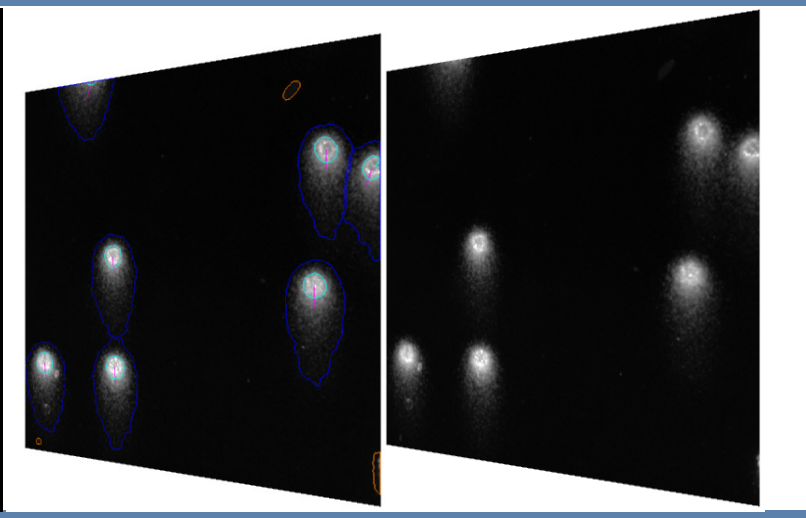
Thermo Scientific Cellomics® COMET

BioApplication Guide



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Chapter

1

Overview of the Comet BioApplication

High Content Screening (HCS) combines biological fluorescent-based reagents, automated optical imaging instrumentation, and an advanced image processing algorithm to automatically extract and quantify useful information from biological systems. This guide provides a description of the Cellomics® Comet BioApplication, an HCS application that helps users quantify DNA damage by quantitating the formation of comet due to electrophoresis in low-melting agarose of nuclei from cells exposed to DNA damaging agents. This guide contains the following chapters:

Chapter 1 provides an overview of the Comet BioApplication and provides examples of biological processes where the BioApplication can be used.

Chapter 2 describes the algorithm used to analyze the results and gives a brief description of input Assay Parameters and Output Features.

Chapter 3 describes some of the Assay Protocol Settings for 10X and 20X objective protocols using the CometBioApplication on biological examples and provides guidance on how to use the BioApplication Events Wizard.

Chapter 4 describes the Protocol optimization tasks that are available in the iDev™ Assay Development workflow.

System Compatibility

The Comet BioApplication described in this document is designed to run on the following platforms:

- ArrayScan® HCS Reader version VTI
- Cellomics® vHCS™ Discovery Toolbox version 1.6.1

Selected images from other sources (e.g., images up to 15-bits) may be imported and used with your platform.

In addition, to running the Comet BioApplication with comets that are on slides, you also need the Cellomics® Slideport™, which is designed to hold up to four standard dimension slides on the ArrayScan HCS Reader stage.

Cell Biology Background

The comet assay, also known as Single Cell Gel Electrophoresis (SCGE) is a commonly used assay to ascertain DNA damage induced in cells by acute exposure to chemicals or radiation. The principle behind the assay is the migration of DNA that is embedded in agarose under

electrophoresis. Nuclear DNA migrates towards the anode and depending on the extent of DNA damage, pH of electrophoresis buffer, temperature, the applied voltage and current, and duration of electrophoresis, the nuclear material has the appearance of a comet when viewed under a microscope (1). There are quite a few variants of the assay, but the commonly used assays are (i) the alkaline electrophoresis comet assay and (ii) the neutral electrophoresis comet assay (2). The comet assay was first reported in 1984 as a method to assess low level DNA damage caused by radiation (3) and the modified alkaline buffer version was first reported in 1988 to study DNA damage induced by x-ray or chemicals such as hydrogen peroxide (4). Typically in these assays cells are exposed to the DNA damaging agent for a defined period of time, then embedded in low-meting agarose (LMA) and layered on agarose on a glass microscope slide. The cells are then lysed to expose the nuclear material, denatured to unwind the DNA and subjected to electrophoresis under alkaline or neutral conditions. The nuclear material embedded in the LMA on the microscope slide is then stained with a fluorescent dye such as ethidium bromide, SYBR Green or DAPI. The slides are then imaged in a microscope and the shape and size of the comets are typically analyzed by image analysis software to ascertain DNA damage in cells caused by compounds or radiation.

The comet assay is used in studies related to genotoxicity, ecotoxicity, biomonitoring and other areas (5). Others have used the comet assay with some success to discriminate between genotoxic and cytotoxic compounds (6**)**. The comet assay is also being proposed as an early screening assay to identify genotoxicity of compounds that are drug candidates (7). Due to the varying nature of the comet assay as evidenced by experimental conditions, different biological model systems (both *in vivo* and *in vitro* systems) used to study DNA damage by comet formation, the applicability of the assay to different areas such as drug discovery, chemical industry, environmental monitoring, ecotoxicity, etc., a set of guidelines was established for the comet assay (8).

In addition to being used for studying general DNA damage, the comet assay is also being combined with fluorescence *in situ* hybridization (FISH) to study specific DNA sequence damage or gene sequence damage (5). There are several reports of combined Comet-FISH analysis to study specific DNA damage and its repair. These include studying the damage and repair of dihydrofolate reductase, methylguanine methyltransferase and the p53 gene in CHO cells and human lymphocytes (9), entire chromosomes using chromosome painting probes in human lymphocytes (10) and chromosomal instability of HER-2/*neu* and p53 in malignant breast cancer cells as compared to normal cells (11), to highlight a few.

BioApplication Overview

The Comet BioApplication is an automated image analysis module for use on the ArrayScan HCS Reader or the Cellomics vHCS Discovery ToolBox. The BioApplication is a completely automated tool for acquiring and analysis of images of comets arising due to DNA damage. The performance of image analysis algorithms to assess DNA damage in comet assays as compared to manual methods was shown to be better for certain measures of DNA damage and comparable to certain other measurements (12). Automated image analysis of comets was first reported 1999 by Bocker *et al* (13). The advantages of automated image analysis of comets as opposed to manual methods are the speed of analysis and consistent performance by the algorithm (14). Thus automated analysis of comet formation as a measure of DNA damage will provide rapid and more consistent results.

The Comet BioApplication is capable of imaging fluorescence in up to 6 individual colors/ channels. A channel refers to the visualization of any fluorescent dye on the ArrayScan HCS

Reader by a combination of appropriate excitation, dichroic and emission filters (please refer to yourArrayScan User's Guide for a detailed description of dye selection). However, for analysis of comets the BioApplication can be configured to acquire and analyze images in a single channel. Nuclei (DNA) subjected to electrophoresis on LMA can be stained with a wide choice of DNA staining nuclear dyes such as Hoechst, DAPI, SYBR Green and ethidium bromide that fluoresce from the blue to red region. Thus for comet analysis, the BioApplication will run as a one Channel BioApplication. The BioApplication can also be used to analyze combined Comet-FISH staining to study specific DNA sequence damage along with general DNA damage. For combined Comet-FISH analysis the BioApplication can be configured to run as a 2 channel or a 3 channel assay with FISH spot analysis being done in Channel 2 and/or Channel 3, and comets being analyzed in Channel 1. The BioApplication may also be used to analyze FISH images only by configuring the BioApplication as a 2 or 3 channel assay, using Channel 1 to counter-stain nuclei and Channels 2 and/or 3 for analysis of localized FISH spots in the nuclei. Table 1 below shows a combination of channels and types of analysis that can be done by the Comet BioApplication.

Type of Analysis	Channel 1	Channel 2	Channel 3**
Comet only	✓	--	--
Comet-FISH	✓	✓	✓/ --
FISH only	✓*** (as nuclear counter stain only)	✓	✓/ --

Table 1: *Configuration of the Comet BioApplication for Comet only, Comet-FISH or FISH only type of analysis. ** Channel 3 need not be used when only a single color FISH stain is present. *** In the case of FISH only analysis, Channel 1 is needed and can be used for a nuclear counter stain.*

Irrespective of whether the BioApplication is configured to perform Comet, Comet-FISH or FISH only analysis, the BioApplication will provide on-the-fly analysis of images as they are acquired on the ArrayScan HCS Reader with appropriate output features relevant for Comet or Comet-FISH analysis. Channels 4 to 6 can be used as gating channels and provide only gating related output features. The Assay Parameter s and output features in the Comet BioApplication are described in greater detail in Chapter 2 of this user's guide.

