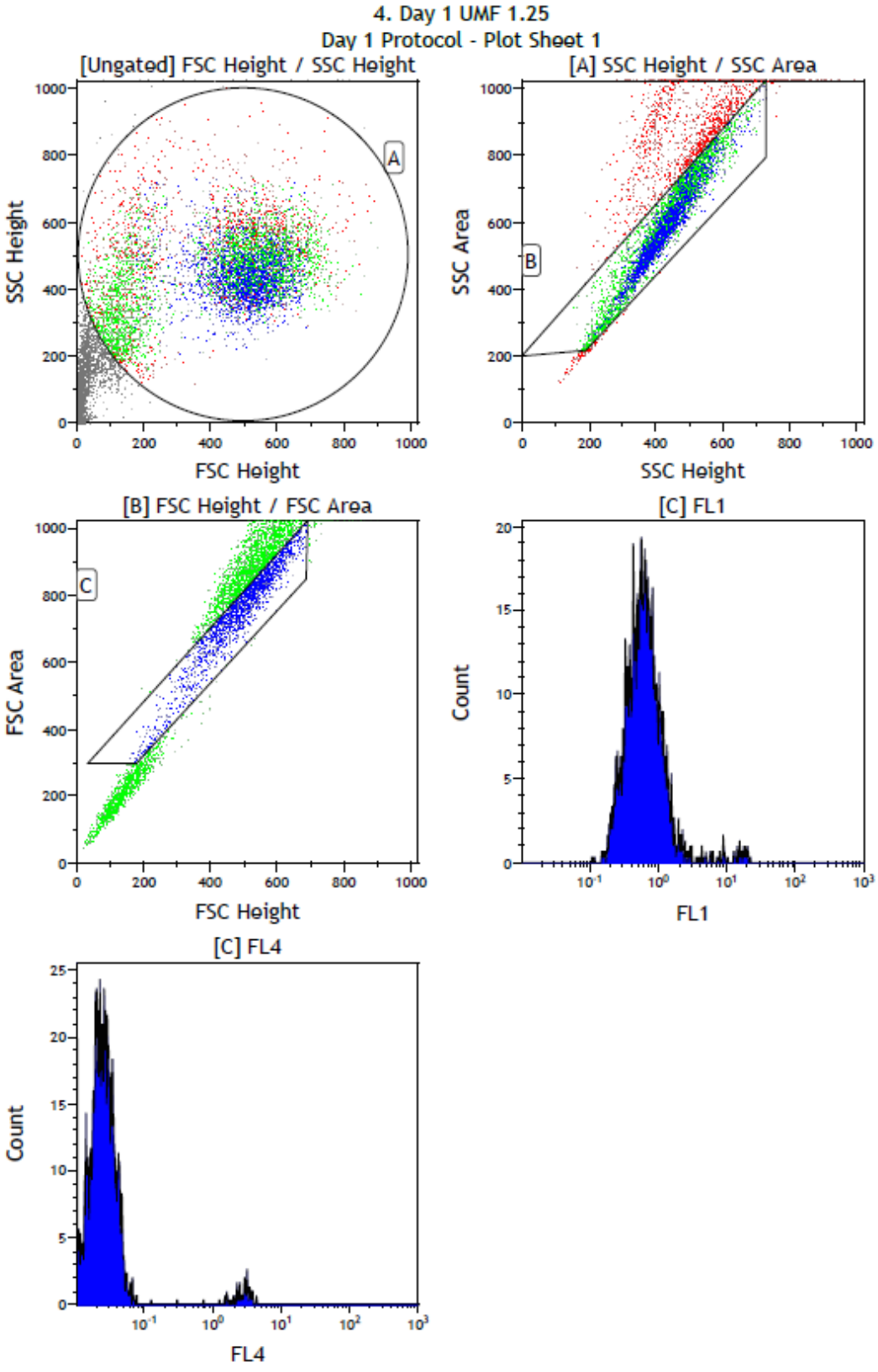


Figure 88: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 24 hours (Set 1).

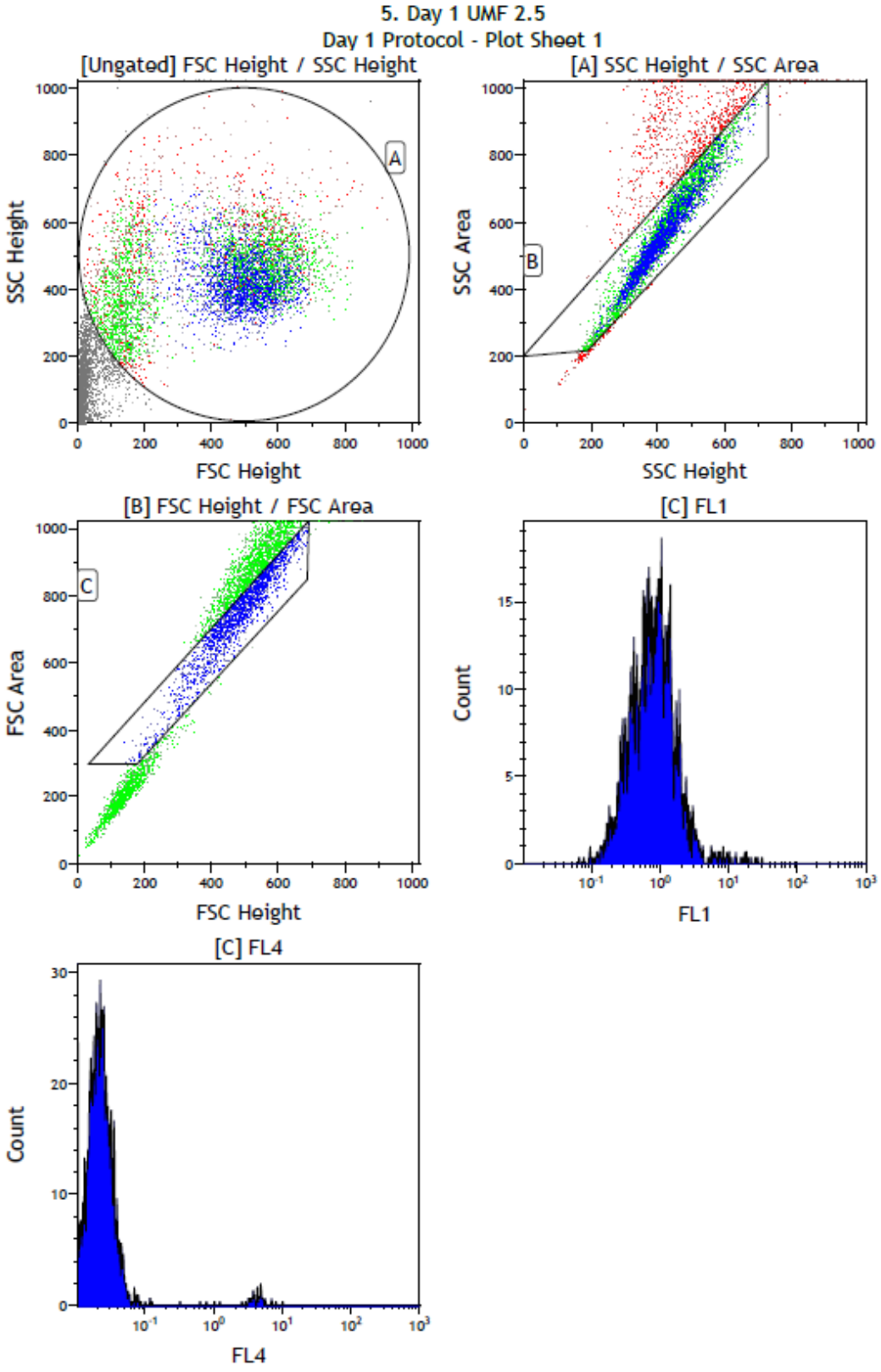


Figure 89: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 24 hours (Set 1).

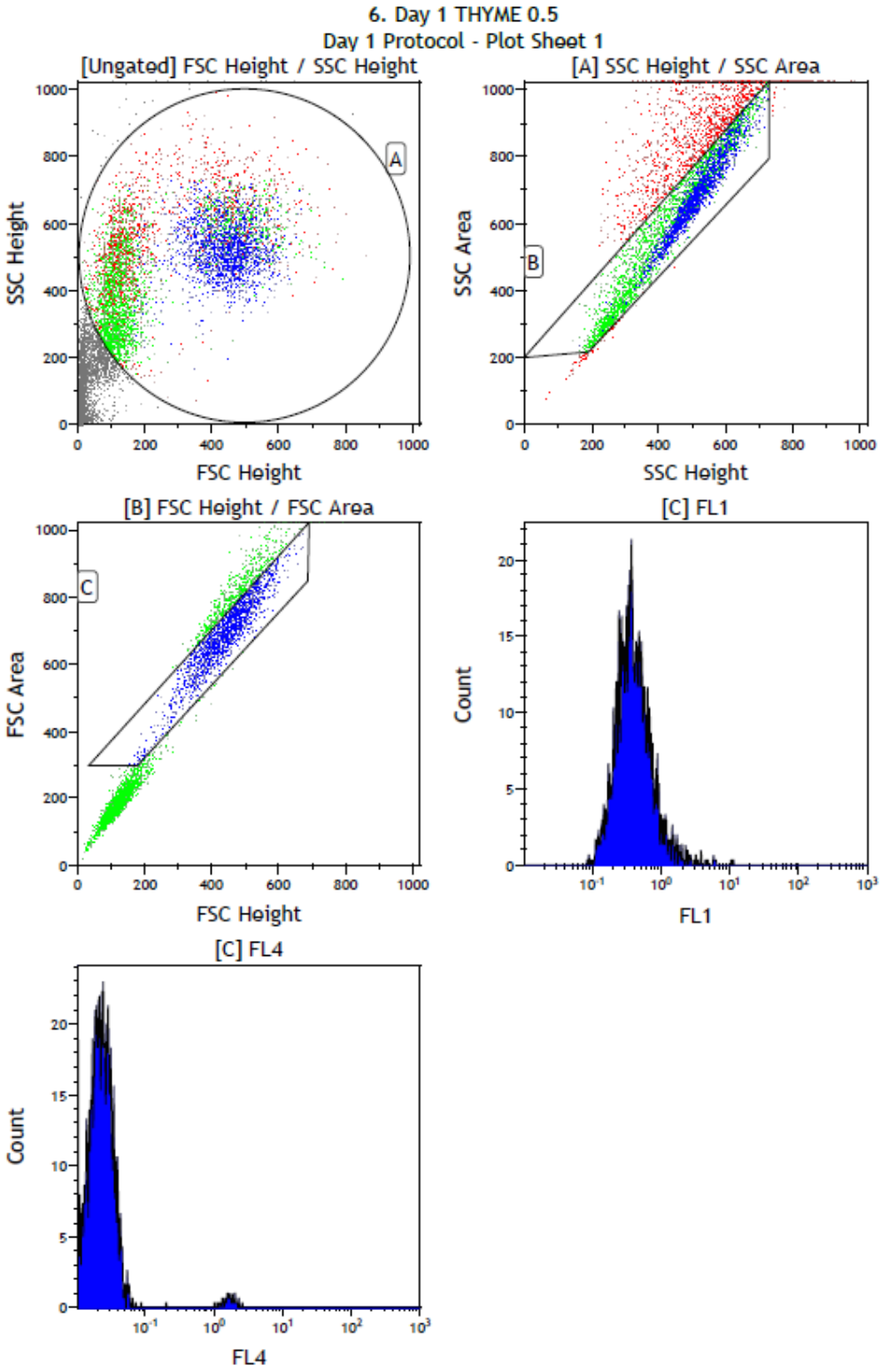


Figure 90: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 24 hours (Set 1).

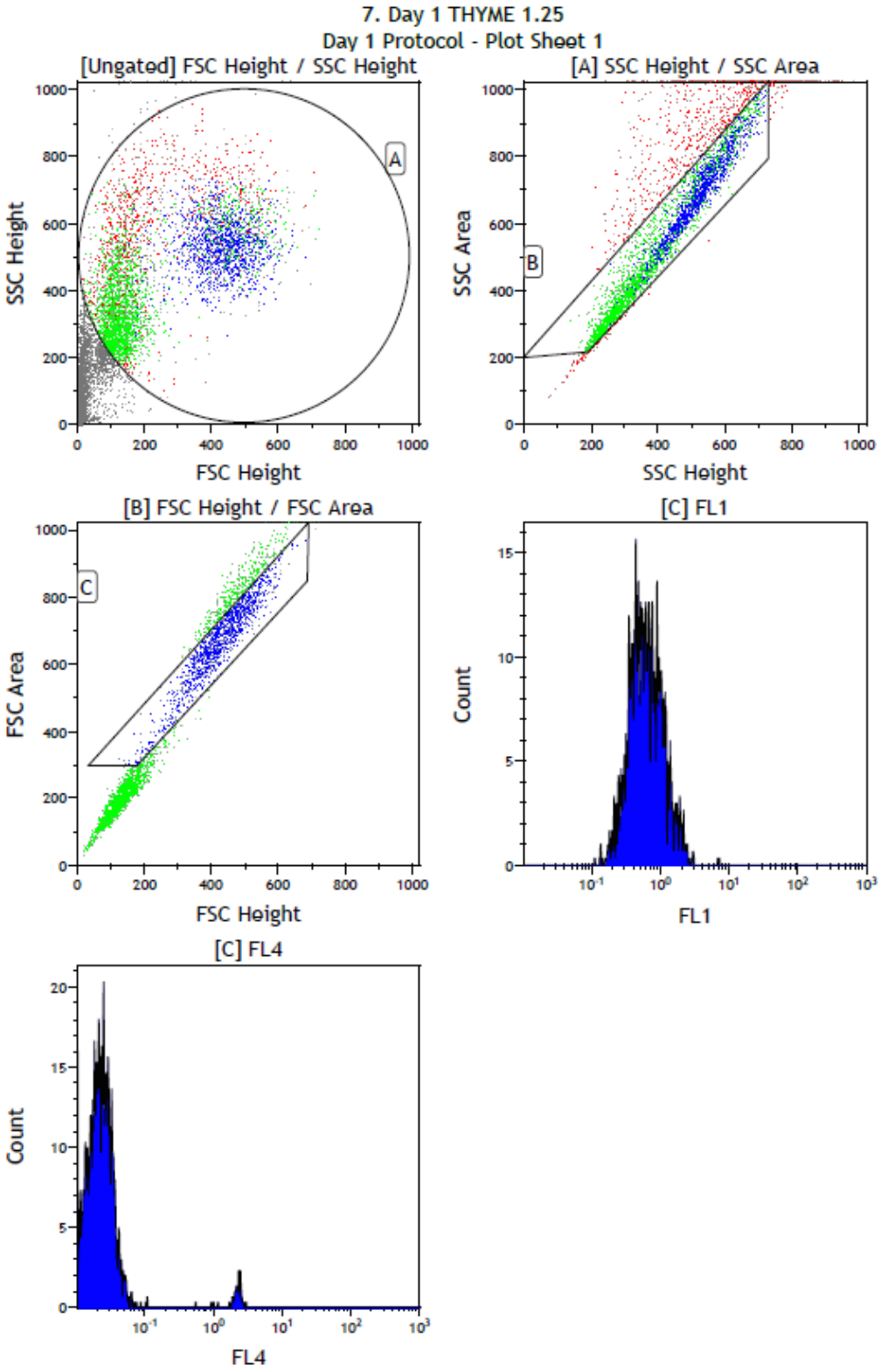


Figure 91: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 24 hours (Set 1).

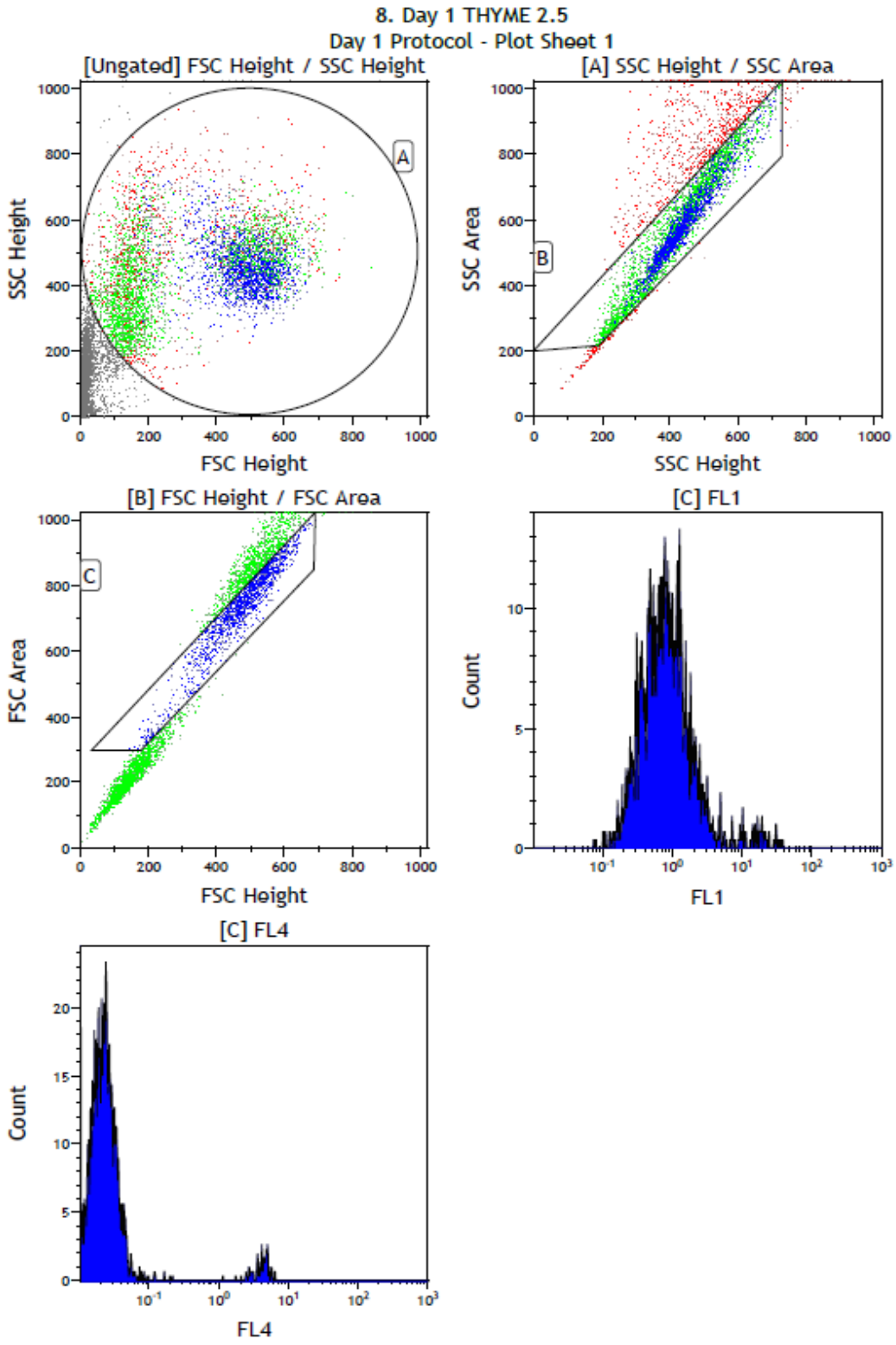


Figure 92: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 2.5% for 24 hours (Set 1).

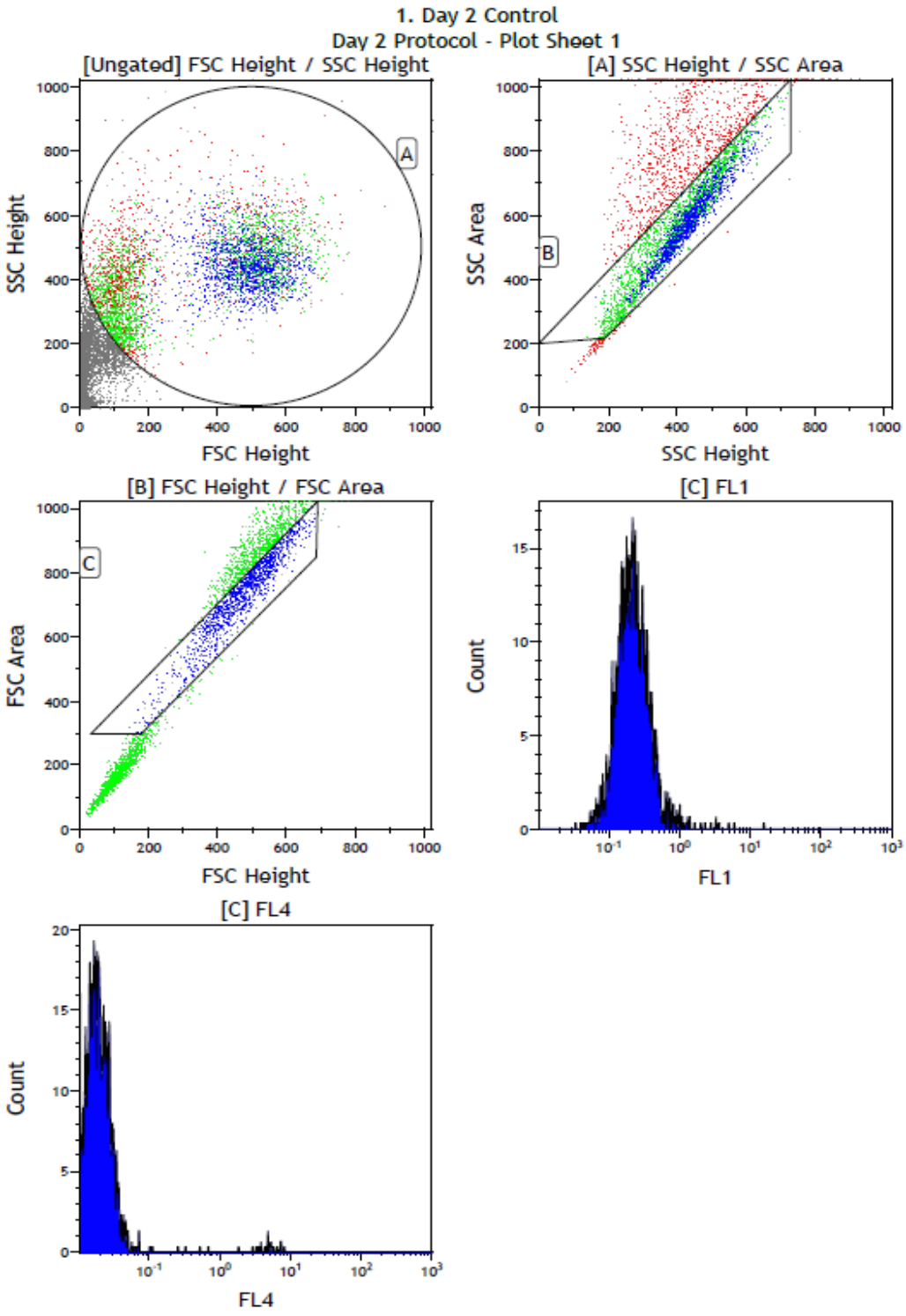


Figure 93: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 48 hours (Set 1).

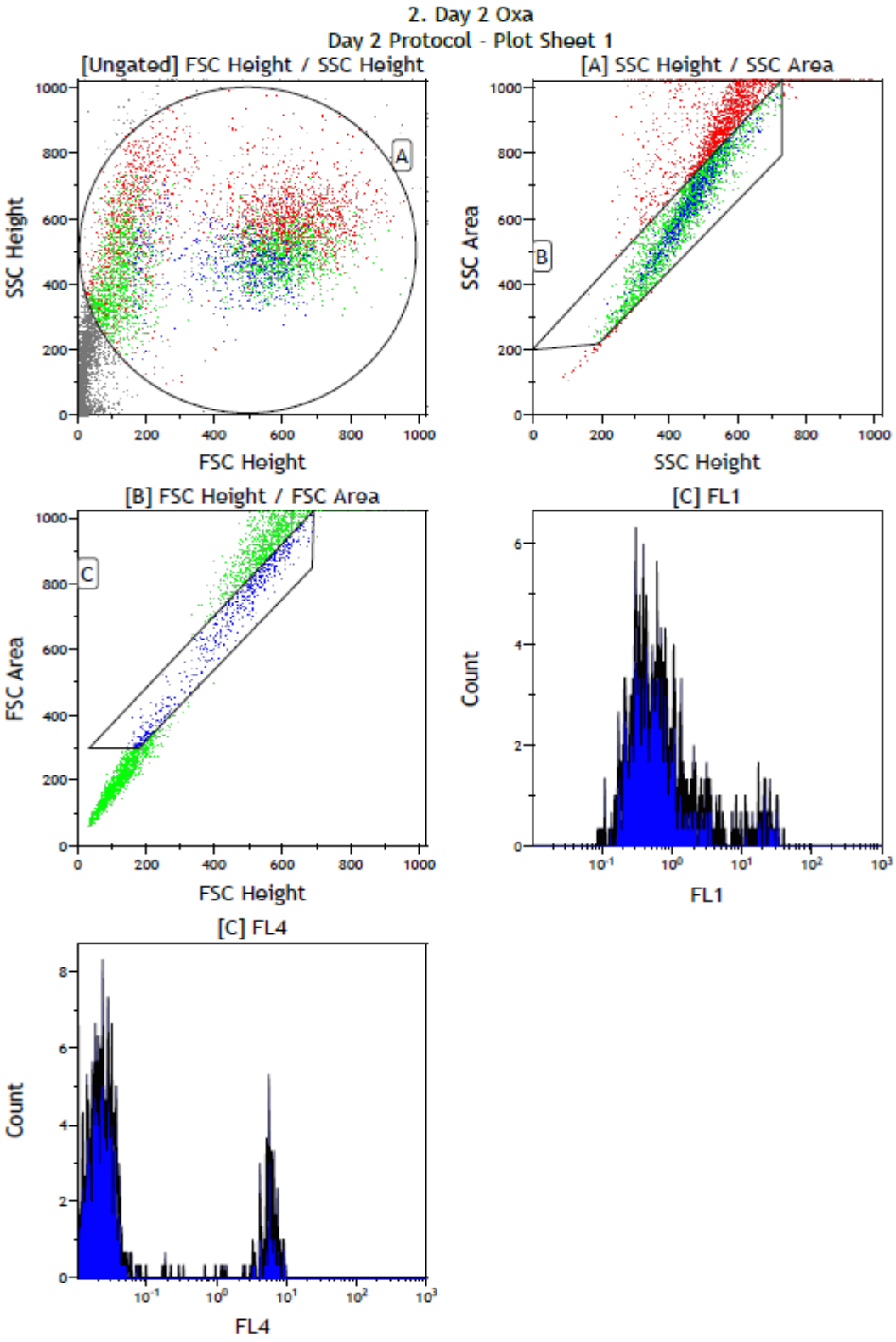


Figure 94: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 48 hours (Set 1).

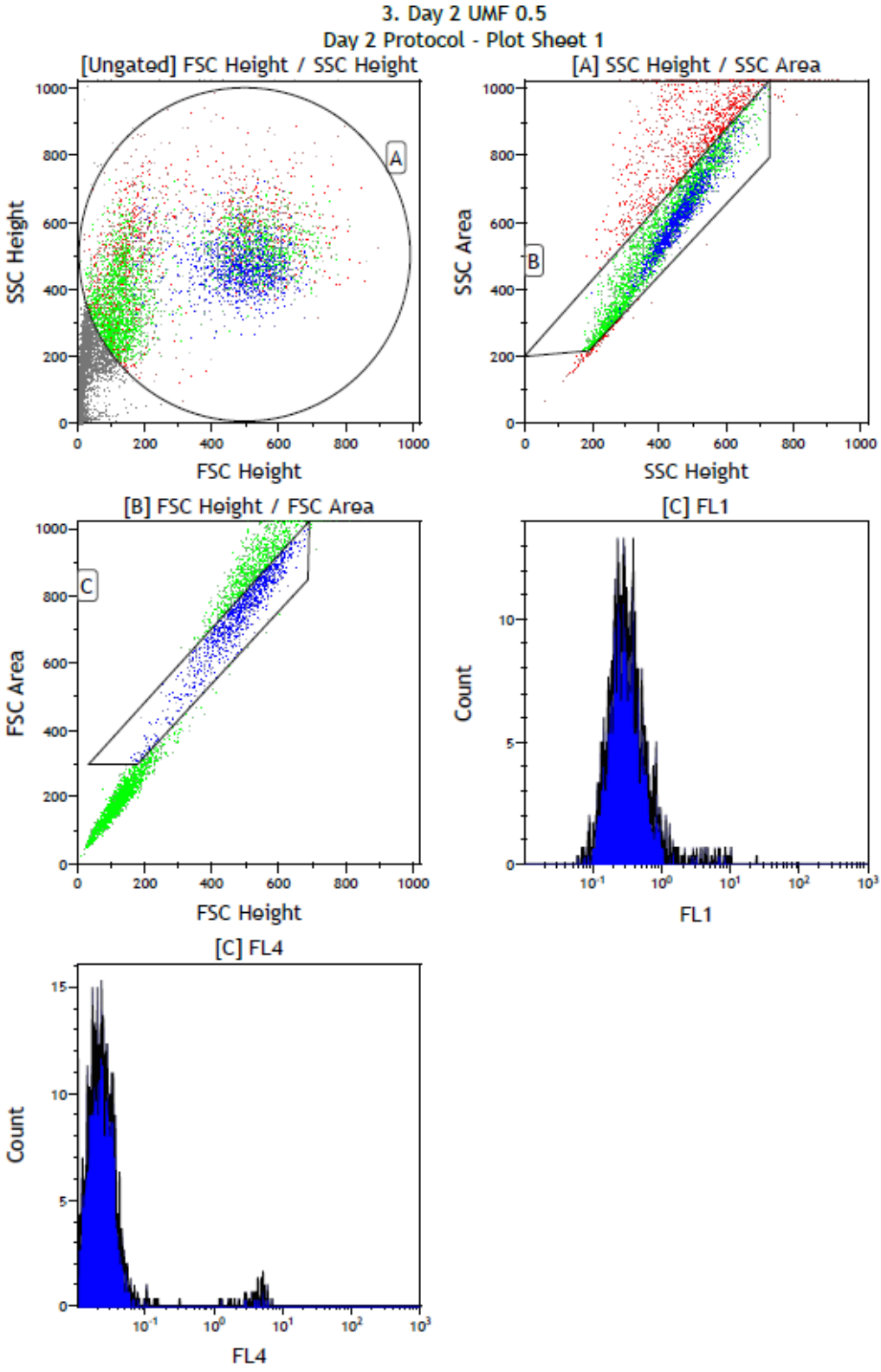


Figure 95: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 48 hours (Set 1).

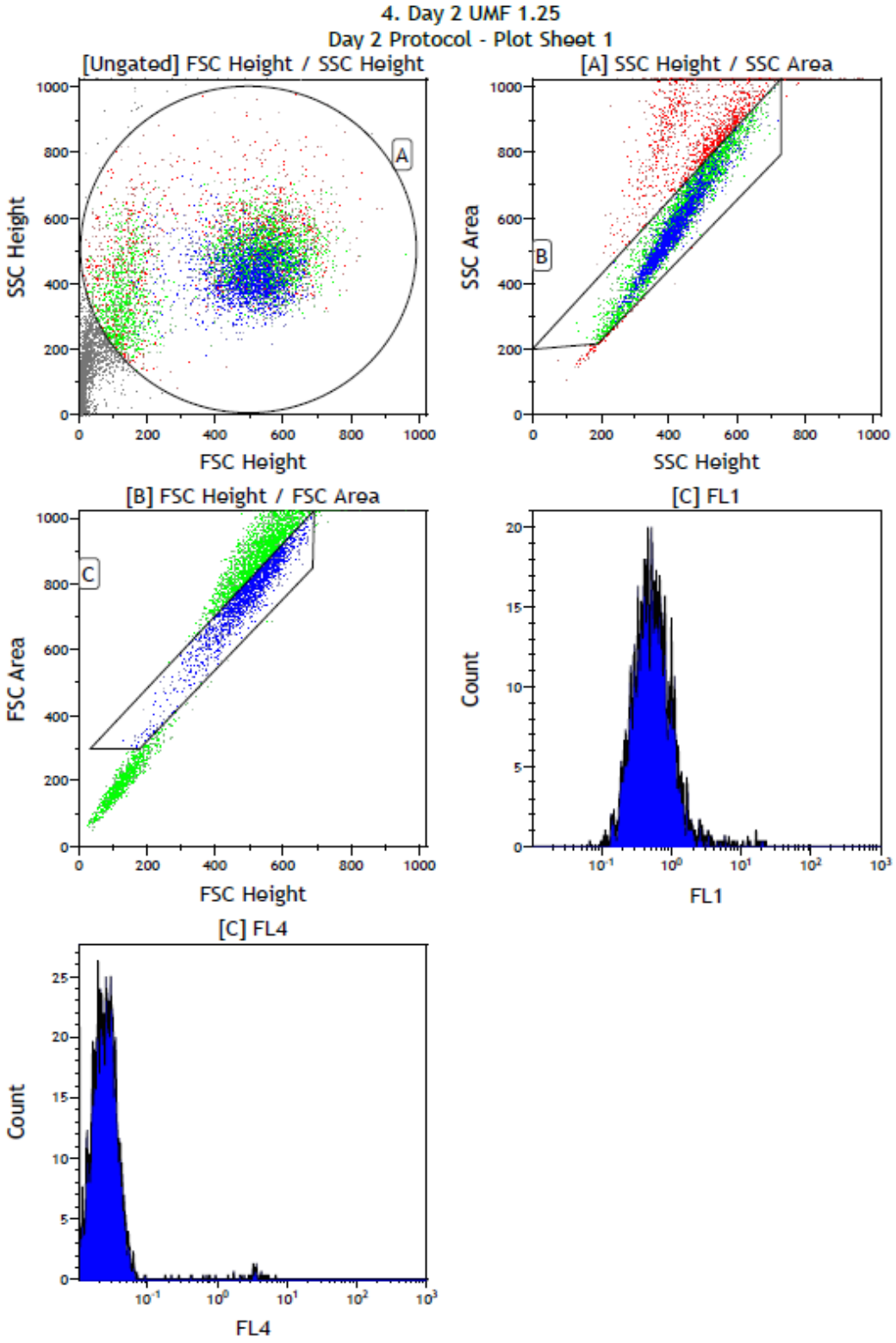


Figure 96: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 48 hours (Set 1).

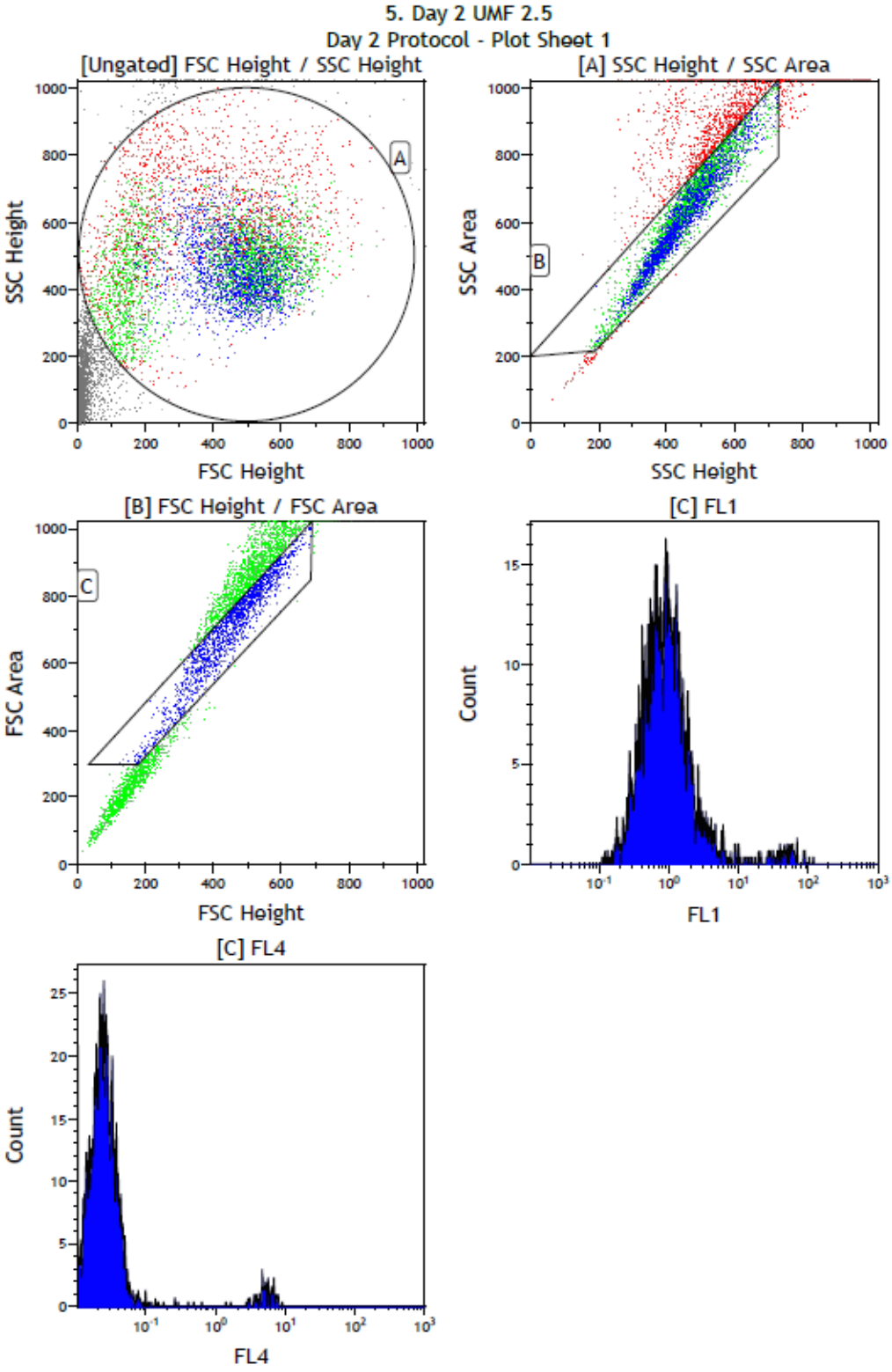


Figure 97: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 48 hours (Set 1).

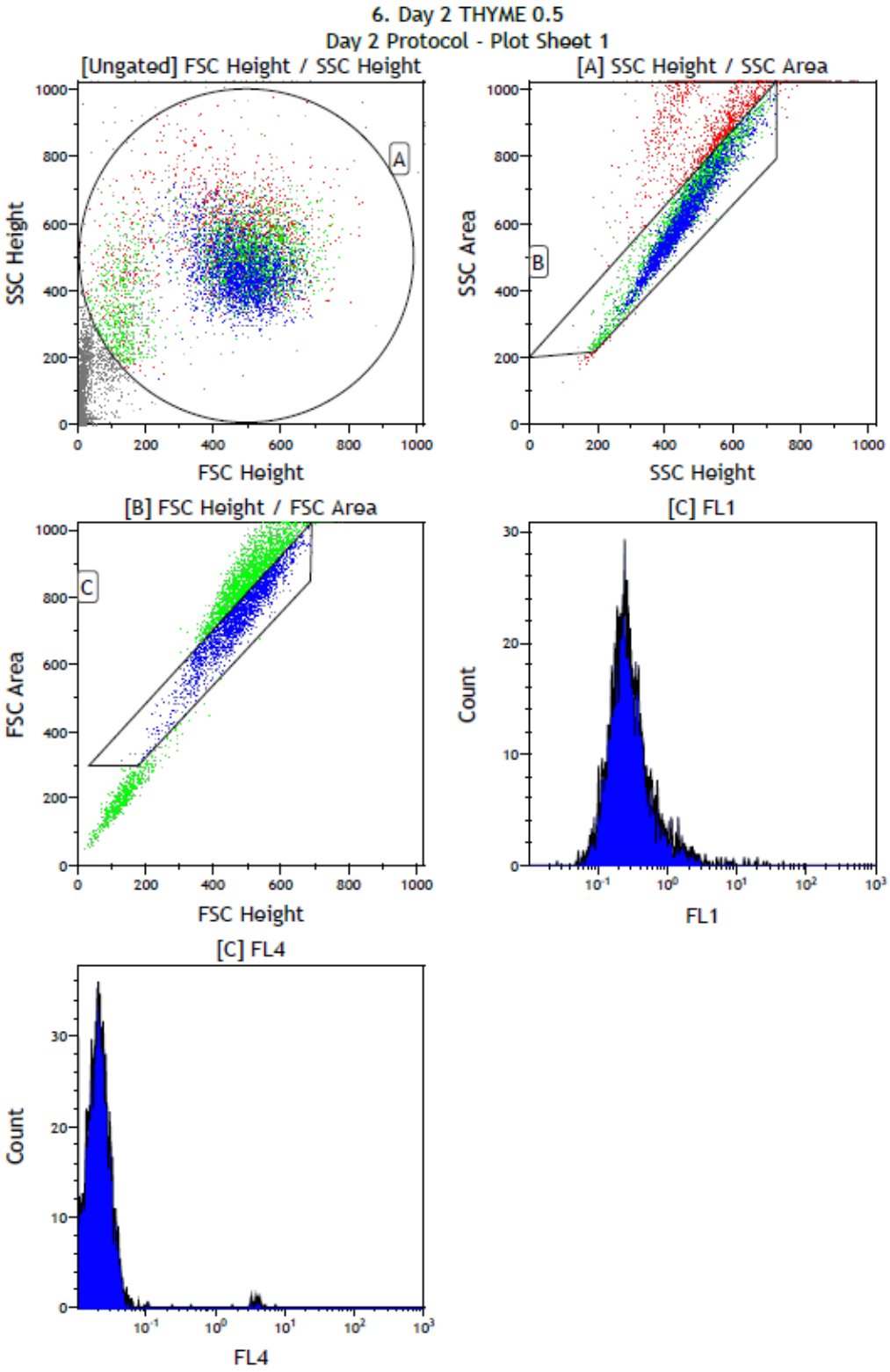


Figure 98: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 48 hours (Set 1).