Figure 1 shows a schematic description (panel A) and an image (panel B) obtained from the ArrayScan VTI HCS Reader running the Comet Assay BioApplication. The image was obtained using a 20X objective of CHO-K1 cells treated with 150 µM 9- aminoacridine for 1 hour. The comets analyzed by the BioApplication are identified by a purple overlay around the DNA stain. The image depicts normal comets, abnormal comets, valid and rejected heads, and a rejected comet that are identified by differently colored overlays. In Chapter 2 of this guide, refer to the section titiled **"CometDataAnalysis"** for a description of "normal" and "abnormal" comets.

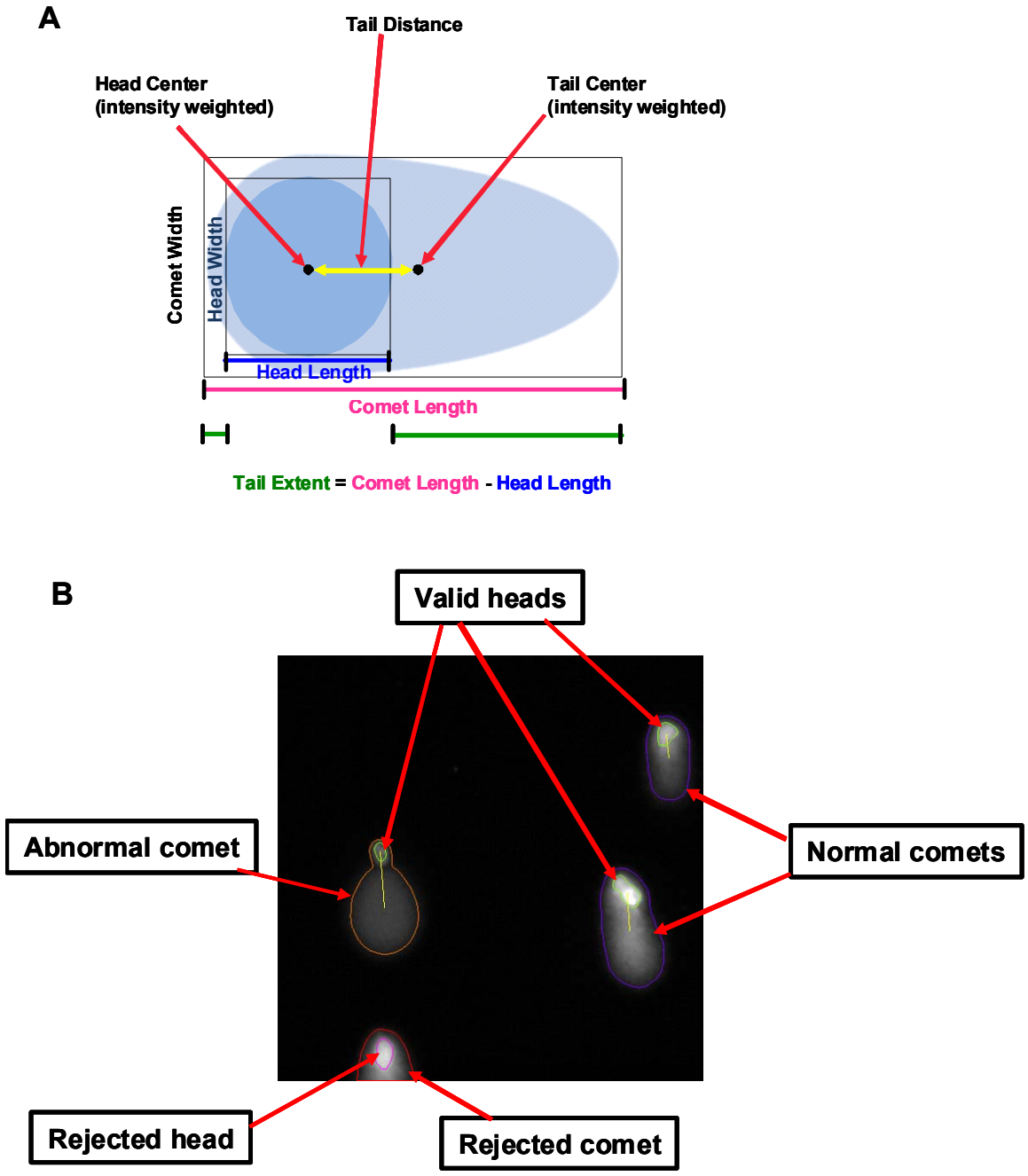

Figure 1: *Panel A is a schematic depicting the various regions of a normal comet. Panel B- comets obtained by treating CHO-K1 cells with 150 μM, 9 aminoacridine for 1hr. The image was acquired on an ArrayScan VTI HCS Reader with a 20X objective. The overlay masks in different colors identify normal comets, abnormal comet, valid head, rejected head, and rejected comet.*

Event Definition

The Comet BioApplication, similar to other Cellomics BioApplications, allows simultaneous definition of up to three Events to enable rapid multiparametric analysis at the level of individual cells across multiple Cell Features. Figure 2 relays these capabilities and how they interact to enable event definition and detection. Operation of this software tool is described in more detail in Chapter 3. Event definition and detection at the level of individual cells of a population requires processing of raw Cell Feature values, i.e., the data extracted from individual cells. Limits are then applied (manually entered or automatically computed via Reference Wells as described in Chapter 2) to identify responder cells for each feature. For the purposes of Event detection, responder cells are defined as those cells showing feature value greater than the upper limit (LevelHigh) or lower than the lower limit (LevelLow) defined by ***Feature*Ch*N*LevelHigh** (***Feature*Ch*N*Status =1**) or ***Feature*Ch*N*LevelLow** (***Feature*Ch*N*Status = 2**). It is critical that the limits be set appropriately for meaningful computation of Events.

Events are defined as Assay Parameters and consist of logical statements employing specific Cell Features. These statements are then applied to classify and count responder and non-responder cells for the Cell Features contained in the Event Definition. Well Features are calculated to provide population statistics for individual Cell Features and frequency of occurrence of a given Event in the cell population. The latter is expressed as the percentage of cells in each well that display a given Event.

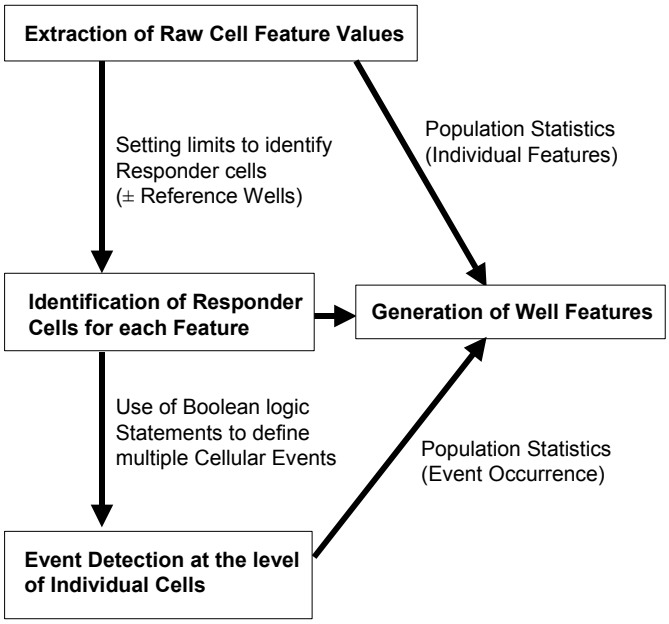

Figure 2: *Schematic showing the process of definition and detection of cellular events*

BioApplication Measurements

The Comet BioApplication measures and outputs several cell and well level features that are relevant for quantitating DNA damage in cells by treatment of cells with compounds that could potentially cause DNA damage or by exposing cells to radiation. Several of these output features such as Olive Tail Moment, Tail Extent Moment, Tail Extent (length), % DNA in Tail are routinely used by researchers studying DNA damage by comet analysis. Additionally, the BioApplication measures and reports a few field level features that are useful to ascertain the general performance of the BioApplication, such as identifying wells or fields that are out of focus or fields that are sparse. Table 1 below lists the types of features that are computed by the Comet BioApplication. Later chapters in this guide provide a more detailed description of cell, field, and well level output features.