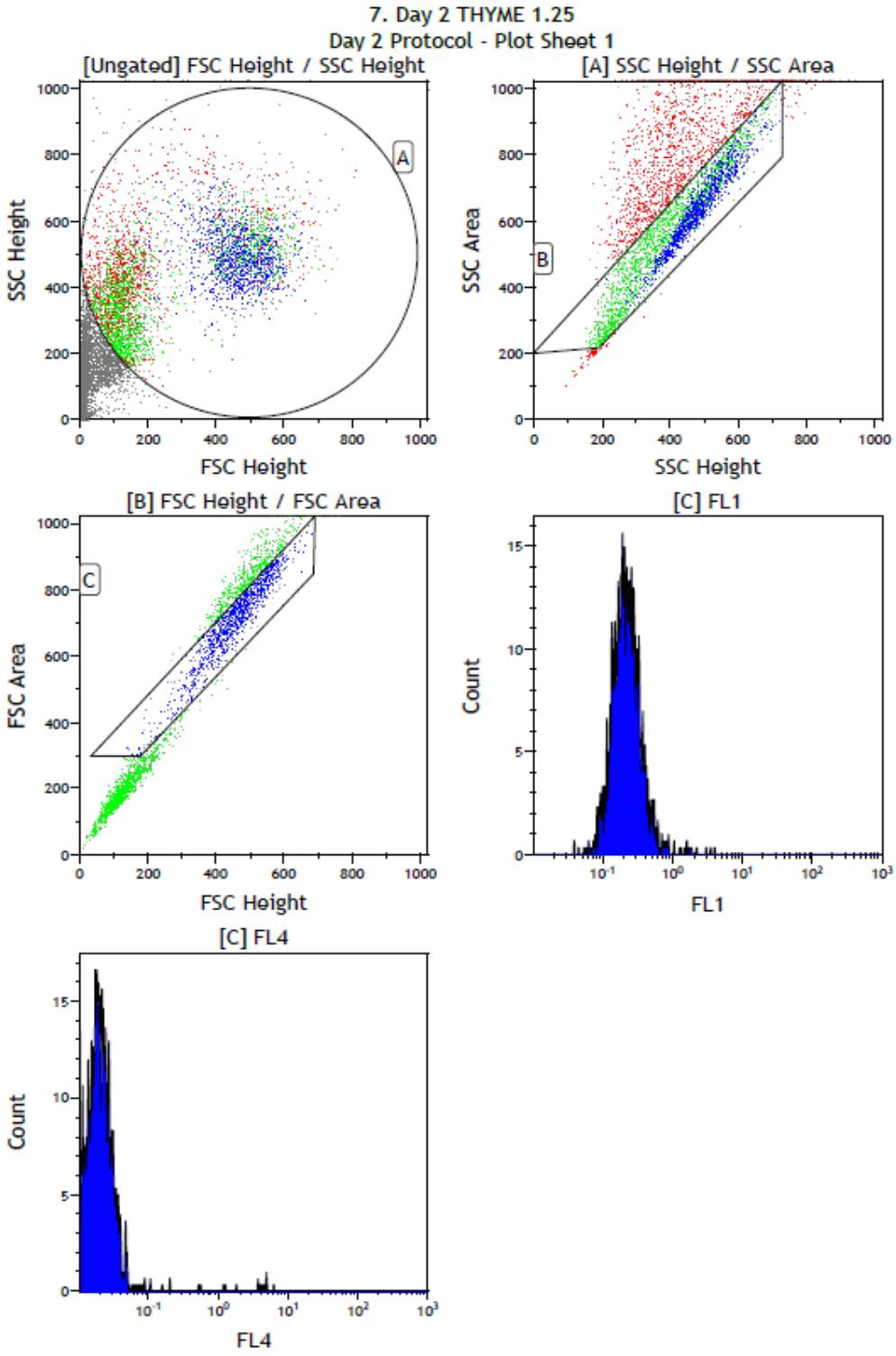


Figure 99: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 48 hours (Set 1).

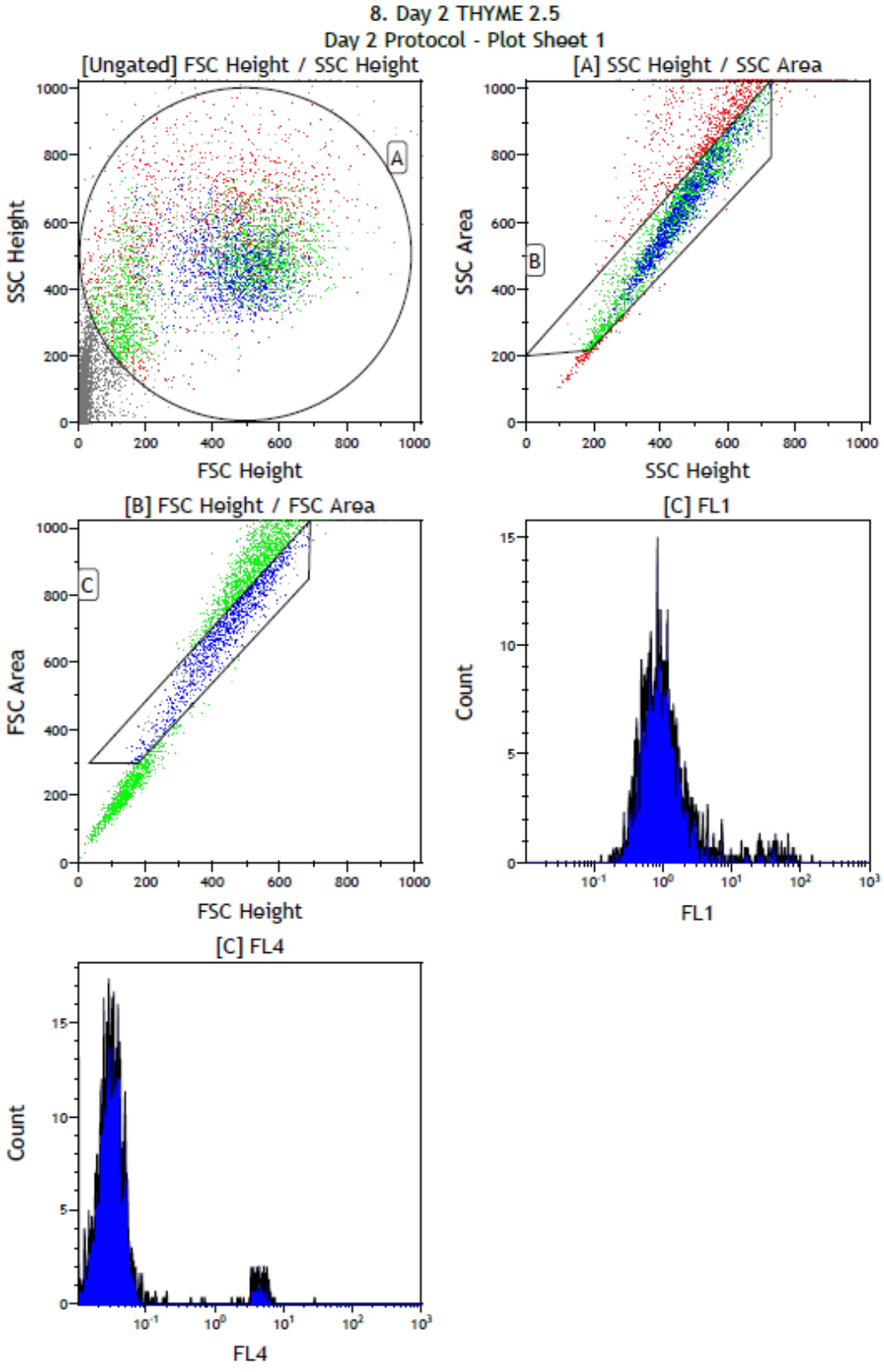


Figure 100: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 2.5% for 48 hours (Set 1).

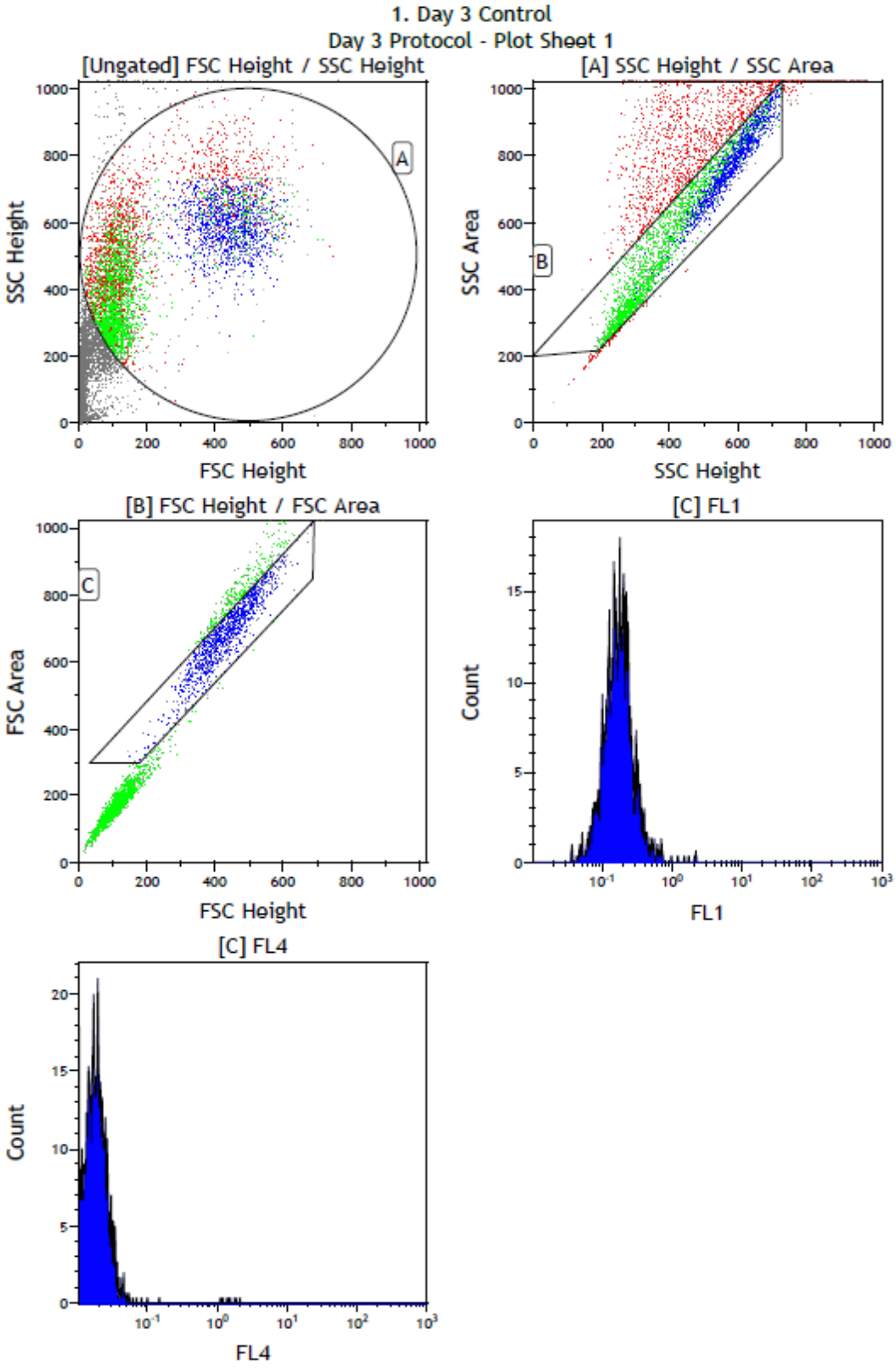


Figure 101: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 72 hours (Set 1).

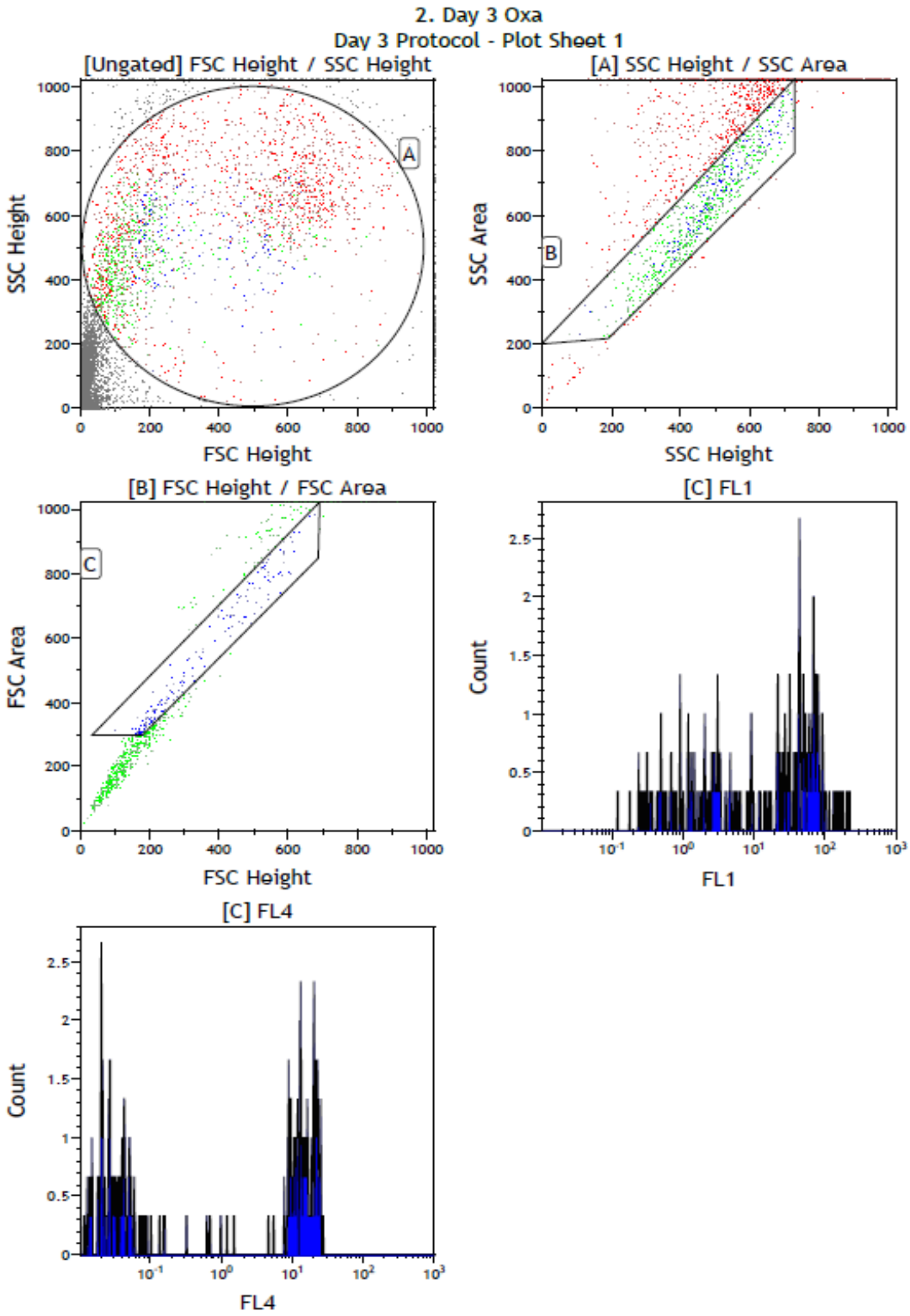


Figure 102: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 72 hours (Set 1).

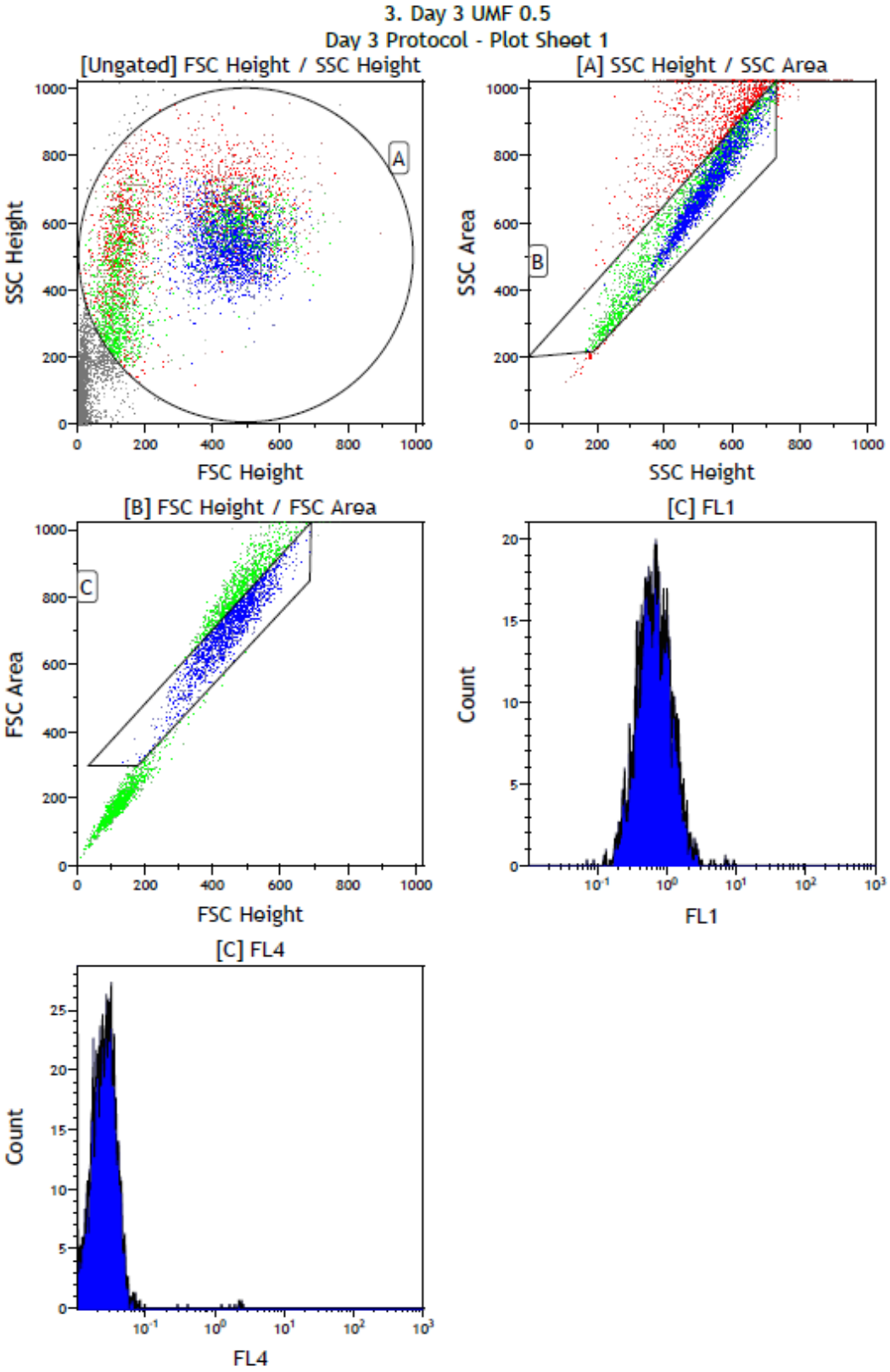


Figure 103: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 72 hours (Set 1).

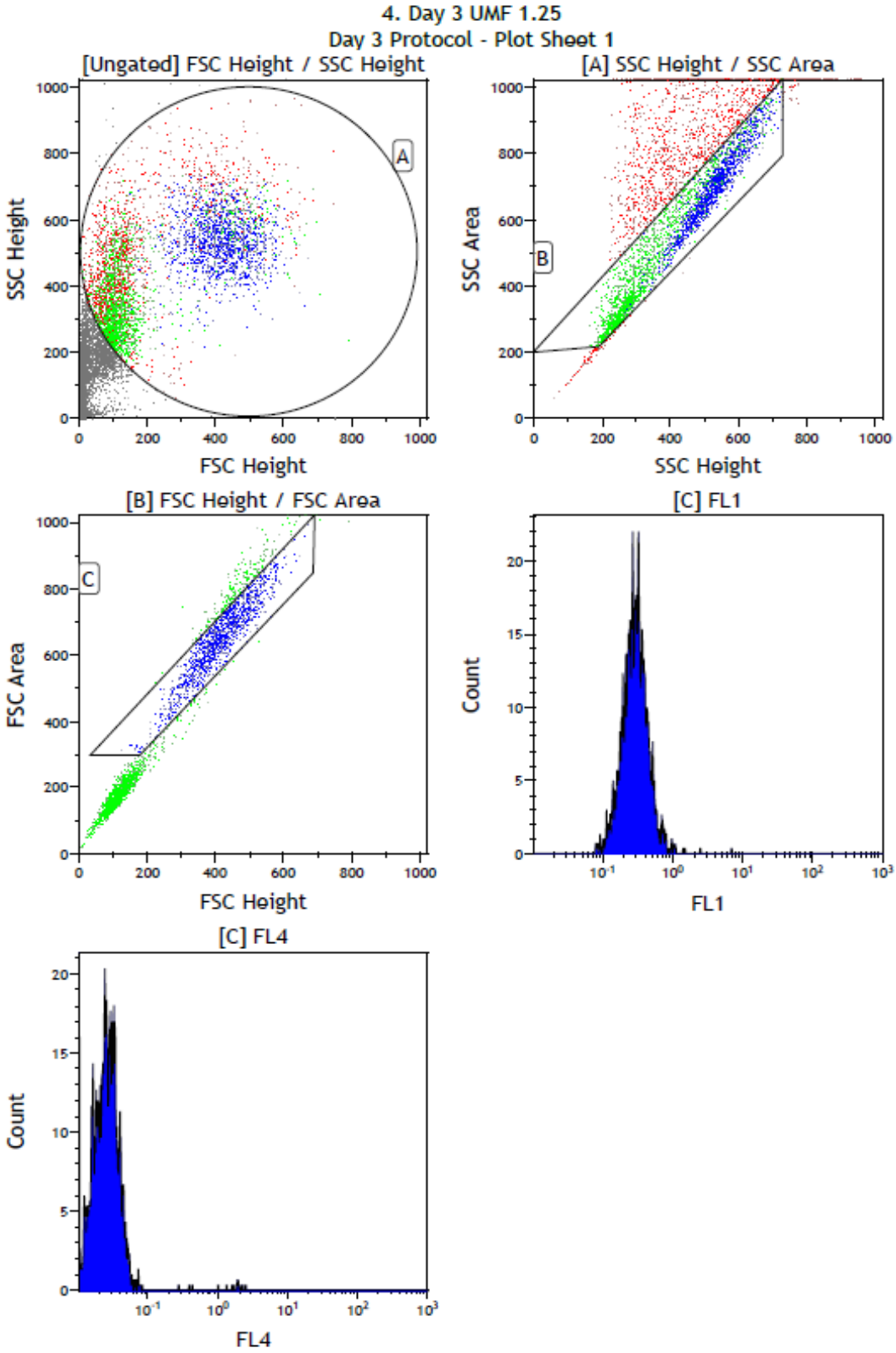


Figure 104: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 72 hours (Set 1).

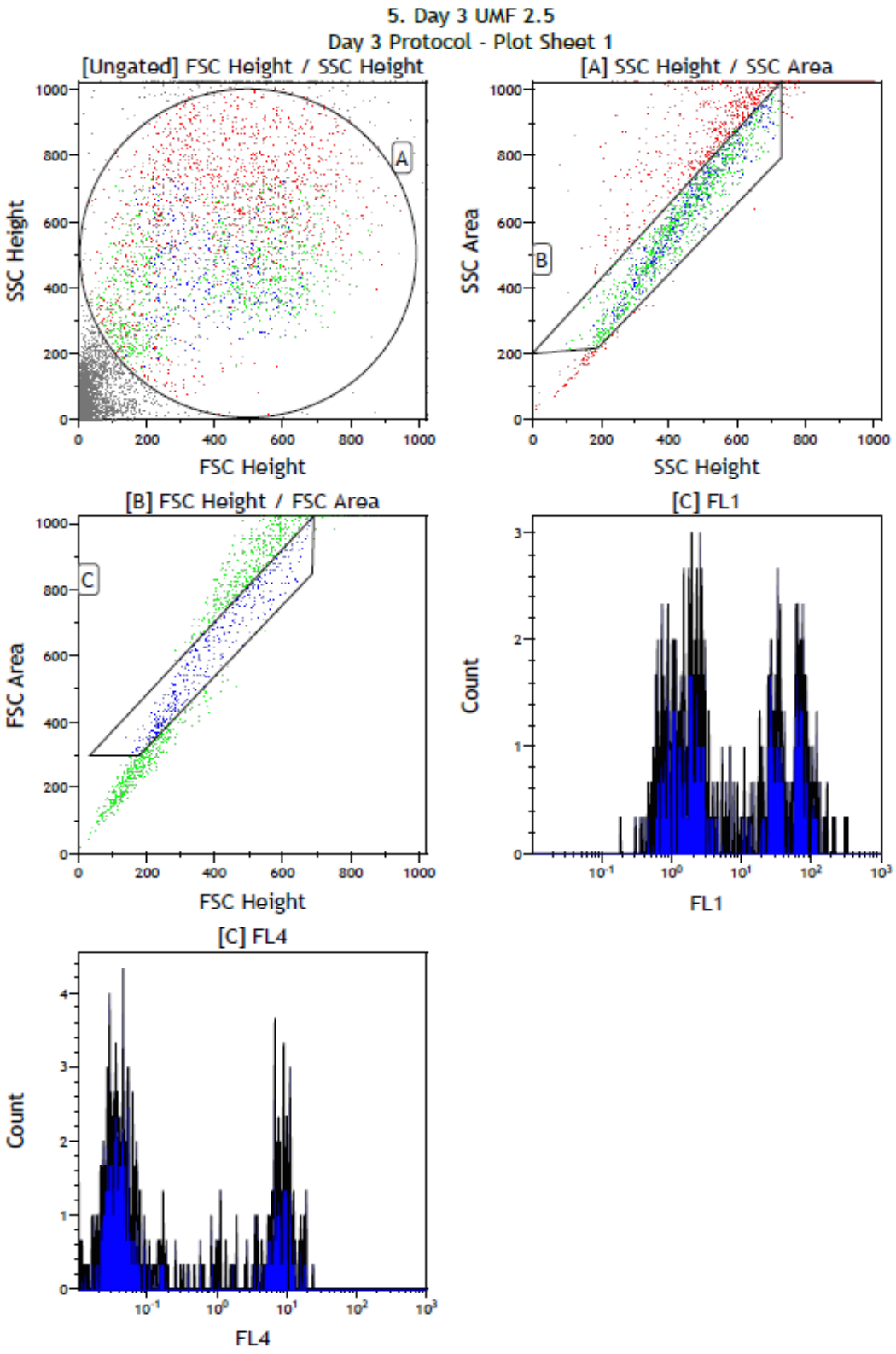


Figure 105: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 72 hours (Set 1).

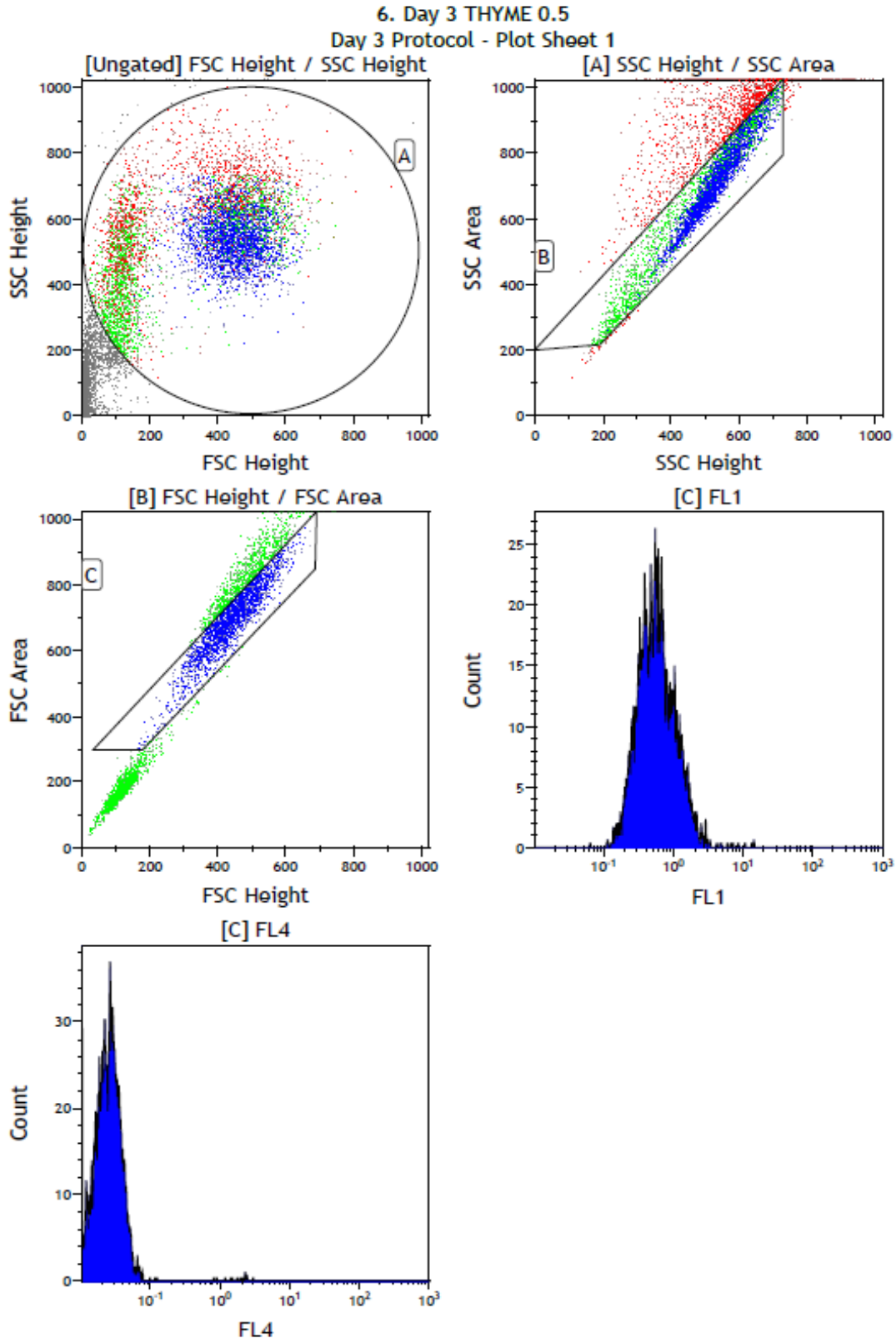


Figure 106: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 72 hours (Set 1).

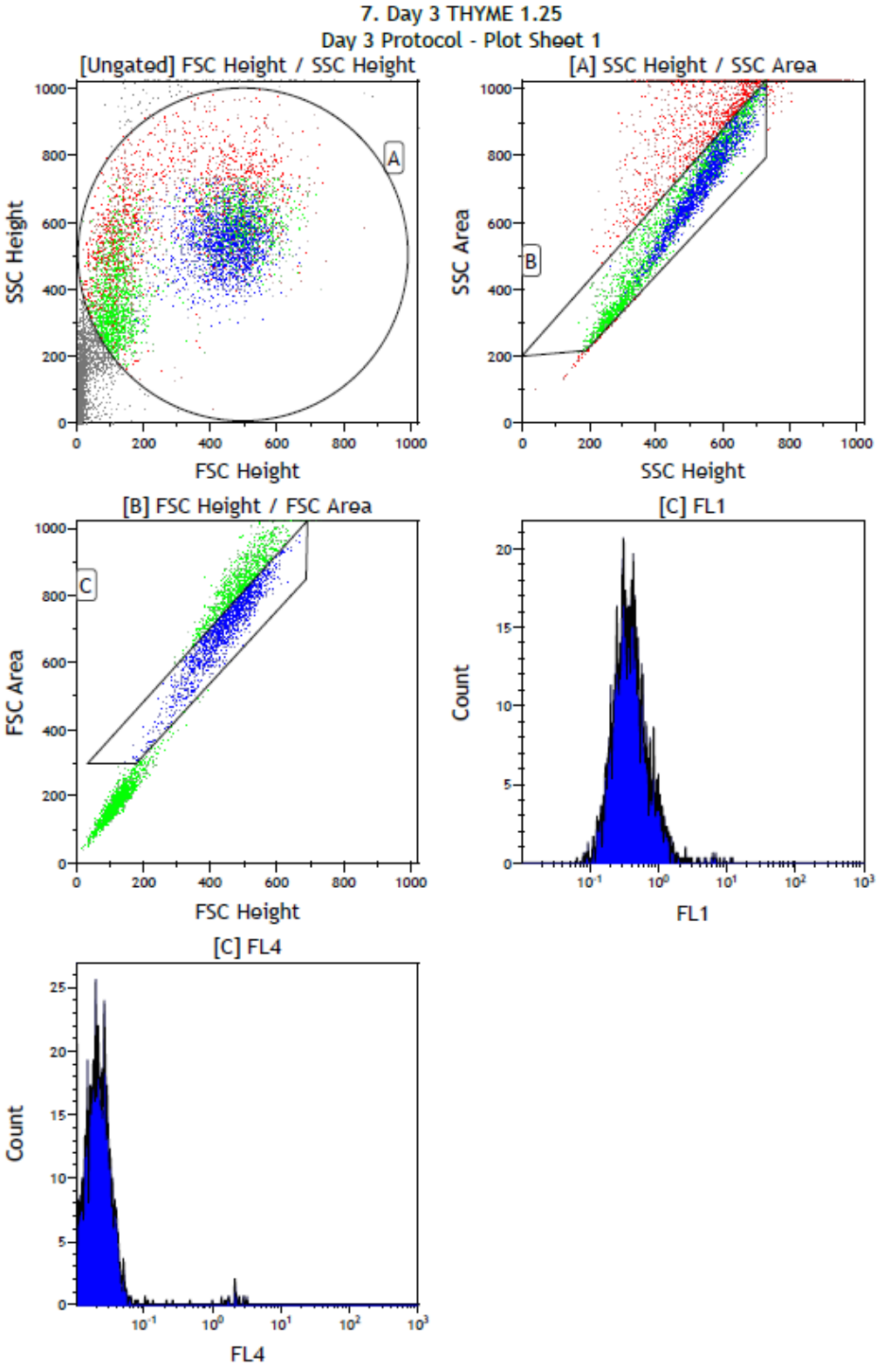


Figure 107: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 72 hours (Set 1).

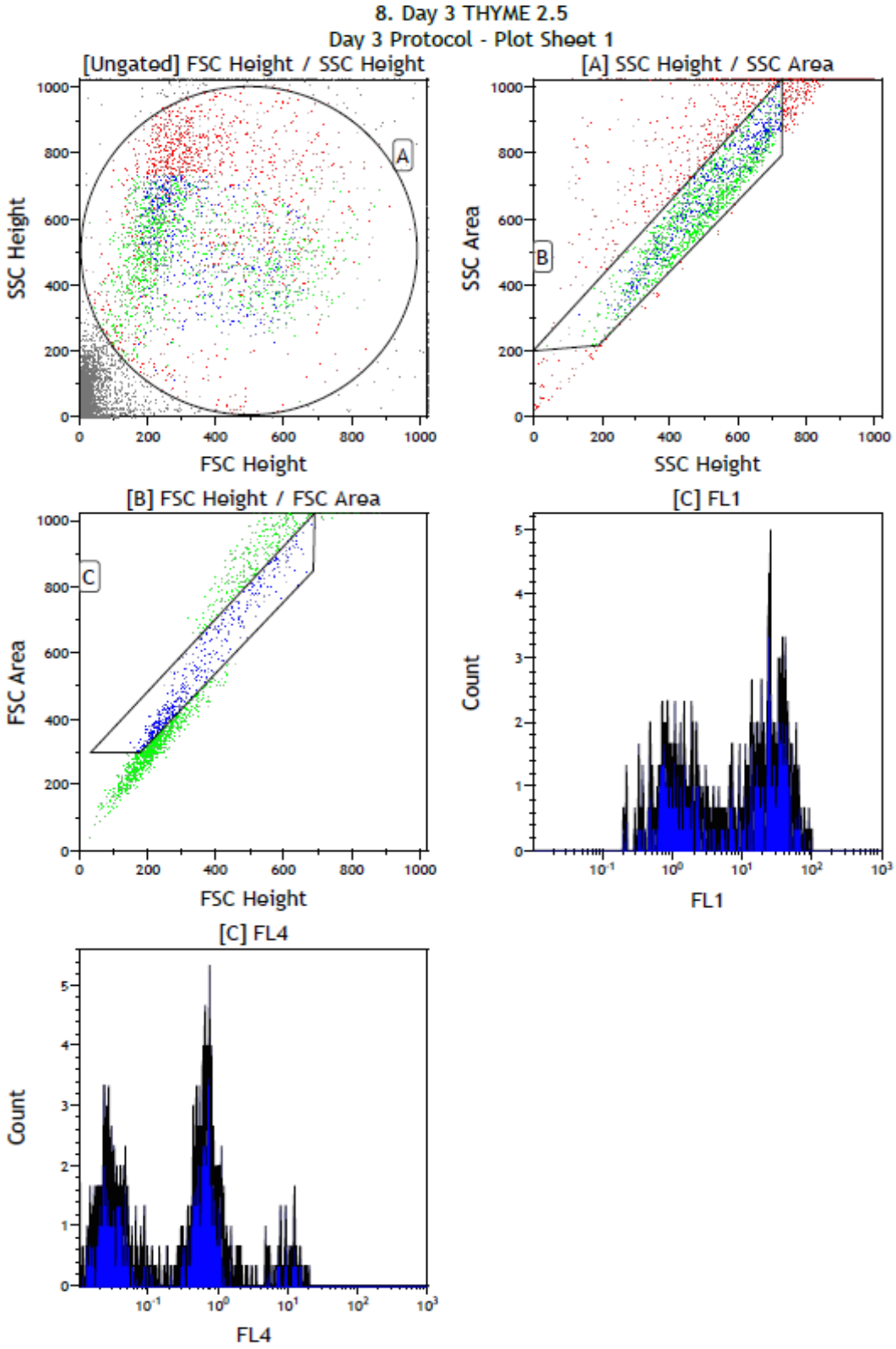


Figure 108: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 2.5% for 72 hours (Set 1).

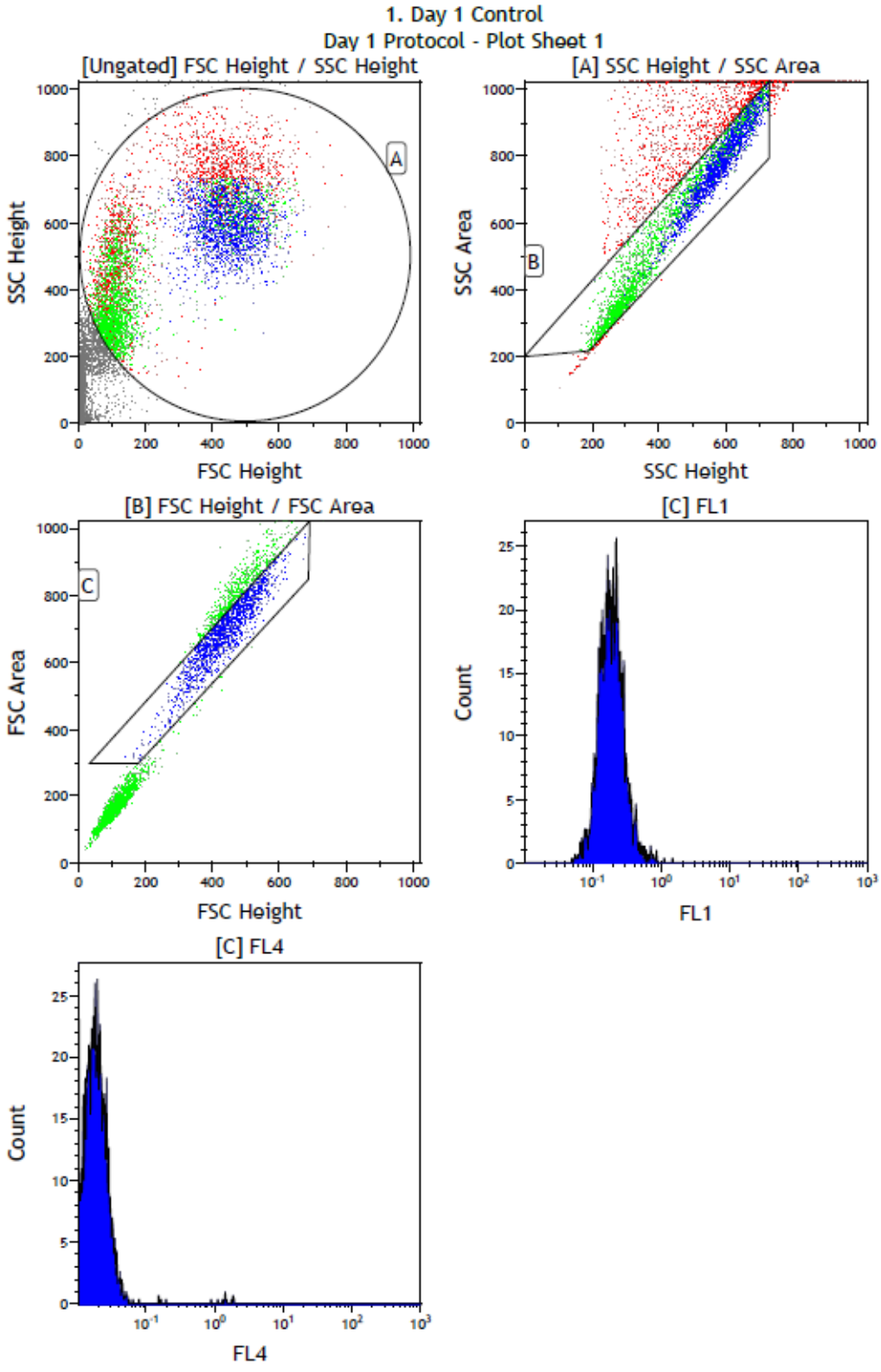


Figure 109: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 24 hours (Set 2).

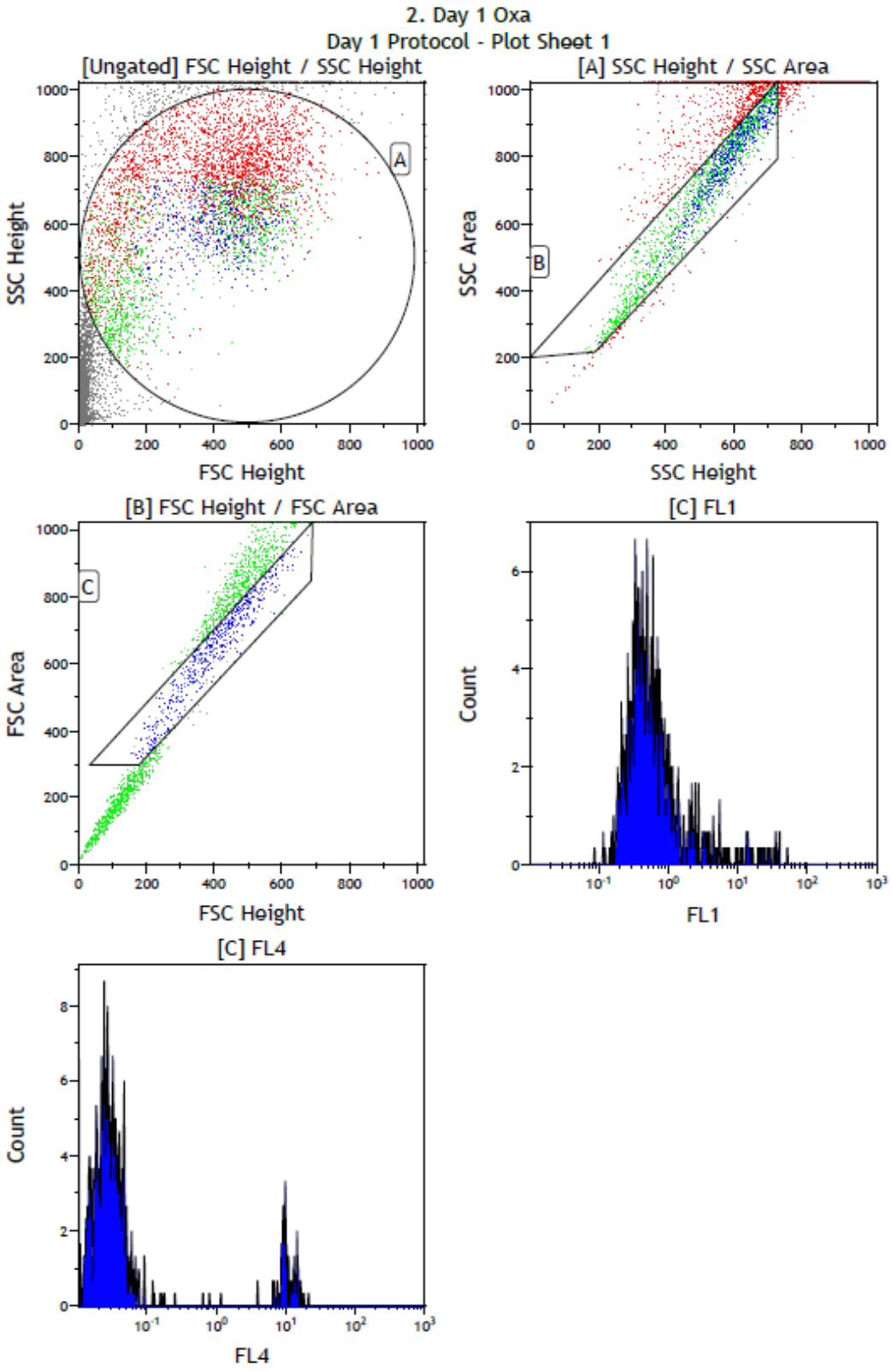


Figure 110: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 24 hours (Set 2).