TYPE	TARGET	OBJECT LEVEL FEATURES	WELL LEVEL FEATURES
Field and Well Level Features Reported for Channel 1			
Count	Comets, Head & Tail	Comet Number	Valid Object Count, Selected Object Count, %Selected Objects Valid Field Count %Selected Objects Per Valid Field, % Normal Comets, Normal Comet Count, % Abnormal Comets, Abnormal Comet Count
Morphology		Area, ShapeP2A, ShapeLWR	MEAN, SD, SE, CV, %HIGH, %LOW
Intensity		Total Intensity, Average Intensity, Tail % Total Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW
Moments	Tail	Tail Extent Moment, Olive Tail Moment	MEAN, SD, SE, CV, %HIGH, %LOW
Field and Well Level Features Reported for Channels 2 and 3			
Count	Spots	Comet, Head and Tail Spots	MEAN, SD, SE, CV, %HIGH, %LOW
Morphology	Spots	TotalArea, AvgArea	MEAN, SD, SE, CV, %HIGH, %LOW
Intensity	Spots	Total Intensity, Average Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW
Field and Well Level Features Reported for Channels 4-6			
Intensity		Total Intensity, Average Intensity, Variation of Intensity (StdDev)	MEAN, SD, SE, CV, %HIGH, %LOW

Table 1: *Types of features computed and reported by the Comet BioApplication. A more detailed description of the features can be found in Chapter 2 of this guide.*

Example Use Cases

The Comet BioApplication can be used to automatically quantitate DNA damage caused by exposure of cells to different compounds or radiation. As an example, here we show the use of the Comet BioApplication to determine the effect of 9-aminoacridine on DNA damage in CHO-K1 cells and compare the effect of 9-aminoacridine and tacrine (9-amino-1,2,3,4-tetrahydroacridine) a compound that is similar to 9- aminoacridine, in CHL cells. 9-aminoacridine is a known DNA intercalating agent that is a mutagen. Tacrine, the reduced form of 9-aminoacridine, is a compound that is known to act as a cytotoxic compound affecting protein synthesis. We wanted to compare the DNA damage causing potential of 9-aminoacridine and tacrine using the Comet BioApplication.

CHO-K1 cells were cultured overnight in 6-well plates at a plating density of 200,000 cells per well. After overnight culture, the cells were exposed to different concentrations of 9-aminoacridine for 1 hour in a tissue culture incubator. Cells were then harvested using a cell scraper into cold buffer, suspended into LMA, and layered on agarose on microscope slides. Following lysis and DNA unwinding under alkaline conditions, the DNA in the LMA layer was electrophoresed under alkaline conditions (pH > 13) at 250 mA for 20 minutes. The gels were then stained with SYBR Green to visualize the DNA. The slides were then mounted on a Cellomics Slideport and scanned on an ArrayScan VTI HCS Reader. Figure 3 (panels A & B) shows the effect of short term exposure of 9-aminoacridine on CHO-K1 cells and Figure 4 shows the data from this treatment. Figure 5 shows the extent of DNA damage measured by the comet assay, from the treatment of CHL cells with 9- aminoacridine or tacrine.

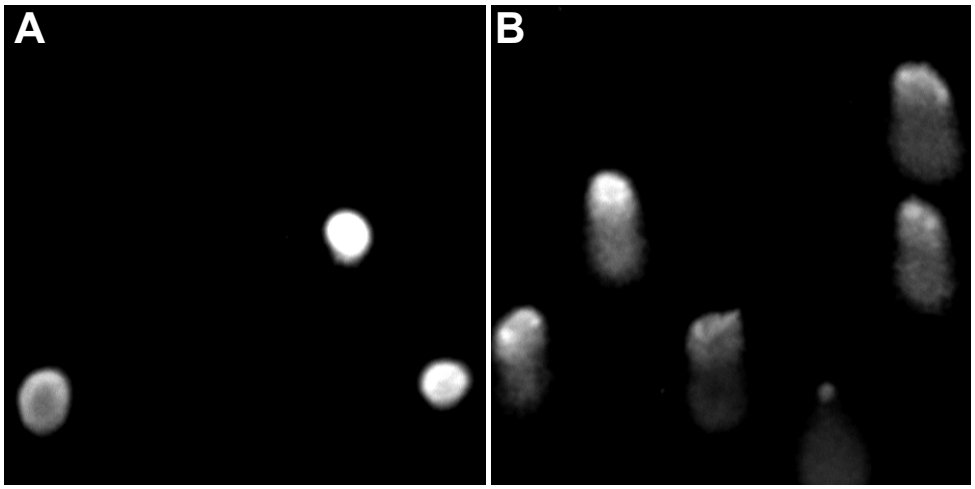

Figure 3: *Images of CHO-K1 nuclear material stained with SYBR Green and acquired on the ArrayScan VTI HCS Reader with a 20X objective. Panels A & B show images of wells treated with 0 μM and 150 μM 9-aminoacridine respectively for 1 hour.*

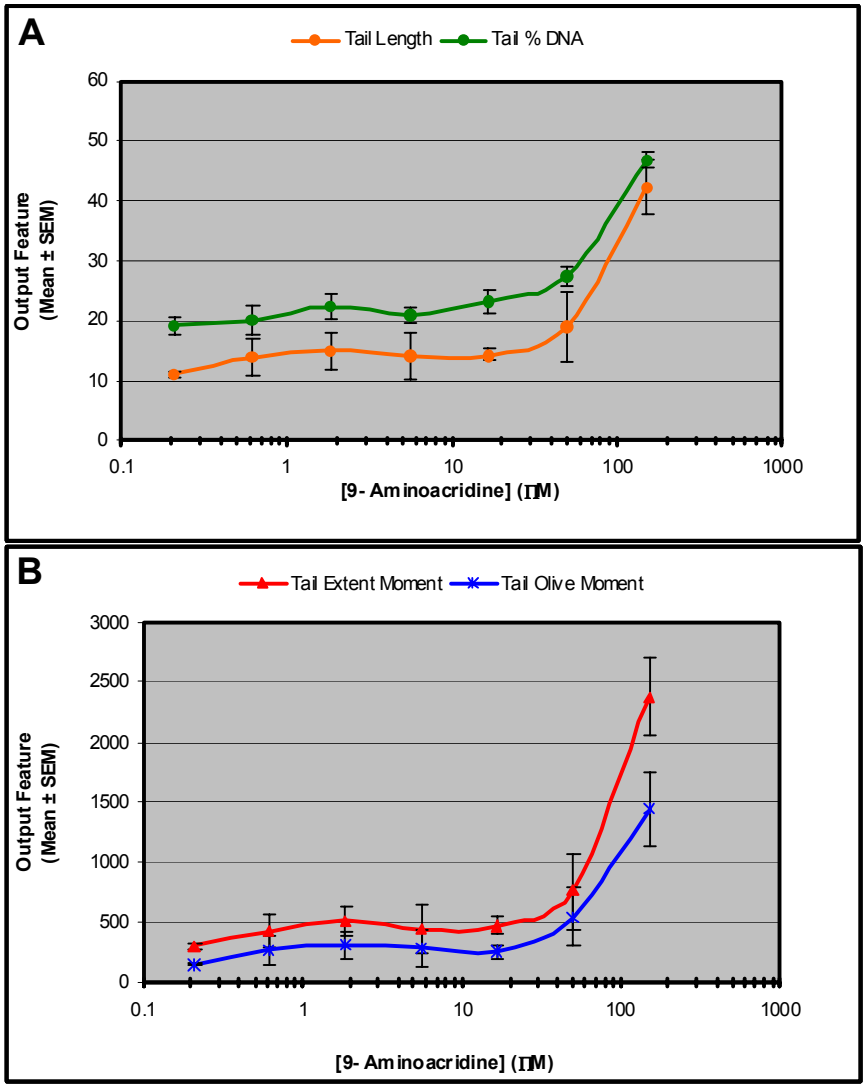

Figure 4: *DNA damage measured by various comet measurements in CHO-K1 cells exposed to varying doses of 9-aminoacridine for 1 hour. Data at each concentration is mean ± SEM from 3 wells. The data was obtained by analysis of images acquired on an ArrayScan VTI HCS Reader with a 20X objective using the Comet BioApplication.*