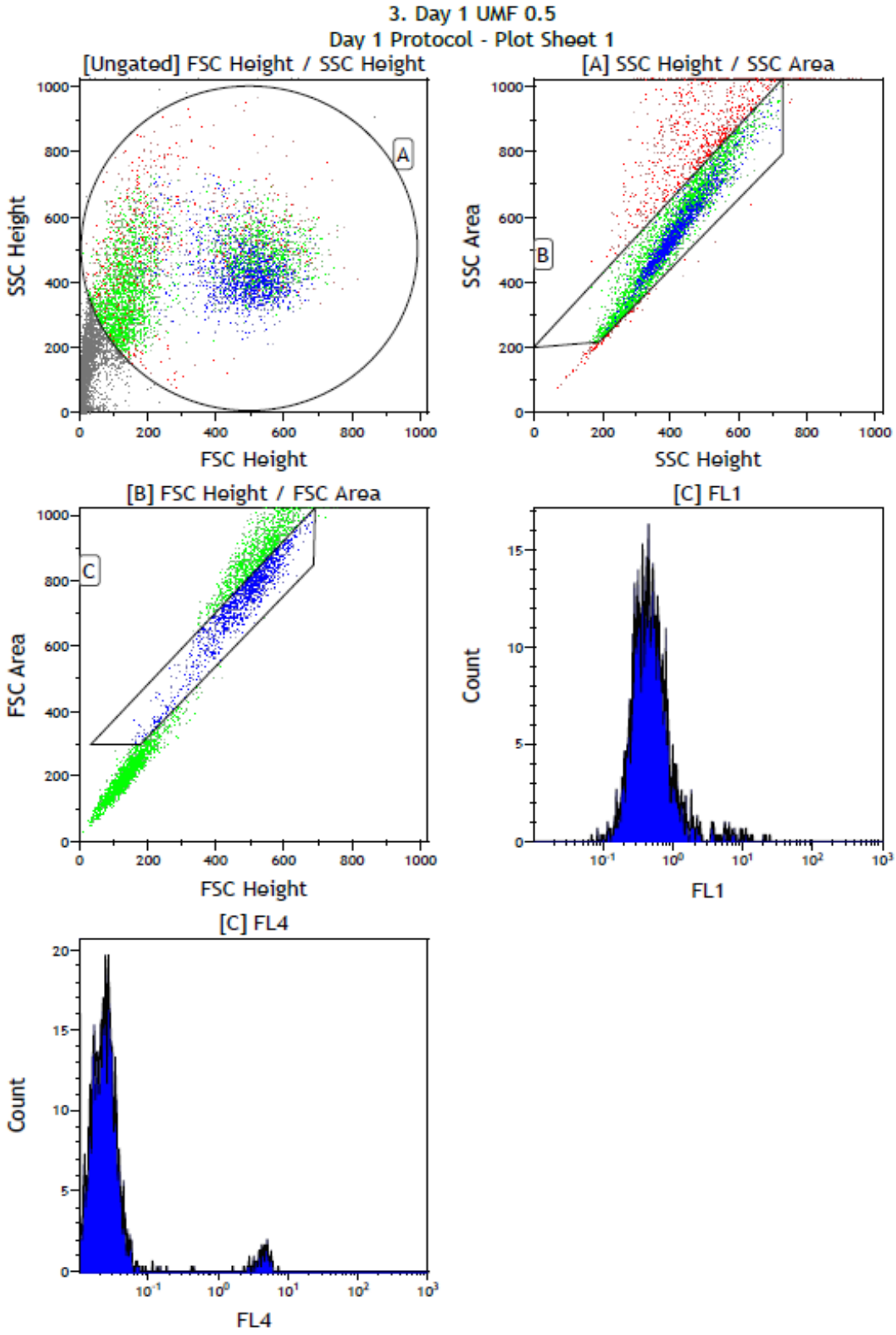


Figure 111: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 24 hours (Set 2).

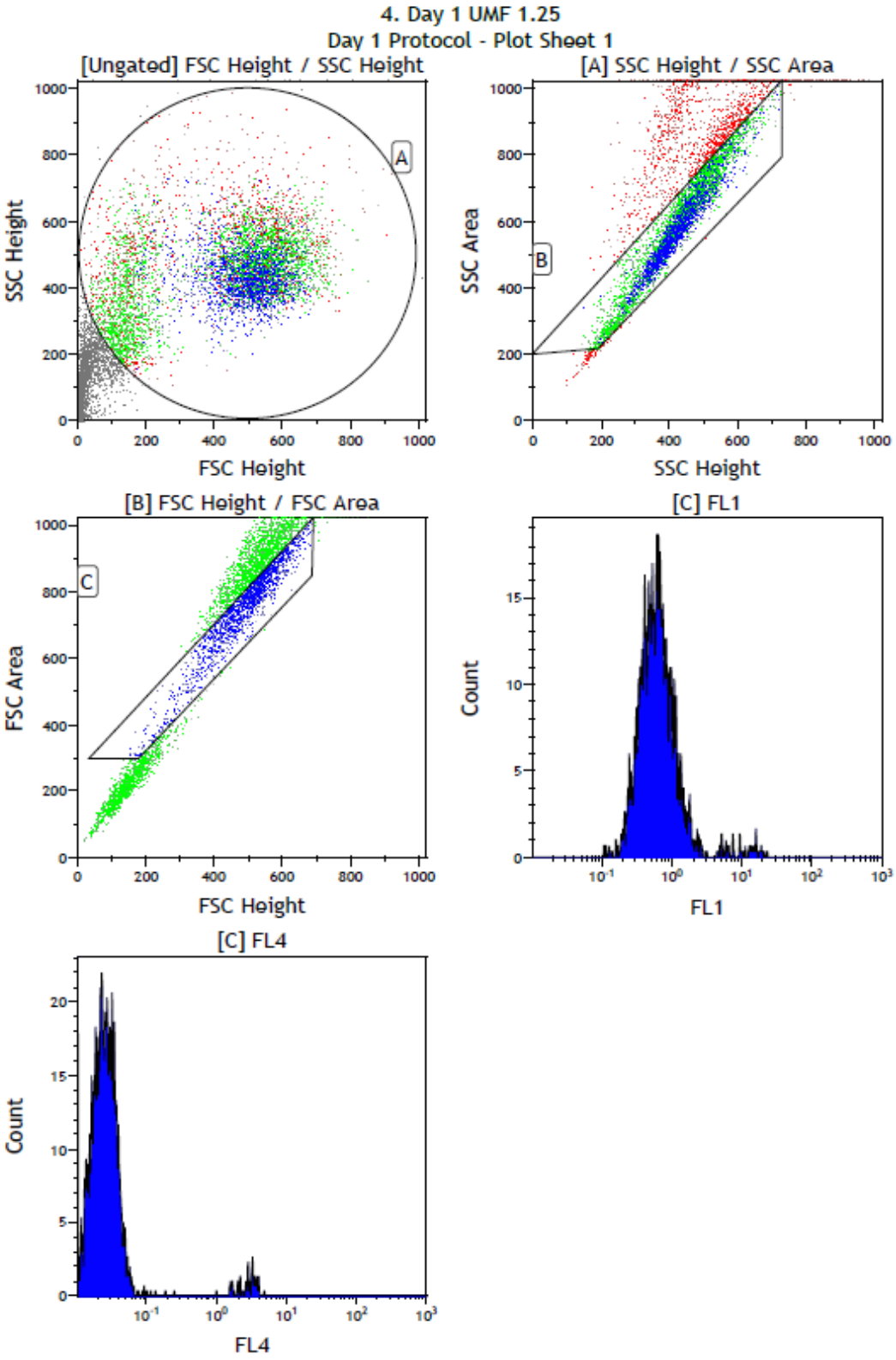


Figure 112: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 24 hours (Set 2).

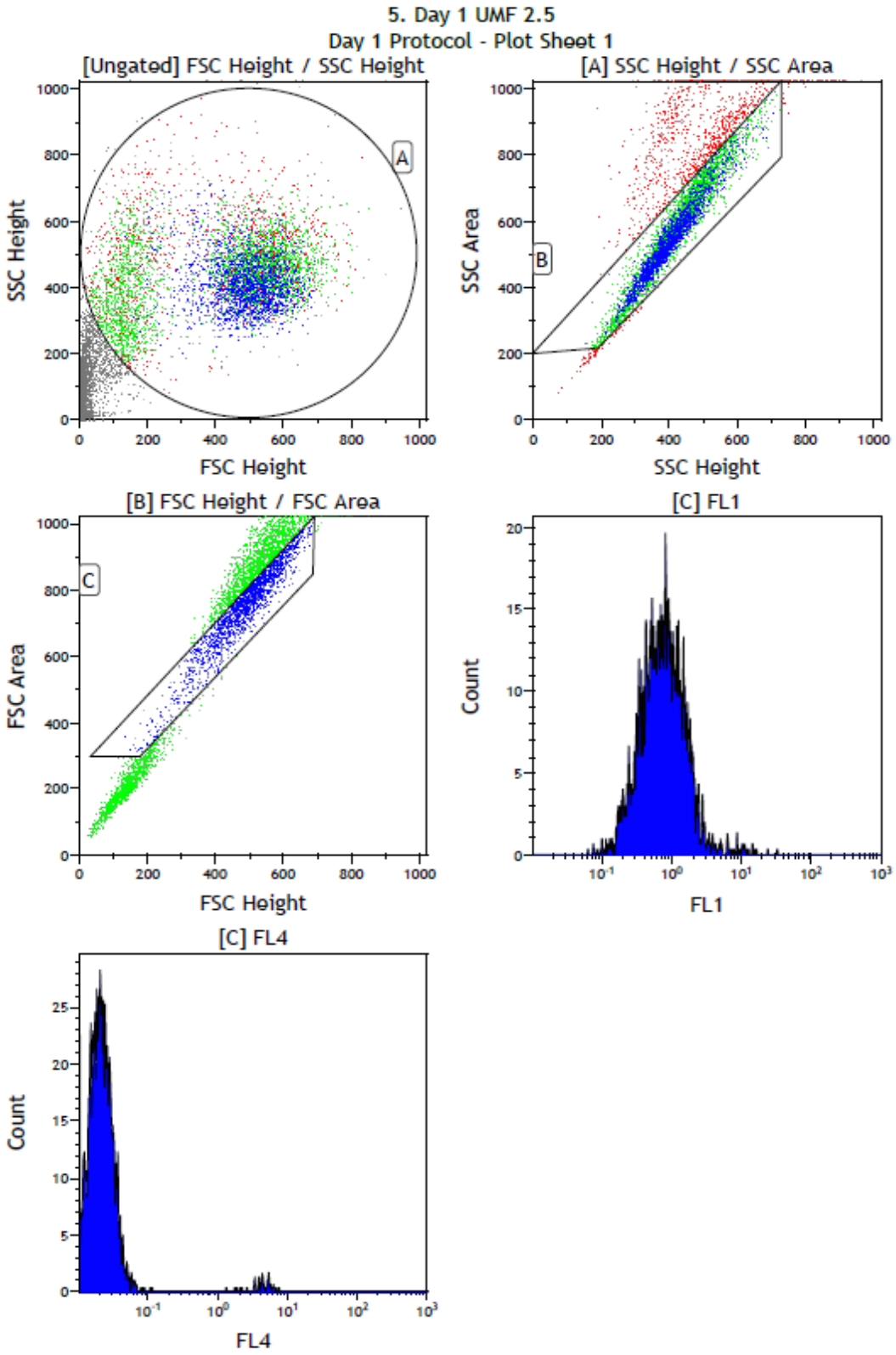


Figure 113: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 24 hours (Set 2).

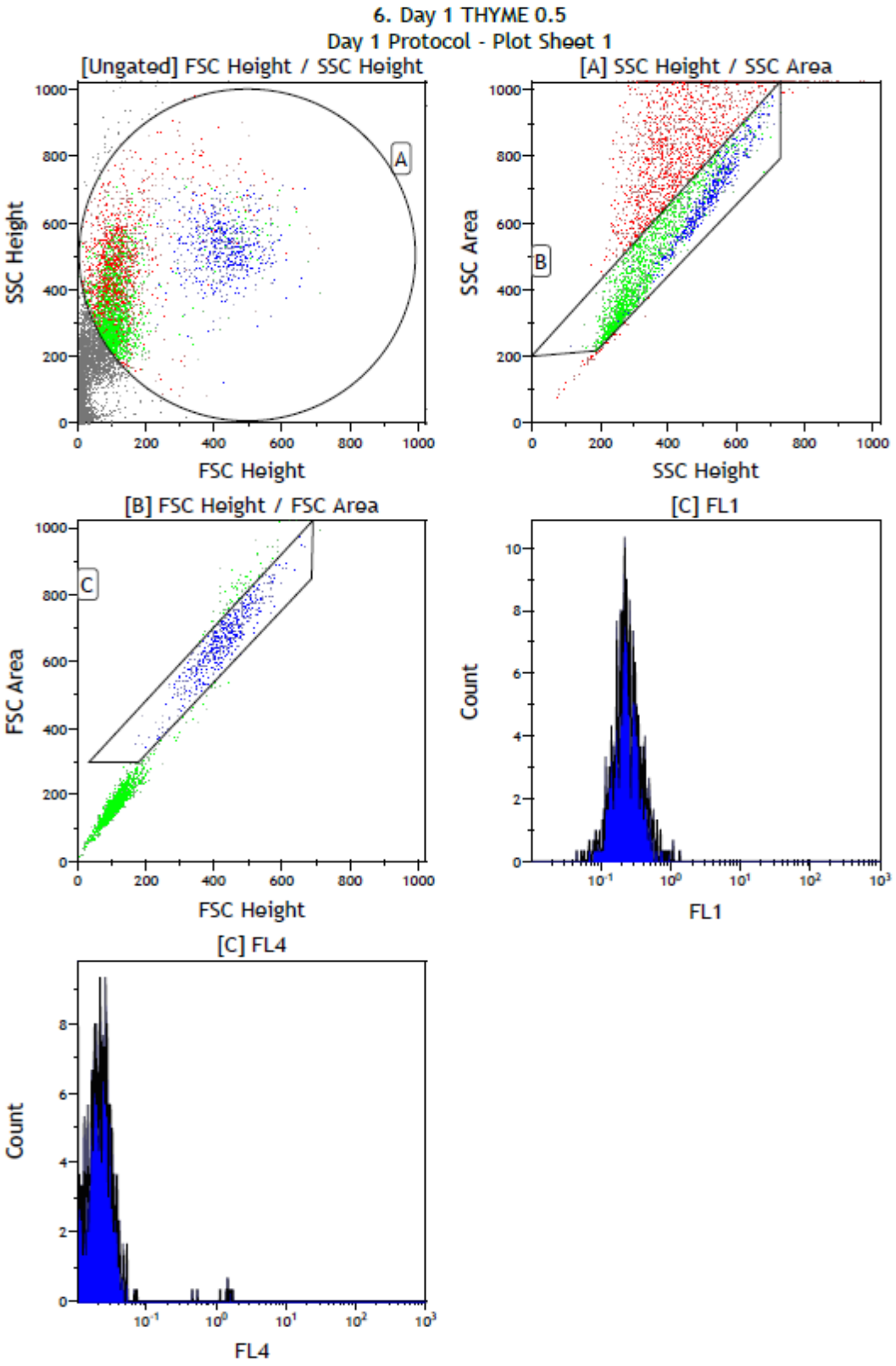


Figure 114: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 24 hours (Set 2).

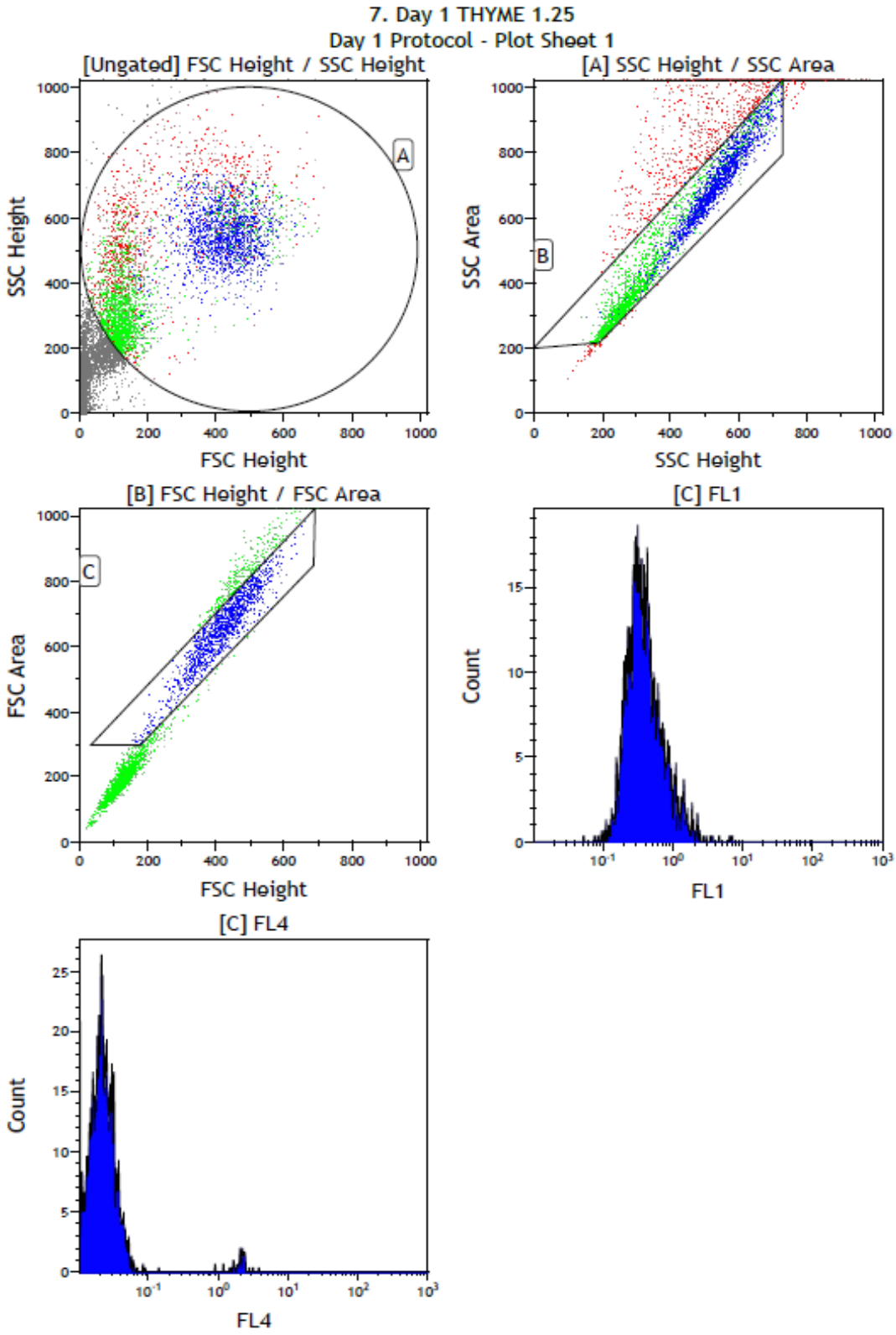


Figure 115: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 24 hours (Set 2).

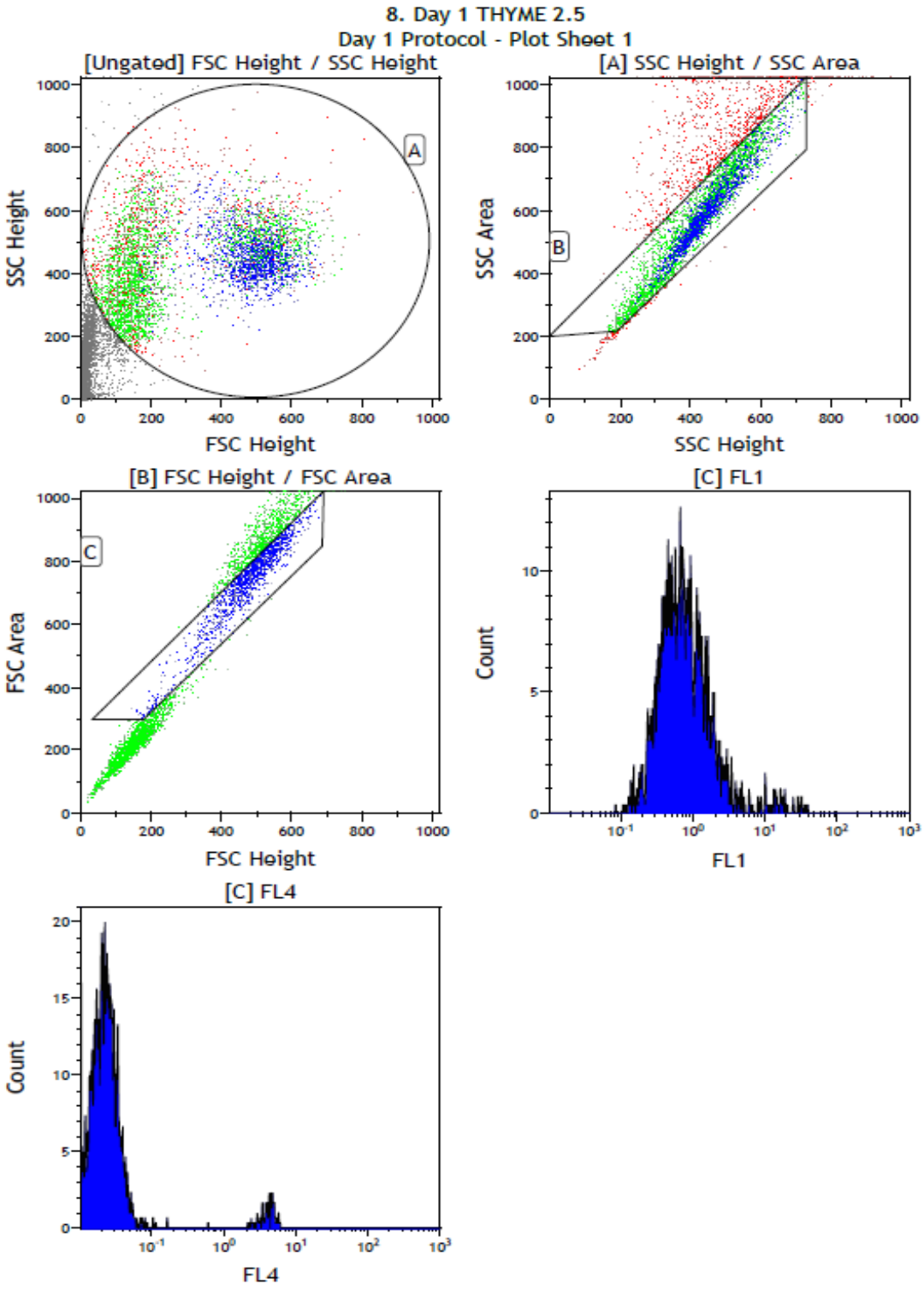


Figure 116: Apoptosis annexin V + PI data plot. WiDr cells treated with THYME 2.5% for 24 hours (Set 2).

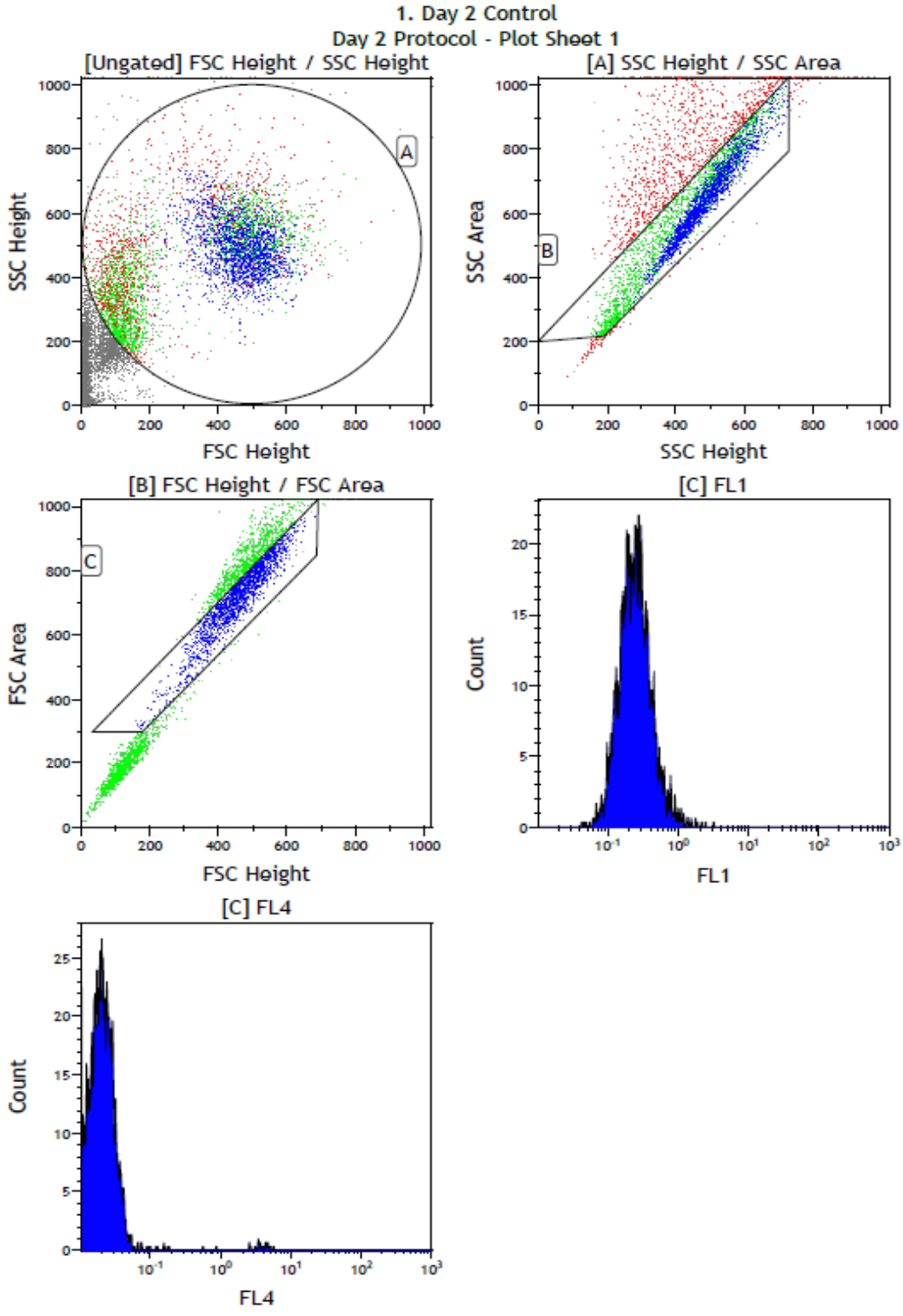


Figure 117: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 48 hours (Set 2).

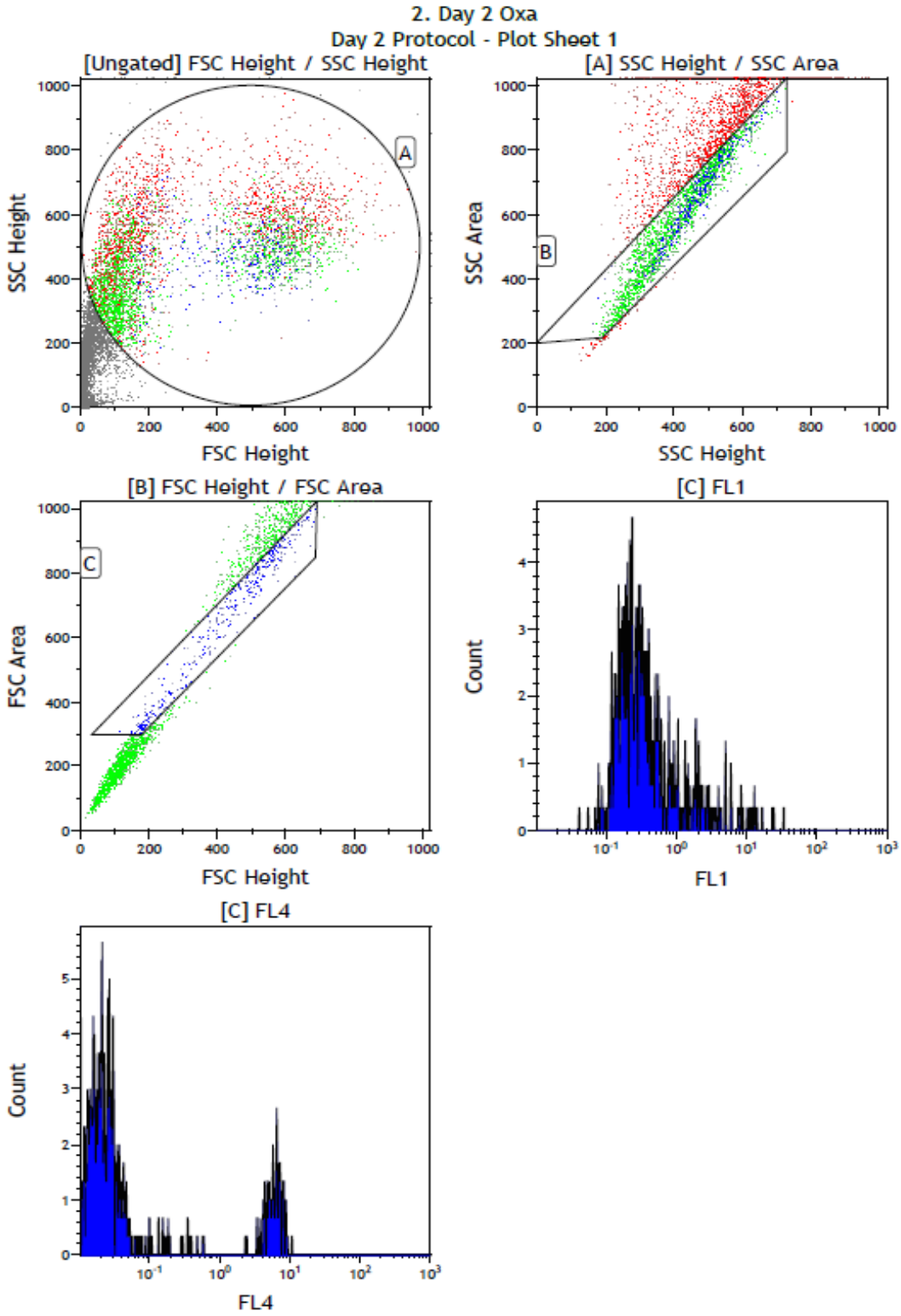


Figure 118: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 48 hours (Set 2).

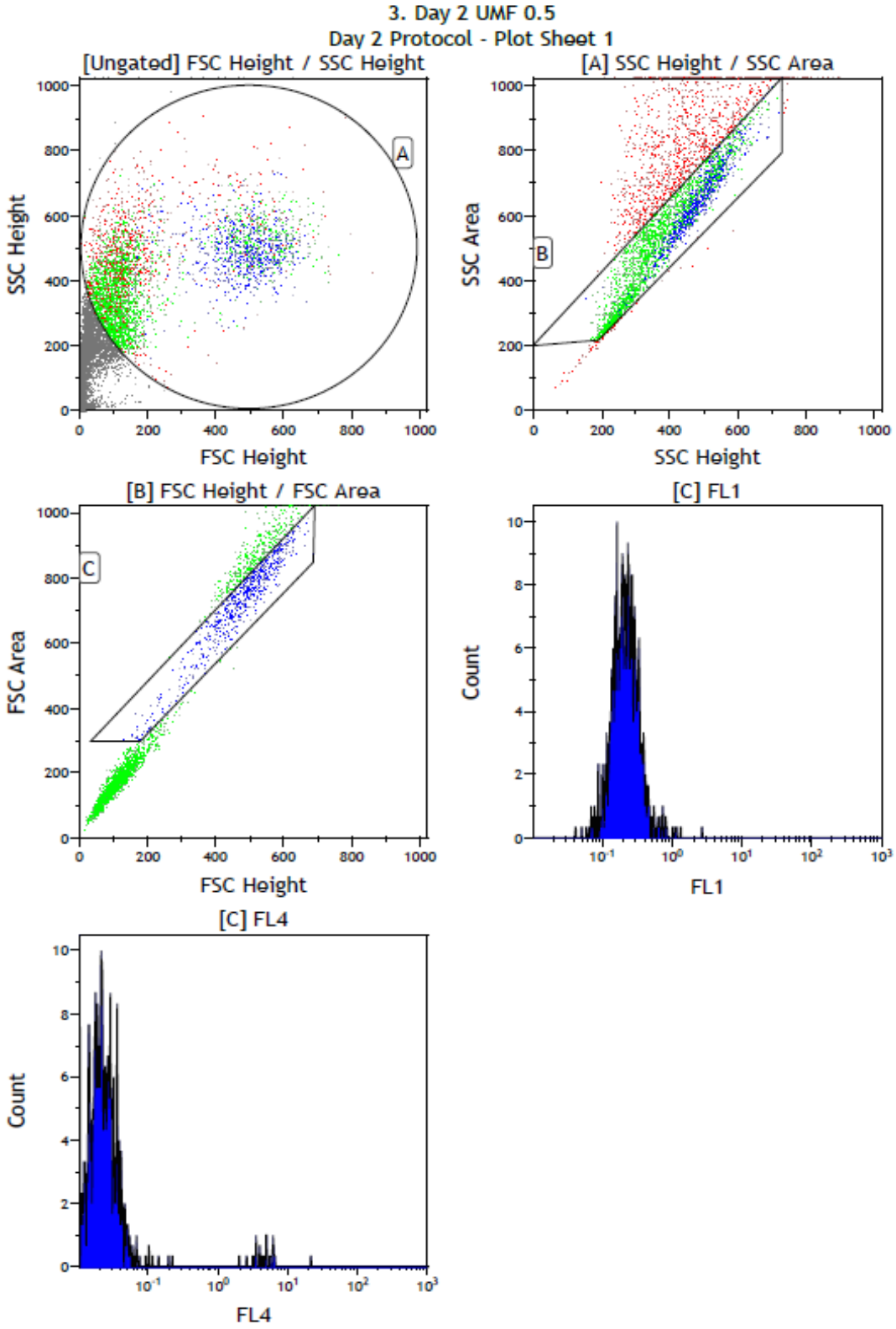


Figure 119: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 48 hours (Set 2).

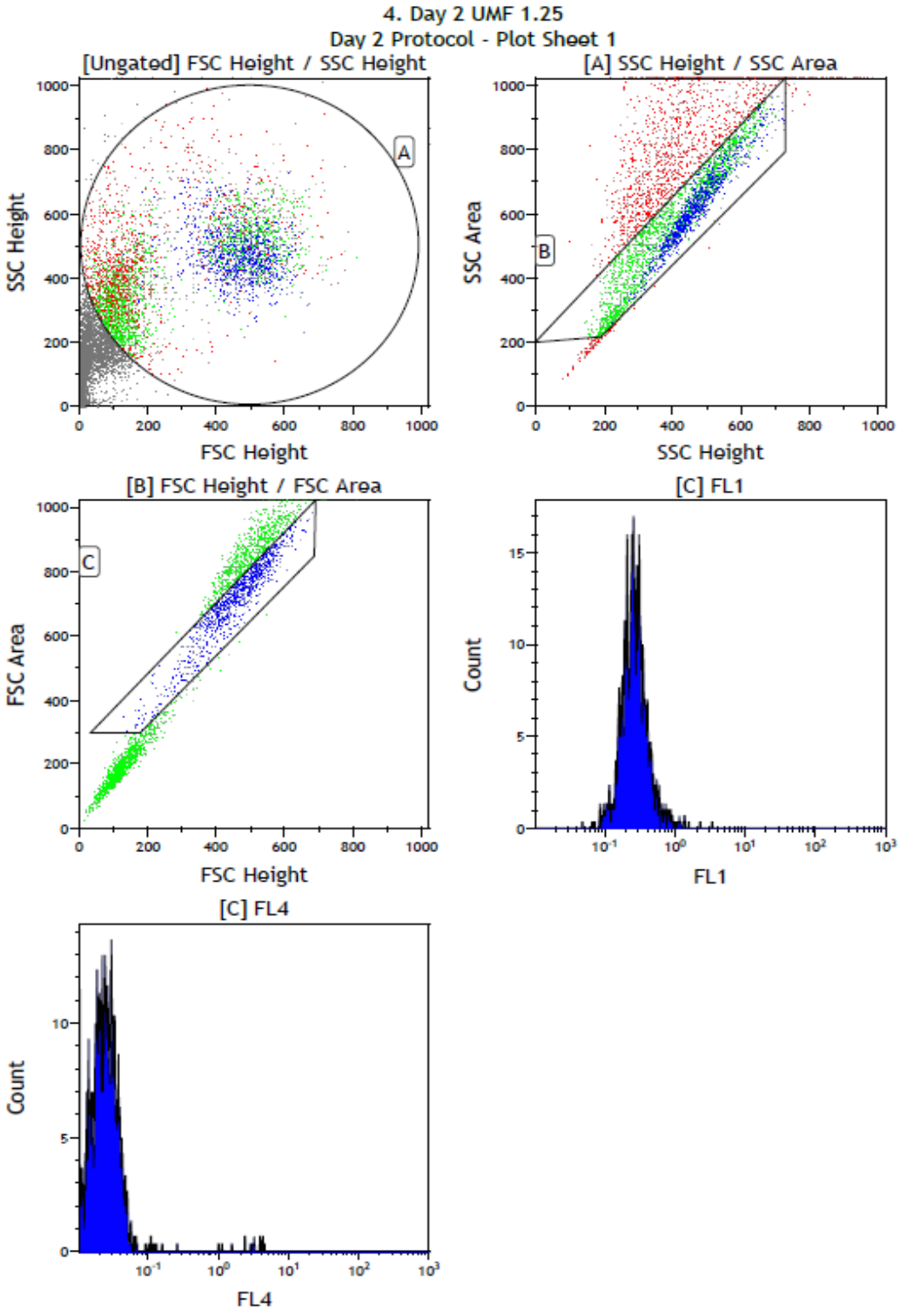


Figure 120: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 48 hours (Set 2).

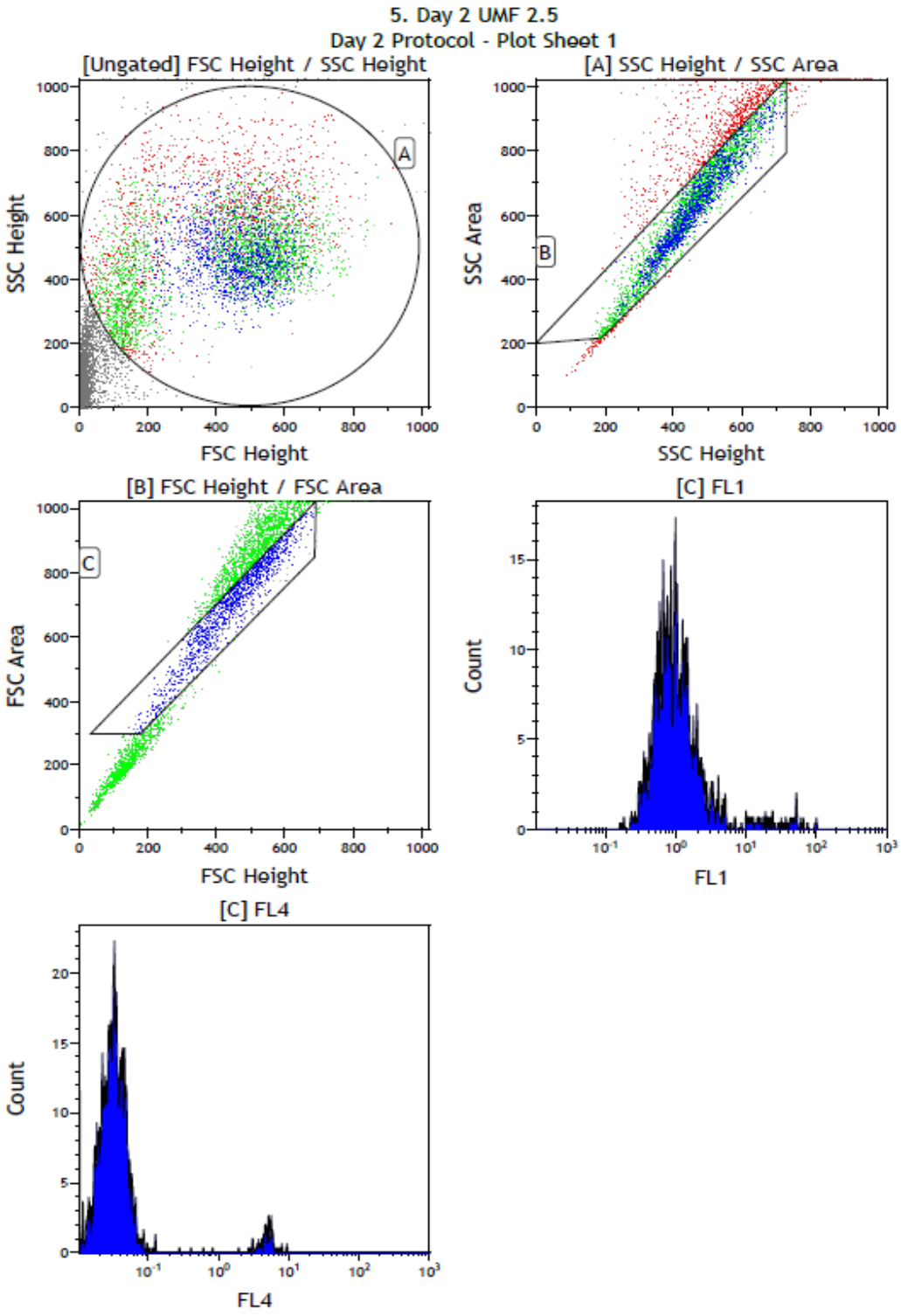


Figure 121: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 48 hours (Set 2).