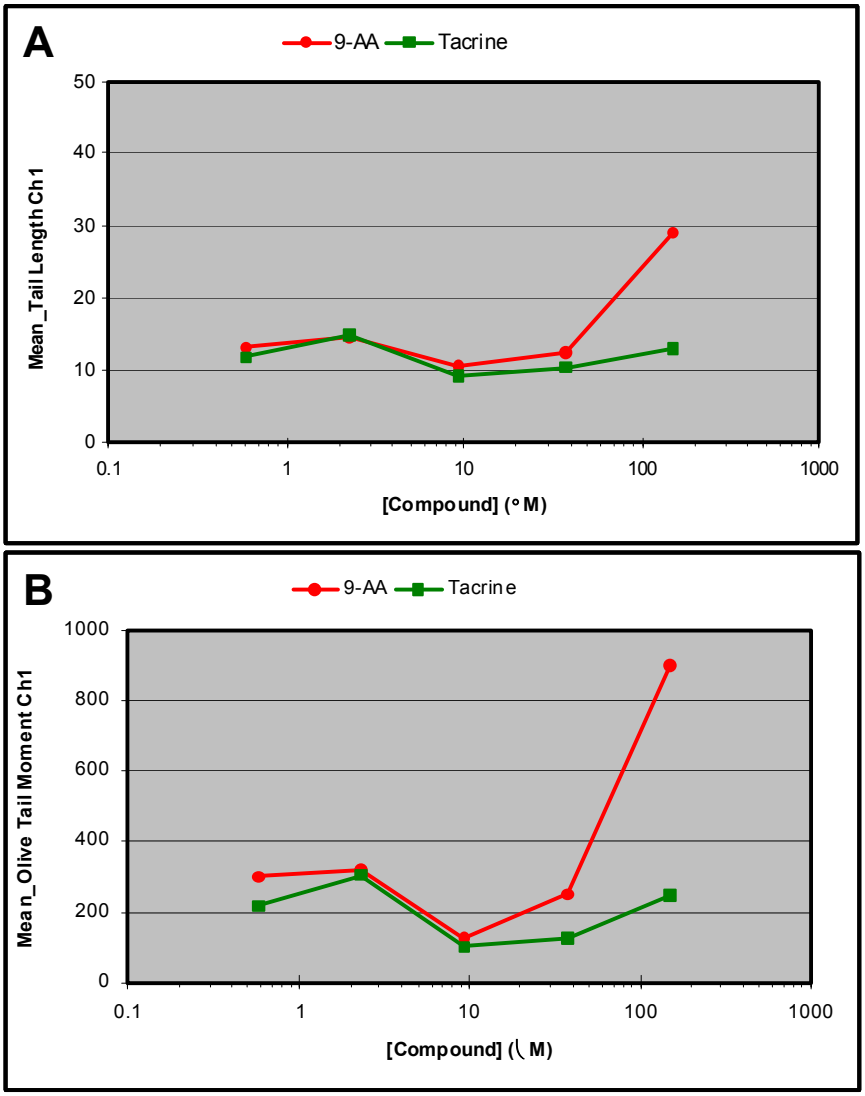

Figure 5: *DNA damage measured by various comet measurements in CHL cells exposed to varying doses of 9-aminoacridine or tacrine for 1 hour. The data is the well output feature from one well per concentration of the compound and was obtained by analysis of images acquired on an ArrayScan VTI HCS Reader with a 20X objective using the Comet BioApplication. Panel A is the effect on the comet tail length and panel B is the effect of the compounds on Olive tail moment.*

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Chapter
2

Description of the Algorithm

NOTE

The Assay Parameters described in this guide function in the same manner regardless of using the Classic or iDev versions of the ArrayScan software. For descriptions of each task for optimizing a Protocol in the iDev Assay Development Workflow, please refer to Chapter 4 of this guide.

Chapter 1 provided a general overview of the Comet BioApplication. This chapter describes how the Comet BioApplication works and describes the adjustable Assay Parameters and Output Features.

Overview

The Comet BioApplication is a simple to use BioApplication designed to quantitate DNA damage at the single cell/nucleus level, in cells exposed to DNA damaging chemicals or radiation. The BioApplication is designed to completely automate the process of acquiring images of fluorescently stained DNA (in the shape of comets) which are embedded in LMA on a microscope slide and analyze the images on-the-fly to quantify DNA damage. The BioApplication provides an objective analysis of all the comets that are imaged, without the introduction of subjectivity or human error. It provides output features that are normally reported by researchers using the comet assay such as Olive Tail Moment, Tail Length, Tail Extent Moment, and % DNA in the Tail. In addition to the above features, the BioApplication also reports a host of other output features including morphological measurements that are relevant for comet assays and allows the user to gain a better understanding of DNA damage induced by their compounds. Since the Comet BioApplication is a cell based High Content Analysis (HCA) tool, it provides individual cell level information allowing users to perform a deeper analysis of cell populations.

In addition to performing analysis of comets, the Comet BioApplication can also perform analysis of fluorescence *in situ* hybridization (FISH) spots on DNA. i.e., allow for combined Comet-FISH analysis. This allows the user to not only quantitate general DNA damage, but also obtain data for specific DNA sequence damage by using appropriately designed FISH probes. Comet-FISH analysis can be done for up to two different DNA sequence probes, each of which is tagged with a different fluorescent dye.

Analysis of images obtained from the ArrayScan HCS Reader or other sources is governed by two sets of input parameters; Assay Parameters and Object Selection Parameters. These input parameters are available for every Channel in the BioApplication. A combination of Assay Parameters and Object Selection Parameters with other settings such as objective type, filter set, minimum number of objects per well, provides an Assay Protocol. While Assay Parameters control the quantitative analysis of the images, the Object Selection Parameters control which objects are chosen for processing. These input parameters can be found in the Create Protocol View and Protocol Interactive View of the ArrayScan Classic software or in

the Protocol Optimization task list of the iDev software. The available input parameters are dependent on the number of channels selected; only the input parameters for the selected channels will be displayed. In the ArrayScan Classic software, the available input parameters will also vary depending on the mode in which you are running: Basic Mode or Advanced Mode. Basic Mode enables you to modify only a few basic input parameters and uses default protocol settings for other parameters in the Assay Protocol, to quantitate DNA damage by assaying comets. The Advanced Mode setting allows you to modify all input parameters that govern the Comet BioApplication.

Description of Assay Parameters and Settings

The algorithm has Assay Parameters that control the analysis of images, which can be adjusted to allow customization of the algorithm to your own biology and conditions. The Assay Parameters can be described in two distinct groups: (i) specifically designed for the Comet BioApplication and (ii) general Assay Parameters that control general aspects of image processing.

Assay Parameters for Comet BioApplication

The following Assay Parameters are specifically designed for the Comet BioApplication, so the user can optimize and specify the best assay protocol settings for optimal analysis of Comets and Comet-FISH. The following is a list of Assay Parameters that are specific to the Comet BioApplication. A description of each of these Assay Parameters is described below.