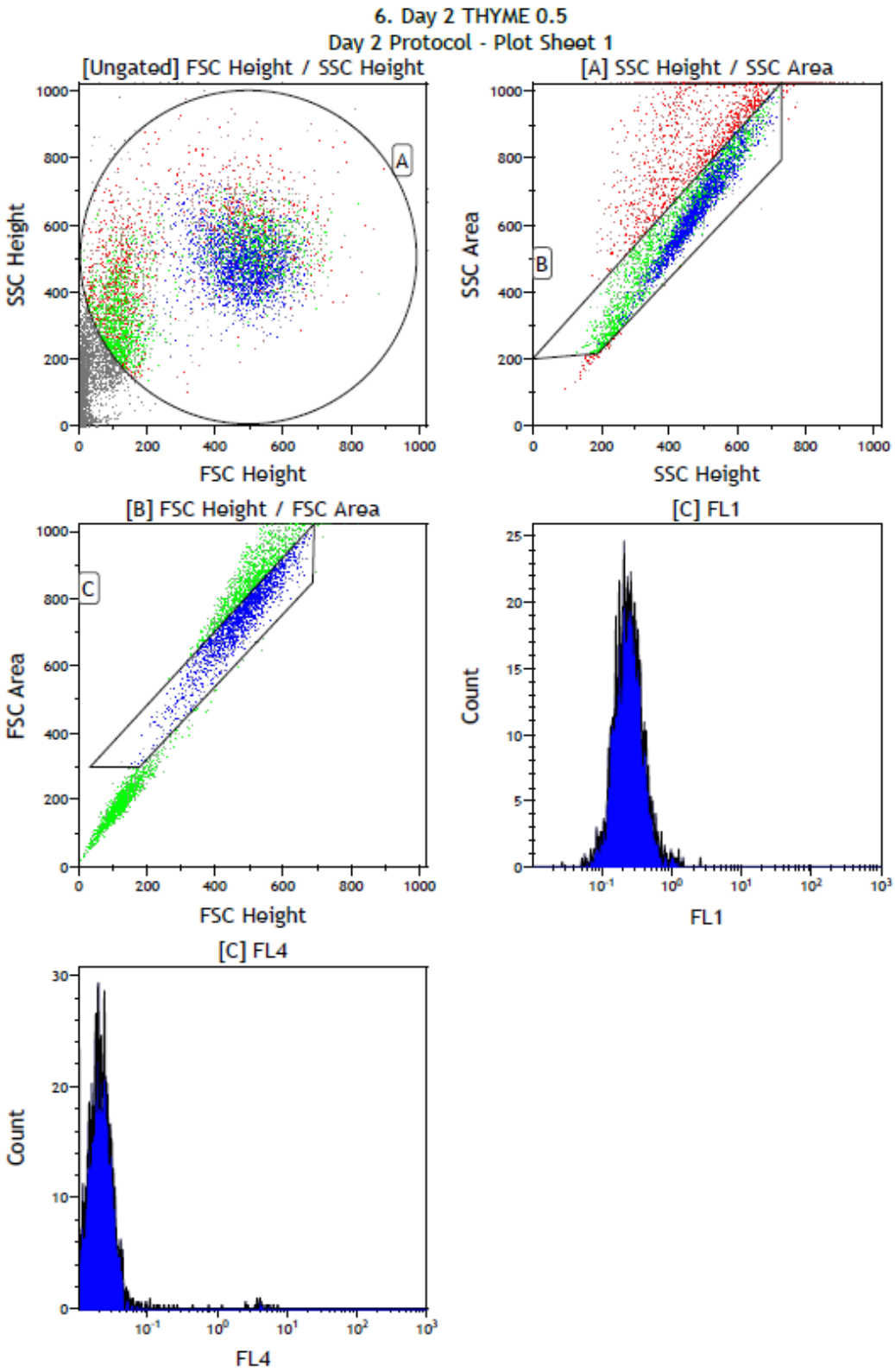


Figure 122: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 48 hours (Set 2).

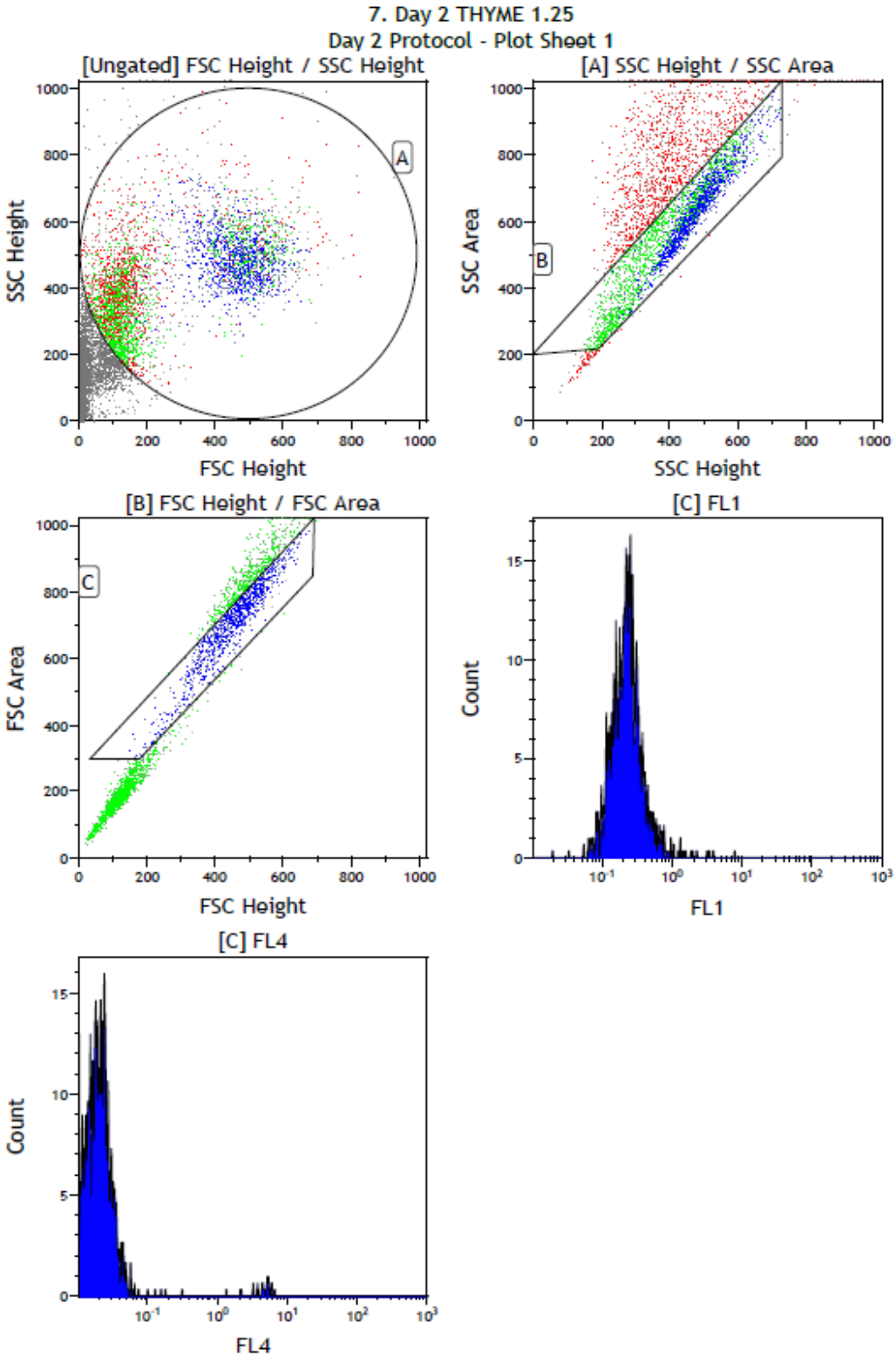


Figure 123: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 48 hours (Set 2).

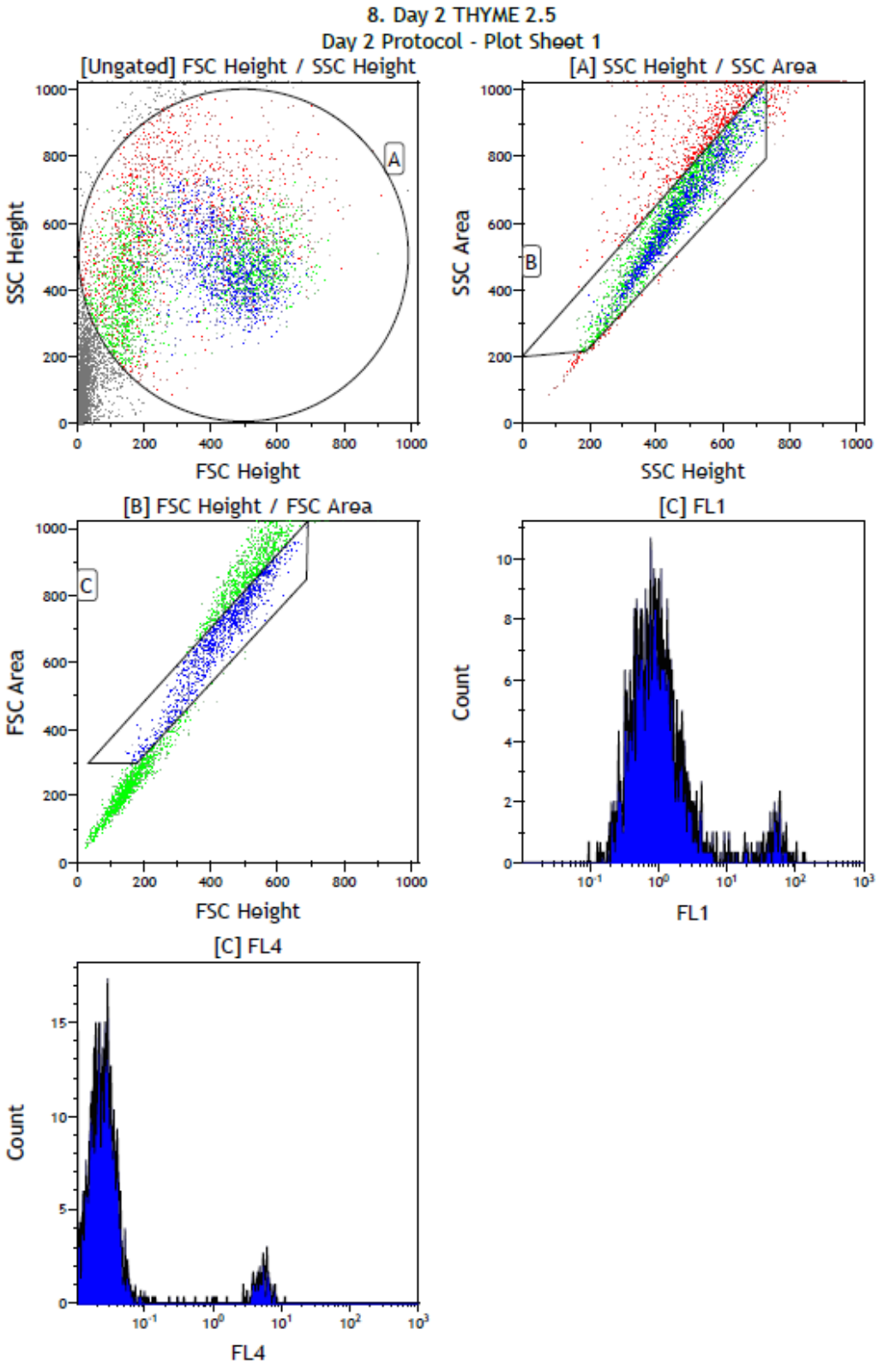


Figure 124: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 2.5% for 48 hours (Set 2).

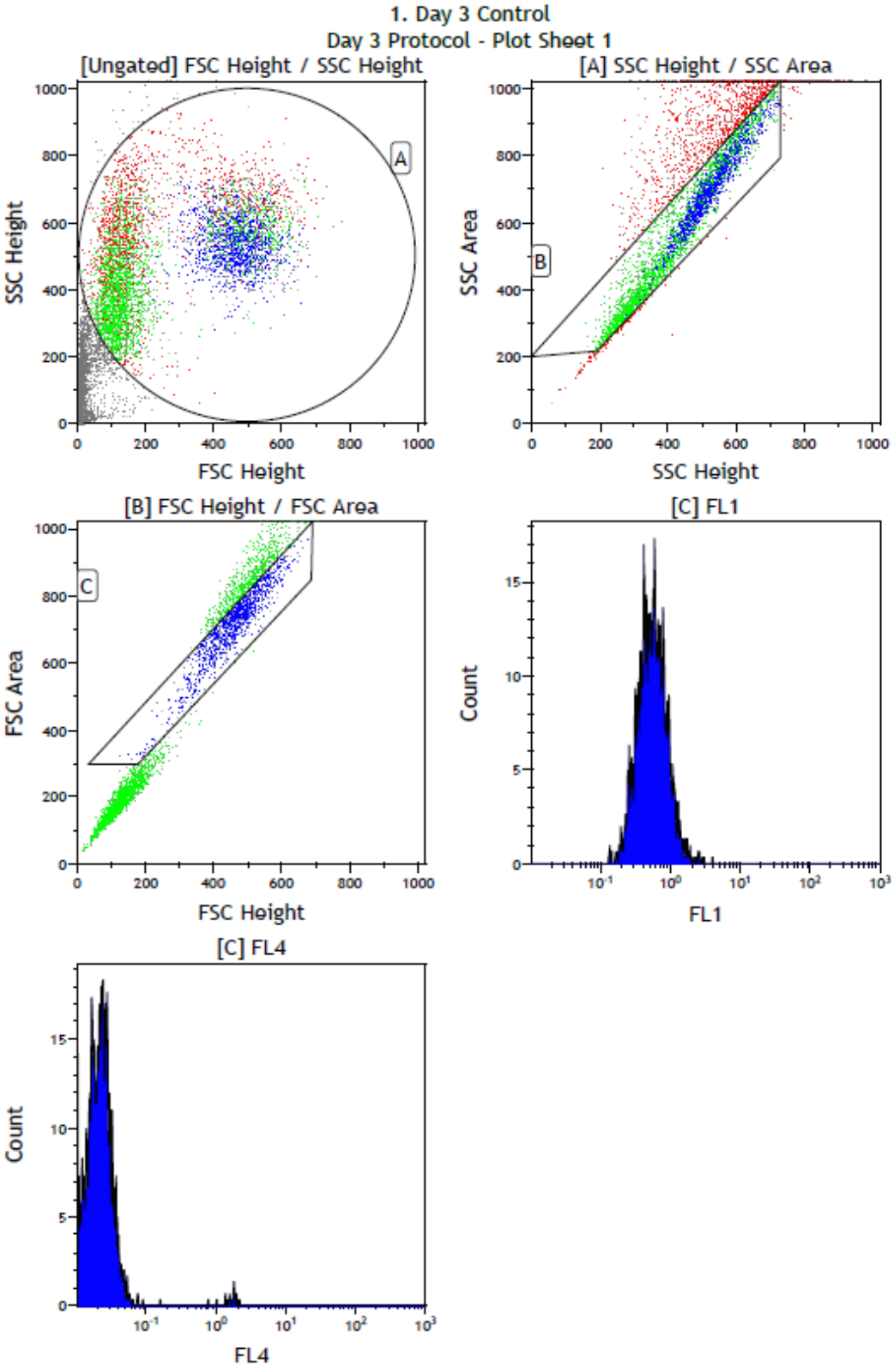


Figure 125: Apoptosis annexin V + PI data plot. Untreated WiDr cells for 72 hours (Set 2).

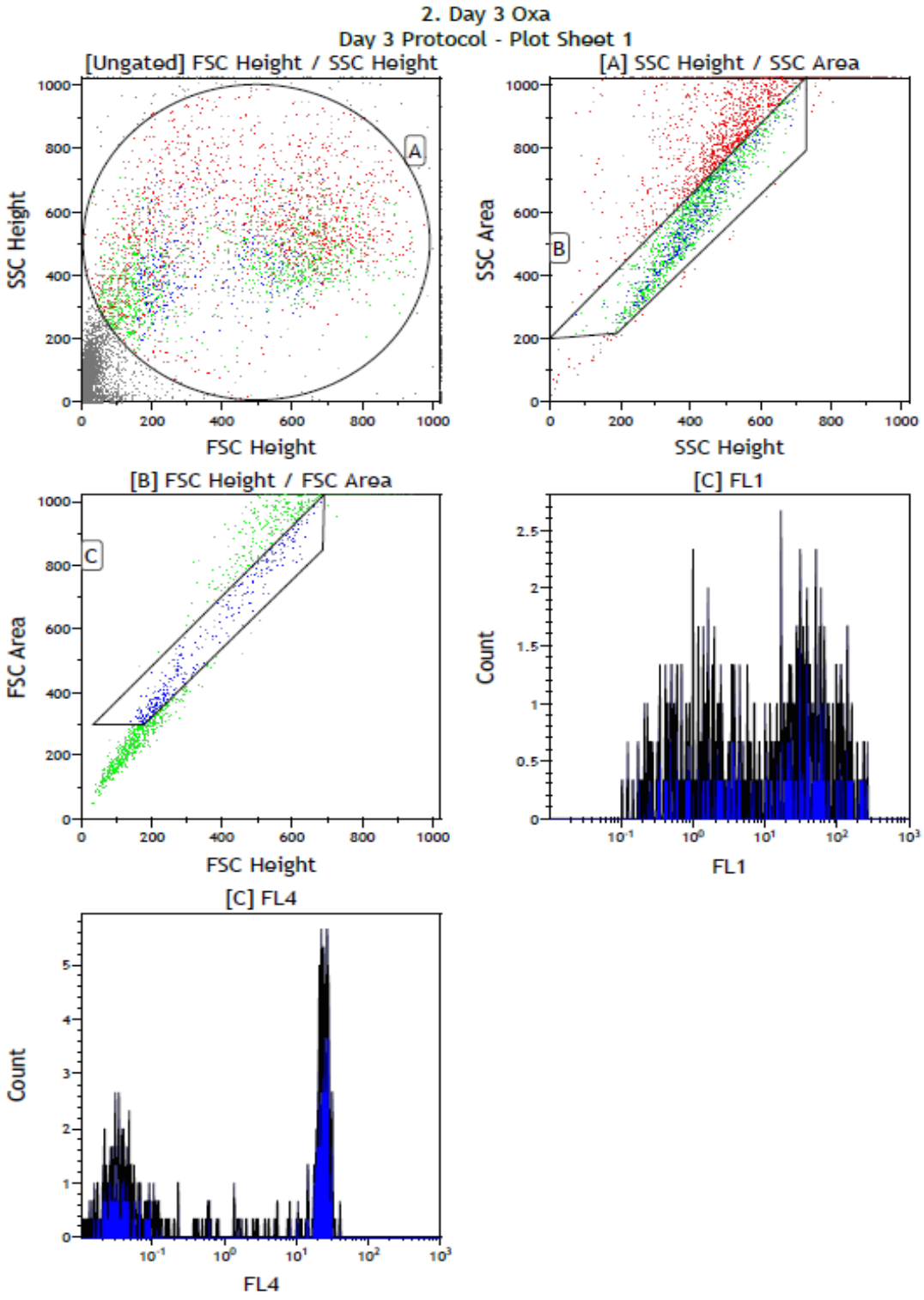


Figure 126: Apoptosis annexin V + PI data plot. WiDr cells treated with oxaliplatin for 72 hours (Set 2).

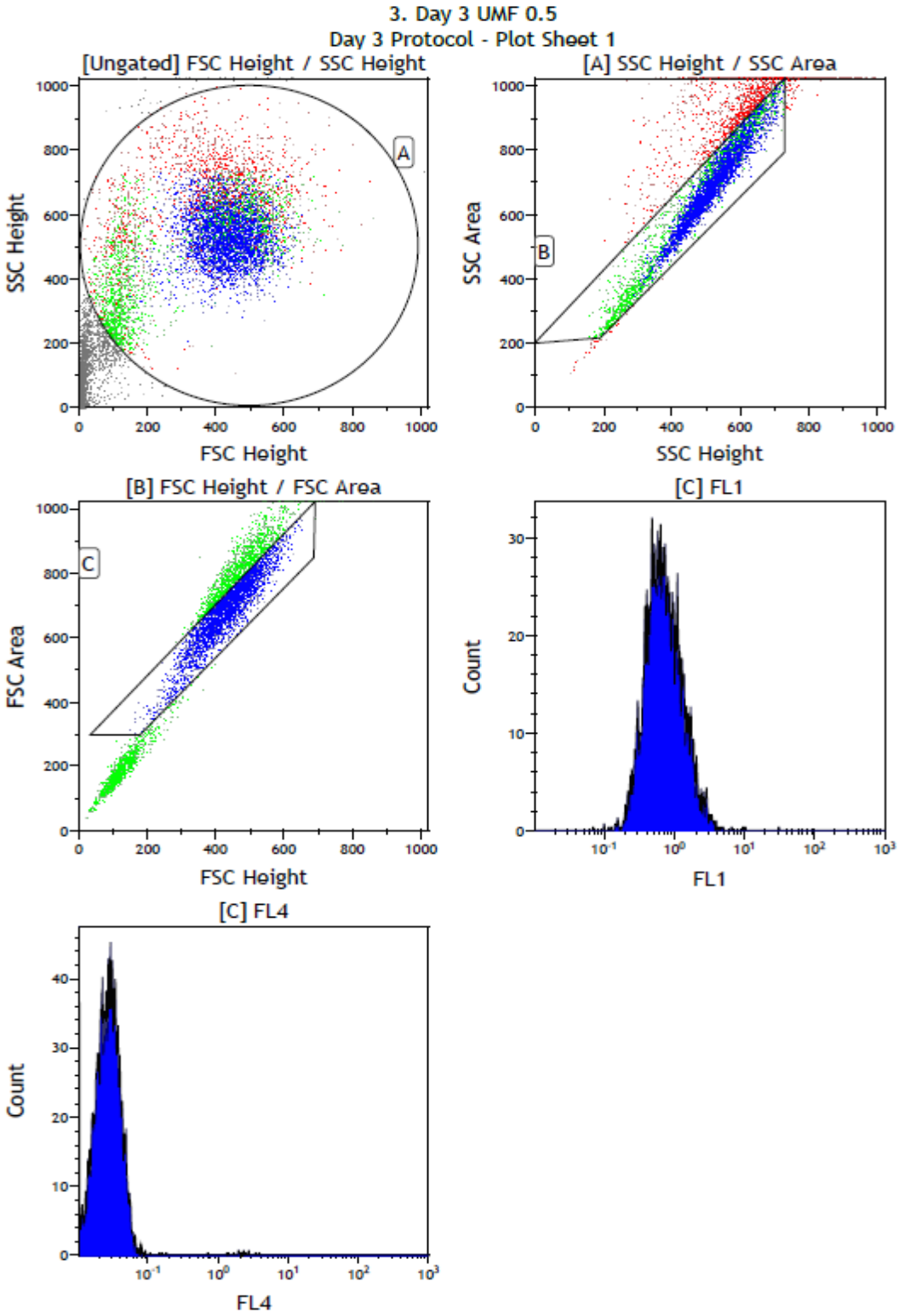


Figure 127: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 0.5% for 72 hours (Set 2).

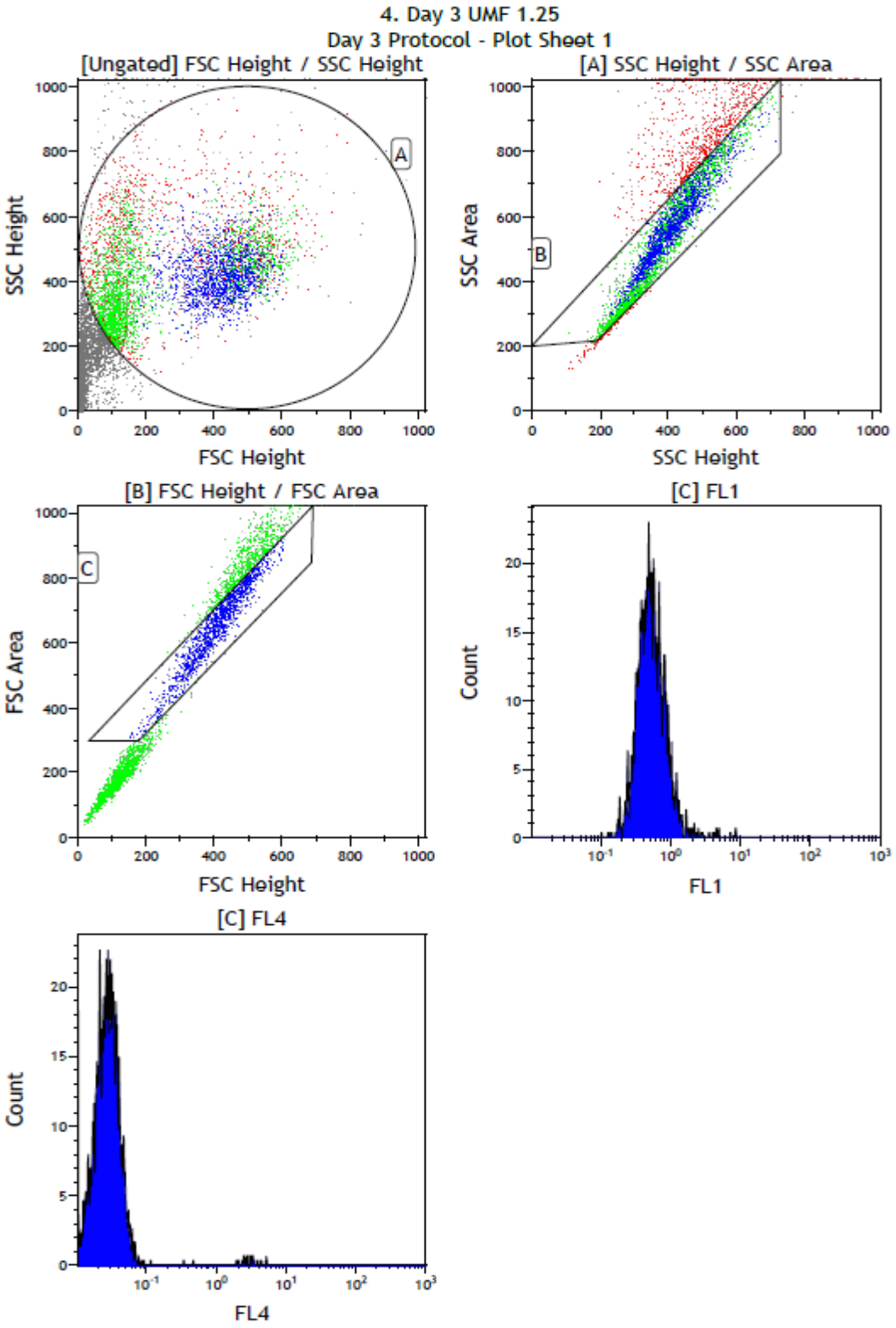


Figure 128: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 1.25% for 72 hours (Set 2).

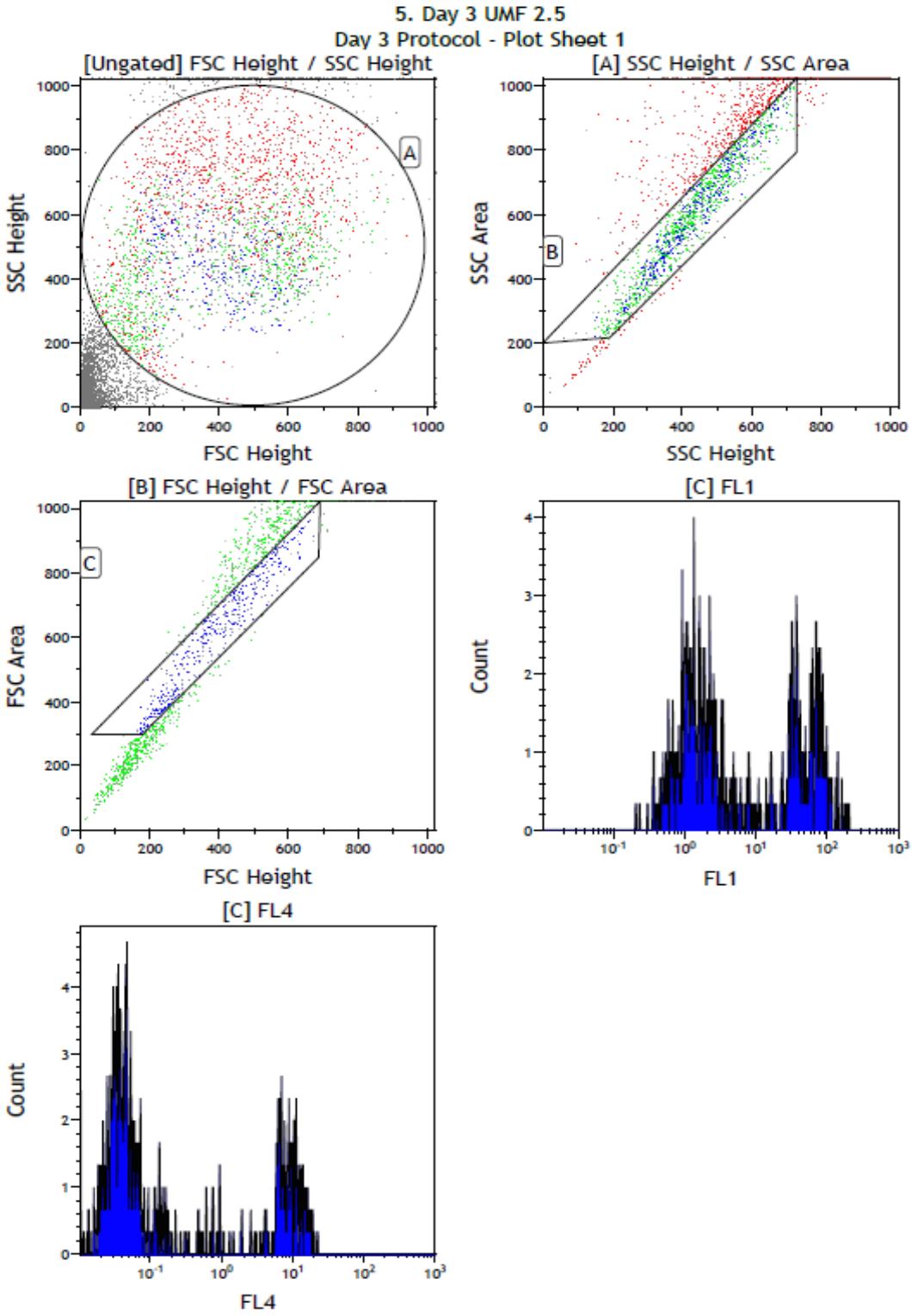


Figure 129: Apoptosis annexin V + PI data plot. WiDr cells treated with UMF+15 2.5% for 72 hours (Set 2).

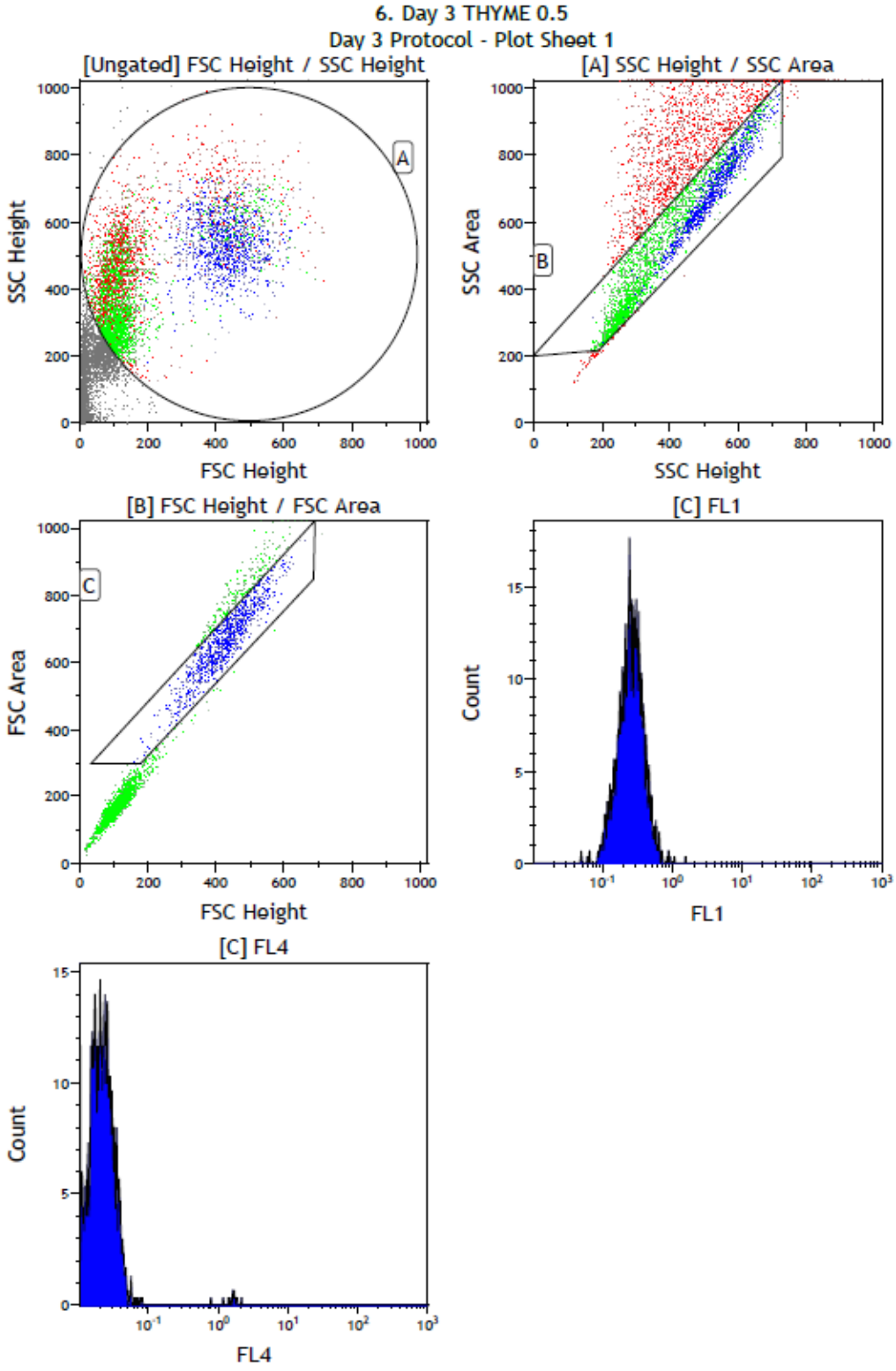


Figure 130: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 0.5% for 72 hours (Set 2).

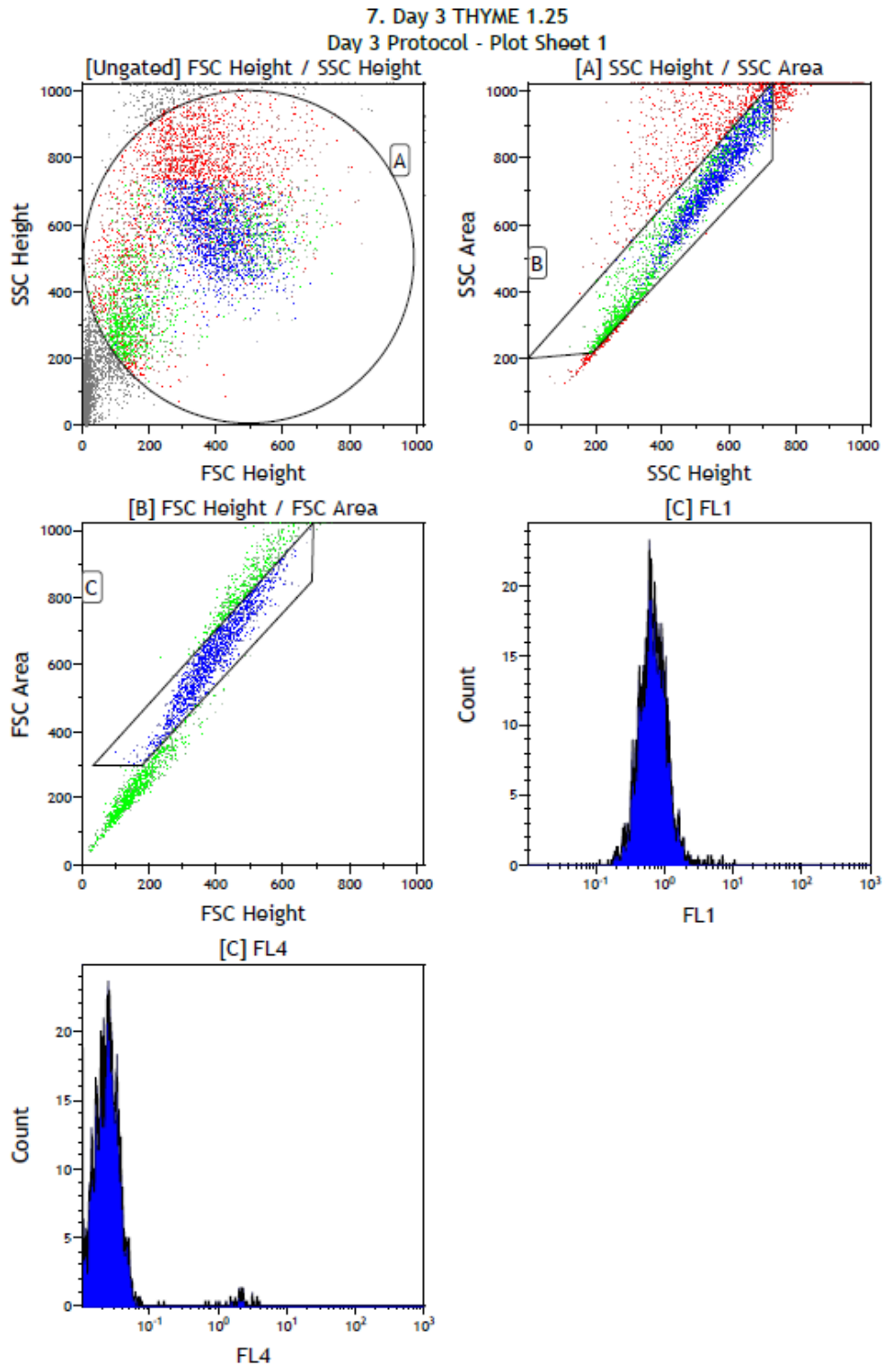


Figure 131: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 1.25% for 72 hours (Set 2).

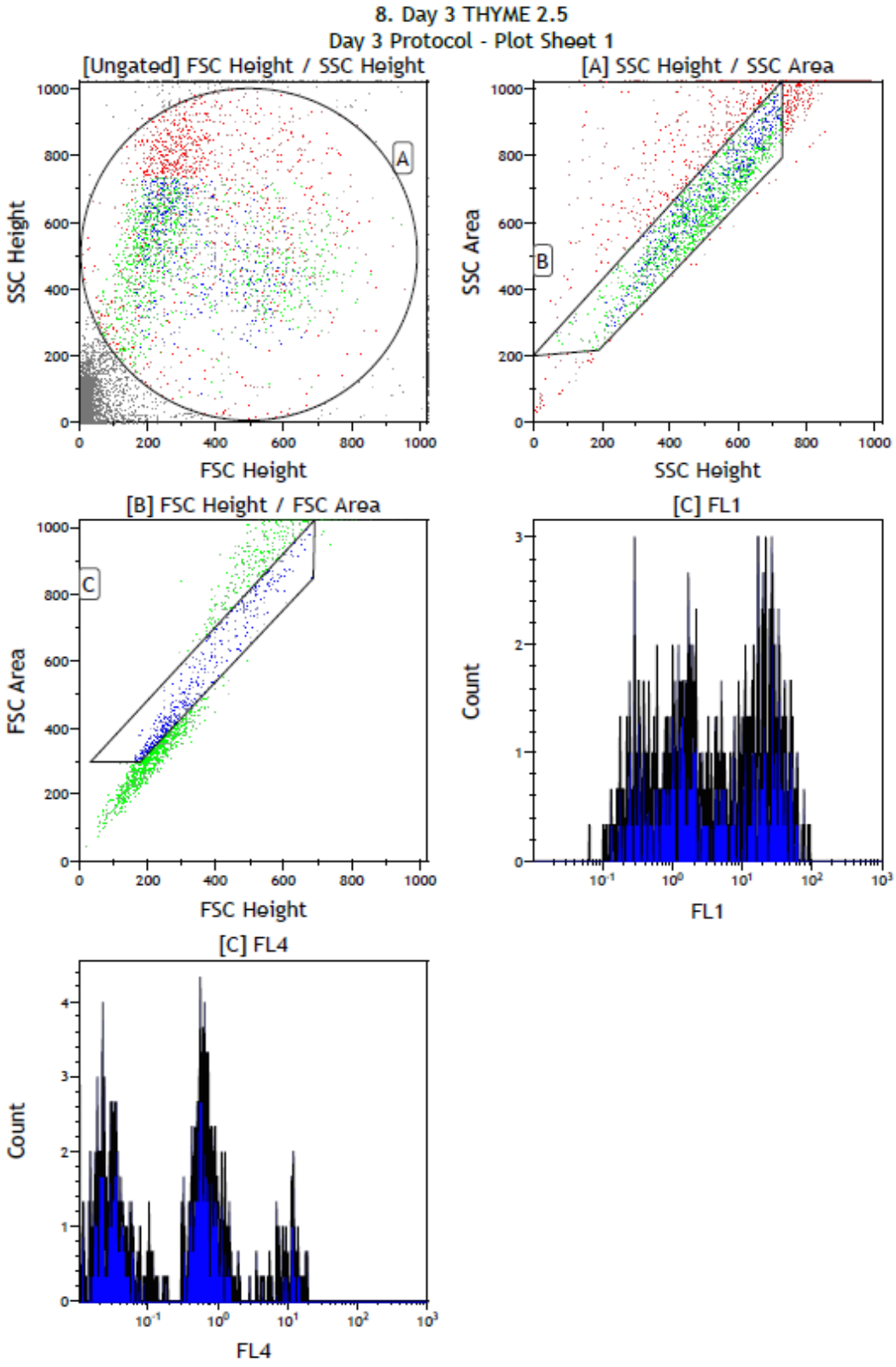


Figure 132: Apoptosis annexin V + PI data plot. WiDr cells treated with Thyme 2.5% for 72 hours (Set 2).