- CometMigrationDirection
- CometDataAnalysis
- CometSmoothFactorCh1
- CometSegmentationCh1
- HeadRegionFractionCh1
- HeadSmoothFactorCh1
- HeadDetectionCh1
- HeadIdentificationModifierCh1
- HeadSegmentationCh1

CometMigrationDirection

The Assay Parameter "**CometMigrationDirection"** defines the orientation of the comets in the ArrayScan Protocol Interactive View and Scan View. This Assay Parameter ensures that your comets will be analyzed properly irrespective of the orientation of the electrophoresis unit that was used for the comet migration with respect to the stage and the camera on the ArrayScan HCS Reader. This Assay Parameter can have values from 0–3. A value of **"0"** indicates that the orientation of the comets is top to bottom in the Protocol Interactive View or Scan View of the ArrayScan software, with the comet head at the top and the tail migrating towards the bottom. A value of **"1"** indicates that the orientation is bottom to top (head at bottom with tail on top). A value of **"2"** indicates that the orientation is left to right (head on the left with tail migrating towards the right) and **"3"** indicates a right to left orientation (head on right and tail towards left). Figure 4 shows the four possible orientations of comets.

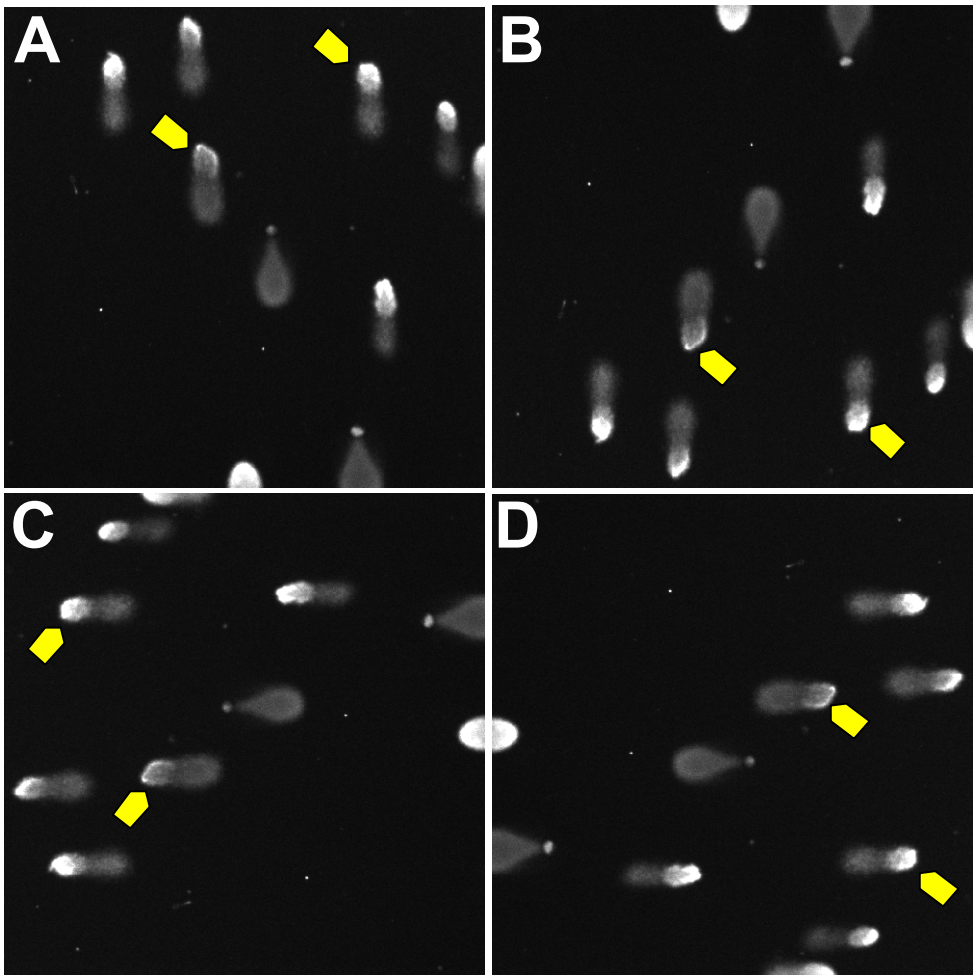
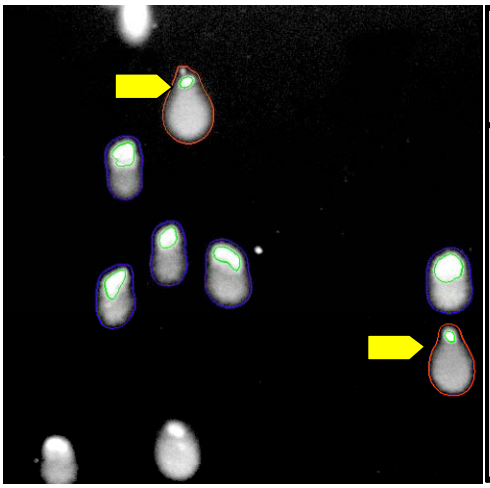


Figure 4: *The above figure depicts the four possible orientations of the comets for analysis by the Comet BioApplication. The Assay Parameter* ***"CometMigrationDirection"*** *will have a value of* ***"0"*** *(Panel A),* ***"1"*** *(Panel B),* ***"2"*** *(Panel C) or* ***"3"*** *(Panel D), for the comets to be analyzed properly. The location of the comet head is indicated by the yellow arrow heads.*

CometDataAnalysis

This Assay Parameter determines whether all of the selected comets or only comets designated as normal comets (based on morphological characteristics) are used in the computation of Well Output Features. The Assay Parameter can have a value of either **"0"** or **"1"**. If the value is set to **"0"**(default), then only normal comets are used in the computation of well average of features, while a value of **"1"** will allow the BioApplication to compute well averages from all selected comets (both normal and abnormal). Figure 5 shows an image of comets that are designated as a normal or an abnormal comet, by the BioApplication and also some of the important well level features in a table form. From the table, it is clear that although the various counts do not change with the two different settings for the Assay Parameter, the well averages for the features (**MEAN_*Feature***) are different with the different settings. When the Assay Parameter is **"0",** the abnormal comets (comets with an orange mask around them) are excluded from well average calculations and when the parameter is set to **"1"** both normal and abnormal comets are included in the well average calculations.



Assay Parameter>	*CometDataAnalysis*	
	0	*1*
SelectedCometCount	7	7
NormalCometCount	5	5
AbnormalCometCount	2	2
MEAN_CometLengthCh1	71.50	74.16
MEAN_TailExtentCh1	40.17	47.40
MEAN_Tail%TotalIntenCh1	61.48	69.99
MEAN_TailOliveMomentCh1	1273.89	1710.15

Figure 5: *Panel on left shows comets from CHO-K1 cells. Comets designated as normal comets are identified with purple masks around them and comets designated as abnormal are identified with orange masks around them (yellow arrow heads). The comet heads are identified by green mask around them in both normal and abnormal comets. The table on the right shows the well averages of some main output features from the field in the left panel when the Assay Parameter* ***"CometDataAnalysis"*** *is set to* ***"0"*** *(normal comets only included in well average computation) or* ***"1"*** *(all comets included in well average computation). Notice that the various counts have not changed with either value for the Assay Parameter.*

The BioApplication automatically characterizes a comet as "normal" or "abnormal" based on its morphological characteristics and the location of the center of the comet. The "abnormal" comets generally arise from cells that are undergoing apoptosis and are shaped like a tear drop (yellow arrow heads in figure above) with a head width that is much smaller than the tail width. Figure 8 shows comets that are classified as both "normal" and "abnormal" comets.

CometSmoothFactorCh1

This Assay Parameter determines the degree of smoothing of the objects (comets) prior to their identification and analysis. Smoothing is useful in defining the boundary of an object in image processing correctly from the background. Figure 6 shows the effect of smoothing operation on comets. The panel on the left shows comets identified and analyzed without any smoothing, while the panel on the right shows effect when the Assay Parameter "**CometSmoothfactorCh1"** was set to **"6"**. When no smoothing is done (Assay Parameter is set to **"0"**) then the entire comet is not analyzed as indicated by comets identified with yellow arrow heads. It is recommended that smoothing be done to have the best possible analysis of the comets. Please refer to the section titled "Object Smoothing" later in this chapter for a general description of object smoothing in image processing.

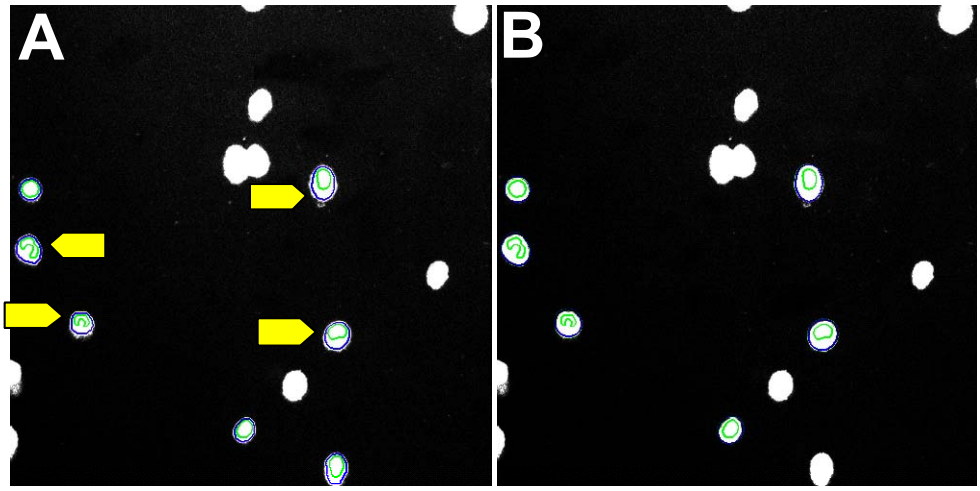


Figure 6: *Panel on left shows comets from CHO-K1 identified and analyzed without any comet smoothing (**CometSmoothfactorCh1= 0**) and panel on right shows the same comets analyzed with smoothing (**CometSmoothFactorCh1=6**).*

CometSegmentationCh1

This Assay Parameter determines if any segmentation will be done on the comet and which method will be used to segment the entire comet. This is especially useful if there are two nuclei (comets) that are close to each other and you want to segment them into two individual comets. The parameter can have negative, zero, or positive values. If the value assigned to this parameter is **"0"** then no segmentation of the comets is done. If the value is negative, then segmentation is achieved using peak intensity method. If the value is positive, then a morphological method is used to segment the comets. Please refer to the section titled "Object Segmentation" later in this chapter for a general description of segmenting objects in image processing.

In Figure 7, the two comets identified by the arrow head are unsegmented (Assay Parameter **CometSegmentationCh1 = 0**) and hence rejected from analysis. The panel on the right shows the same field with segmentation enabled (**CometSegmentationCh1 = 6**) and the two comets are selected for analysis.

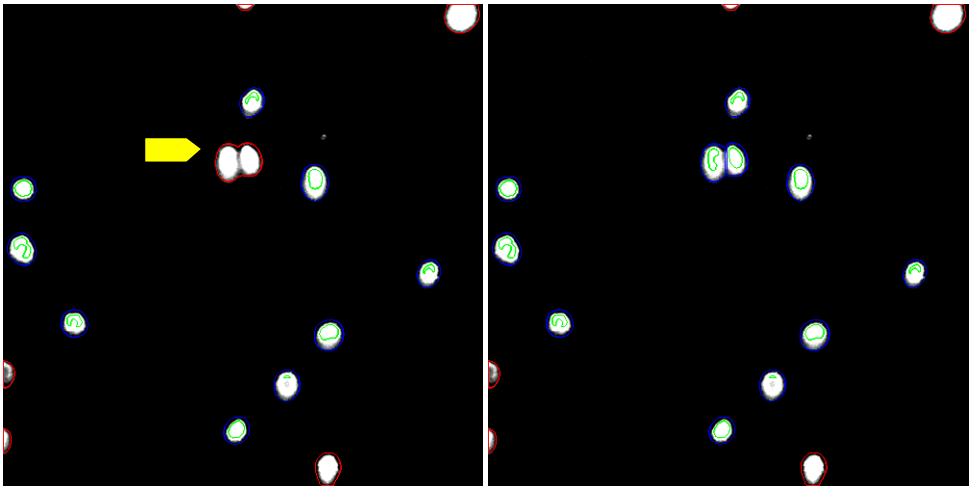


Figure 7: *Two comets from CHO-K1 cells that are close to each other (yellow arrow head, left panel) are rejected from analysis when the Assay Parameter* ***CometSegmentationCh1 = 0****. The same two comets are selected for analysis (right panel) when* ***CometSegmentationCh1= 6****.*

HeadRegionFractionCh1

This Assay Parameter determines the fraction along the length of the comet, where the comet is split into its head and tail regions. The Assay Parameter can have a fractional value from 0 to 1. A value close to "**0"** will split the comet high up in its head region, while a value close to "**1"** will split the comet further into the tail region. If you have no sign in front of the numerical value (a positive value) for the Assay Parameter, then the line demarcating the head-tail region is **not** displayed as a part of the comet mask. If however, a negative sign is placed before the numerical value for the Assay Parameter, then a line displaying the line of demarcation of the head-tail split region is displayed as a part of the comet mask. In Figure 8, the Assay Parameter "**HeadRegionFractionCh1"** is set to a value of **"0.3, 0.6 and 0.9"** in the top panels (A, B, C; positive value, hence line across comet is not seen). In the bottom panels, the value is set to **"-0.3, -0.6 and -0.9"**(D, E, F respectively) and hence the line is seen across the comets that are selected for analysis.

NOTE

The default value for the **HeadRegionFractionCh1** is "0.5". This was based on comet shape and size from CHO-K1 cells. You may have to change this Assay Parameter for other cell types, depending on their comet morphological characteristics.

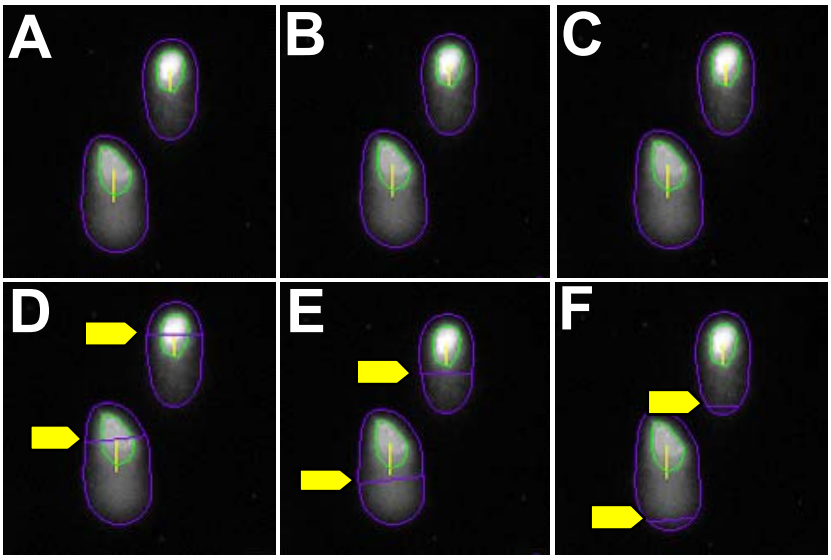


Figure 8: *The Assay Parameter* ***"HeadRegionFractionCh1"*** *can be used to demarcate the head and tail regions of the Comet. In the images shown above of nuclear comets from CHO-K1 cells, the parameter was set to* ***0.3, 0.5 or 0.9*** *(panels A, B, C respectively) or* ***-0.3, -0.6 or -0.9*** *(panels D, E and F respectively). The line of demarcation (identified by yellow arrow heads) between the head and tail regions is displayed along with the comet mask only when a negative sign is placed in front of the numerical value for the Assay Parameter.*

HeadSmoothFactorCh1

This Assay Parameter determines the extent of smoothing of comet heads prior to their detection. The parameter can have values from 0-127. When set to **"0",** no smoothing of the comet head is done. In Figure 9, the comet head region (identified by green masks) is defined more correctly on the right panel (**HeadSmoothFactorCh1** is **"6"**) than on the left panel (**HeadSmoothFactorCh1** is **"0"**). The comets that show clear differences are indicated by yellow arrow heads. Please refer to the section titled "Object Smoothing" later in this chapter for a general description of object smoothing in image processing.

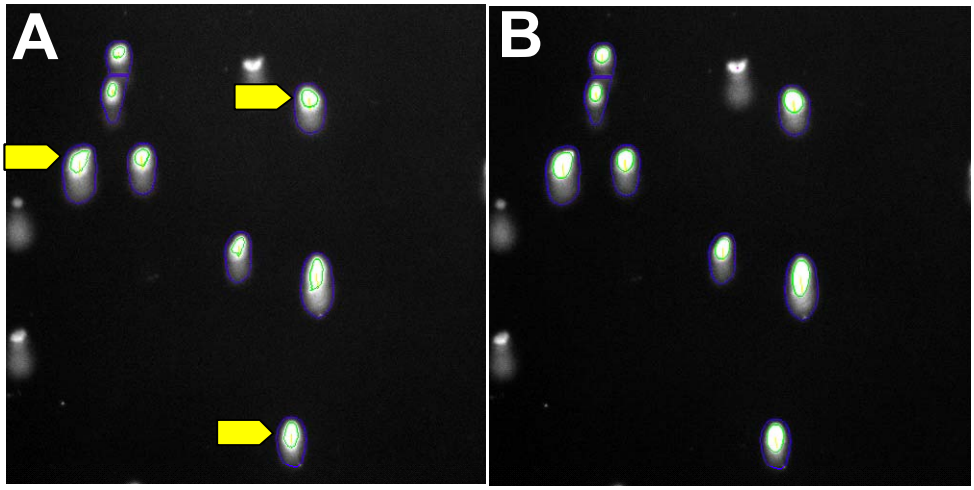


Figure 9: *The Assay Parameter* ***"HeadSmoothFactorCh1"*** *is useful in defining the boundaries of the head regions of comets (green overlay) from the background itself. Panel A shows the comets and their heads when the parameter is set to* ***"0"****, while panel B shows the same comets and their heads when the Assay Parameter is set to* ***"6"****. The heads showing maximum difference in the head overlay are identified by yellow arrow heads.*

HeadDetectionCh1

This Assay Parameter is useful in identifying the comet head. This parameter can have positive, negative, or **"0"** as value. If the value is set to **"0"** then head detection is not applied, while positive or negative values apply different methods of detecting comet heads. In several cases, with a well defined comet head that is clearly distinguishable from the rest of the comet, this Assay Parameter can be set to **"0"** and heads can still be successfully identified. This is shown in Figure 10.

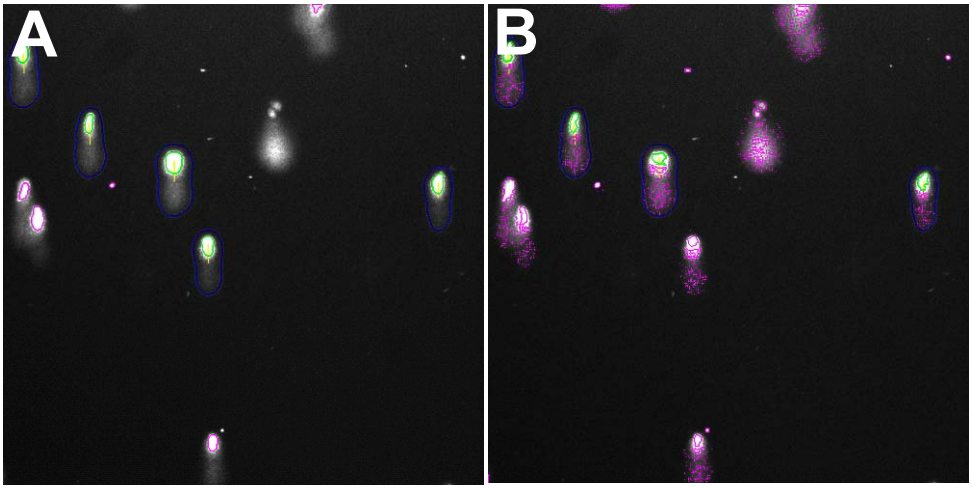


Figure 10: *The Assay Parameter **"HeadDetectionCh1"** is useful in identifying comet heads. The panel on the left (A) shows comet heads (green overlay) in CHO-K1 cells with the Assay Parameter set to "**0**". The panel on the right (B), shows the same comets when the Assay Parameter was set to **"2"**. The pink overlays identify objects identified and rejected as comet heads. Thus it is apparent that when the comet head appears well defined, the Assay Parameter **"HeadDetectinCh1"** can be set to **"0"**.*

HeadIdentificationModifierCh1

The **HeadIdentificationModifierCh1** is an intensity threshold parameter that allows for better identification of comet heads from its background. Sometimes the comets head may not be differentiated in terms of intensity from the rest of the comet and adjusting this Assay Parameter will help in identifying the comet head successfully. The Assay Parameter can have values ranging from -1 to 10. More negative values will identify comet heads that are less clearly differentiated from the rest of the comet (head intensity that is comparable to the rest of the comet), while more positive values are used to identify brighter comet heads from the rest of the comet. In Figure 11, the left panel shows comet heads (green overlays) identified with a negative value (**HeadIdentificationModifierCh1= -0.5**), while the center panel shows the same comet heads with overlays when the Assay Parameter is set to **"0"**. The panel on the right shows the comet heads identified appropriately when the Assay Parameter is set to **"1"**. This setting appears to be most appropriate for this image set, with the comet head overlays matching the periphery of the comet heads closely.

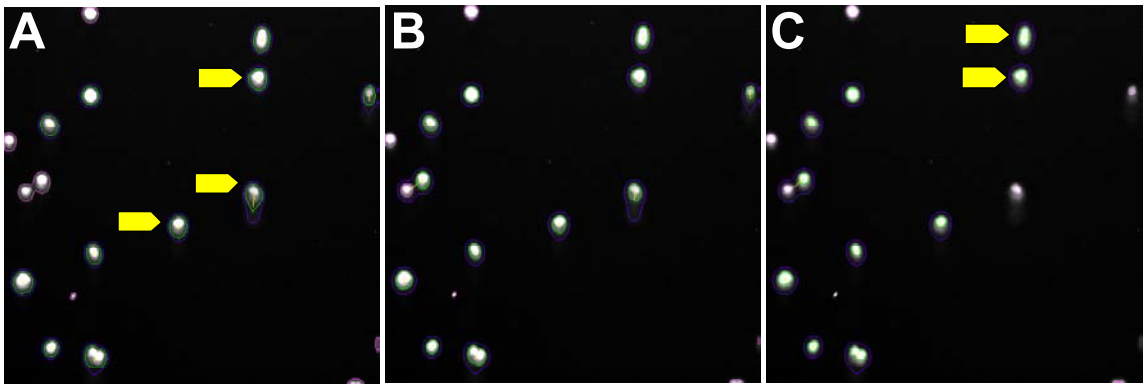


Figure 11: *Comet heads identified with different settings of the Assay Parameter* ***"CometIdentificationModifierCh1"****. The arrows indicate comet heads whose green head overlays progress from identifying too much of the background as the head when the Assay Parameter is negative (A), to identifying the head correctly when the parameter is positive (C).A value of* ***"0"*** *(panel B) identifies some background (comet itself) as the comet head.*

HeadSegmentationCh1

The Assay Parameter **"HeadSegmentationCh1"** is useful to segment two or more heads that are close to each other in a single comet. A typical example of segmenting two heads in a single comet is shown in Figure 12. On the left panel the Assay Parameter **HeadSegmentationCh1 = 0** and on the right panel the Assay Parameter is **"-2"**. The yellow arrow head identifies the two heads that are unsegmented in the left panel, while segmented in the right panel. Also notice in the right panel that one of the two segments is rejected (pink overlay) and hence is not included in the cell or well level calculations. Please refer to the section titled "Object Segmentation" later in this chapter for a general description of object segmentation in image processing.

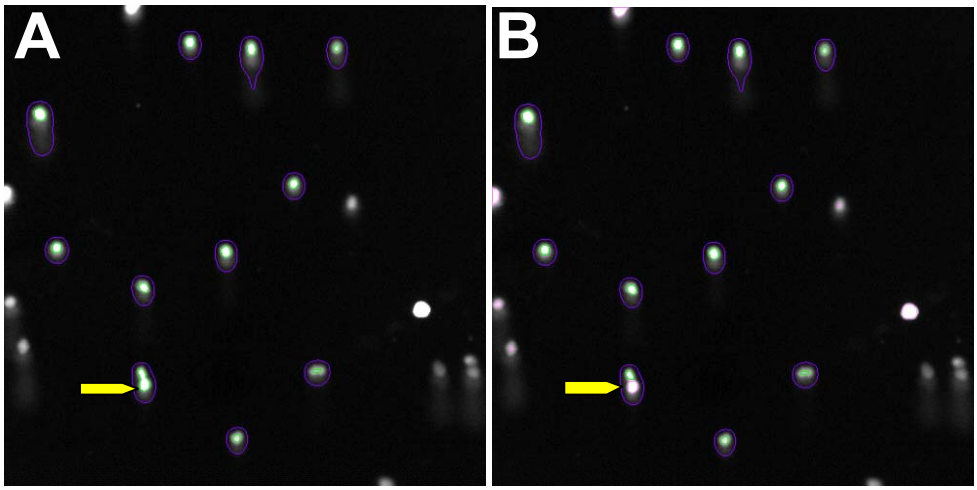


Figure 12: *Two comet heads identified by yellow arrow head in a single comet are unsegmented (panel A) or segmented (panel B) based on two different settings of the Assay Parameter* ***"HeadSegmentationCh1"****. In the former case, the value for the Assay Parameter was* ***"0"*** *while in panel B, the values was* ***"-2"****. Notice in panel B, that one of the two heads is rejected (pink overlay) from analysis.*

Assay Parameters for Comet-FISH

The Comet BioApplication can also be used to characterize specific DNA sequence damage by combining FISH with comets. Thus the Comet BioApplication allows for identification and analysis of FISH spots in Channels 2 and 3. The main Assay Parameters for analysis of FISH spots are:

- SpotTypeCh2/Ch3
- SpotSmoothFactorCh2/Ch3
- SpotDetectionCh2/Ch3
- SpotSegmentationCh2/Ch3

SpotTypeCh2/Ch3

This parameter determines the nature of the spots that are to be analyzed by the BioApplication in Channel 2 and/or Channel 3. This parameter can have a value of either **"0"** or **"1"**. A value of **"0"** indicates that the spots are bright spots on a dark background (typical of florescent staining) and a value of **"1"** indicates that the spots are dark spots on a bright background (as obtained when samples are imaged using the Brightfield Module on the ArrayScan HCS Reader). Irrespective of the type of spots, they are analyzed similarly by the BioApplication.

Although it is unlikely that the Assay Parameter **SpotTypeCh2/Ch3** will be set to **"1"** for FISH analysis, this option allows users to carry out non fluorescent spot analysis in Channels 2 and/or 3.

SpotSmoothFactorCh2/Ch3

This Assay Parameter determines the extent and method of smoothing prior to identification and analysis of spots in Channel 2 and/or Channel 3. If the value for the Assay Parameter is set to **"0"** then smoothing is not applied to the spots. Please refer to the section titled "Object Smoothing" later in this chapter for a general description of object smoothing in image processing.

SpotDetectionCh2/Ch3

This Assay Parameter determines the method used to detect spots during FISH analysis in Channel 2 and/or Channel 3. This parameter can have negative, positive or **"0"** value. If the value for this Assay Parameter is set to **"0"**, then spots are not detected. Thus it is imperative that a proper value be assigned for this Assay Parameter so FISH are analyzed appropriately. The method of spot detection applied depends on whether this parameter has a positive or negative value. Please see under "Spot Detection" later in this chapter for a general description on detecting spots in image processing.

SpotSegmentationCh2/Ch3

This Assay Parameter determines the method and extent of segmentation to individually discriminate spots that are too close to each other or are touching each other. Like the other two Assay Parameters discussed above, this Assay Parameter can also have negative, positive or **"0"** value. If the value is **"0"** then no segmentation of spots is done in Channel 2 and/or Channel 3. The method of spot segmentation applied depends on whether this parameter has a

positive or negative value. Please refer to the section titled "Object Segmentation" later in this chapter for a general description of object segmentation in image processing.

General Assay Parameters

These Assay Parameters control general aspects of image processing and its analysis. This section also provides a description of general image processing steps such as background correction, smoothing, and segmentation. Specific Assay Parameters that govern these operations on comets, heads, and FISH spots have been described earlier. The general image processing Assay Parameters are:

- Reference Well Control
- Units for Morphological Measurements
- CometType
- Background Correction
- Object Smoothing
- Spot Detection
- Object Segmentation
- Rejecting Border Objects

Reference Well Control

The two general Assay Parameters controlling the use of Reference Wells are: **"UseReferenceWells"** and **"MinRefAvgCometCountPerField"**. The **"UseReferenceWells"** Assay Parameter allows the user to choose whether reference wells are to be used to determine the population characterization limits. If **"UseReferenceWells = 1"**, then the Assay Parameter **"MinRefAvgCometCountPerField"** must be set. This is the minimum number of comets selected per field required for acceptance of data from that field for calculation of Reference Well Features. If this minimum number of comets is not met from all of the fields/well designated as Reference Wells, then the default *LevelHigh* and *LevelLow* values from the Assay Protocol will be used.You can enter a number that closely matches the average number of selected comets needed to obtain statistically valid results for the different output features. There are additional Assay Parameters for Reference Well processing specific for particular features which will be described in later sections.

Wells that are to be used as Reference Wells also need to be specified when the Assay Parameter **"UseReferenceWells = 1"**. In the ArrayScan software, choose **Tools**>**Change Reference Wells** and select Comet.V4 under assay to change/define wells for the Comet BioApplication. Refer to your ArrayScan VTI User's Guide for more details.

Units for Morphological Measurements

The user has the option of either choosing Micrometers or Pixels as the unit to report morphological measurements. This is done by the **"UseMicrometers"** Assay Parameter. When set to **"0**," the area of selected comets is reported in pixels. Otherwise if set to **"1"**, the area is reported in micrometers. This information is calculated automatically from the chosen magnification and camera resolution settings.

Comet Type

The "**CometTypeCh1**" Assay Parameter allow you to specify whether the comets of interest are brighter or darker than the background of the image. See above for a description of **SpotTyeCh2/Ch3** for defining type of spots in Channels 2 and/or 3 to be analyzed in combined Comet-FISH assays. All of these parameters can have a value of either "**0**" or "**1**". Setting the value to **"0"** (typical setting for fluorescently stained comets) lets the application consider bright objects on a dark background as potential objects, while a value of **"1",** causes dark areas within an image to be considered as potential objects (Table 2).

Setting	Detects
0	Objects that are brighter than the background (most typical)
1	Objects that are dimmer than the background

Table 2: *Binary settings for "**CometTypeCh1"** and "**SpotTypeCh2/Ch3"** Assay Parameters*

Typical fluorescence microscopy images show bright objects on a dark background. The signal that comes from an object is proportional to the amount of fluorescent label in it. Thus, the objects of interest will have intensities that are above the background intensity, and applying the object intensity threshold will identify pixels higher than the threshold as object and those lower than the threshold as background.

In some assays, dark objects are of interest, such as an unlabelled nucleus within a fluorescently labeled cell body or cytoplasm, or in images obtained with the Brightfield module on the ArrayScan VTI HCS Reader. In this case the dimmer areas of the image are considered objects of interest to be identified and measured.

Irrespective of whether you are analyzing bright objects on a dark background or dark objects on a bright background, the strategy for object identification/detection is similar. You will always optimize the threshold value so that it separates objects from background based on intensity.

Background Correction

The Assay Parameter that accomplishes correcting background from images before they are analyzed is "**BackgroundCorrectionCh*N***". Prior to image analysis, the non-cellular background can be computed and subtracted from the image separately in each channel, if desired, as shown in Figure 13. This parameter is available for channels 1-3 and can be set independently, depending on the background intensity in each channel. If the value is set to "**0**", for any channel then no background correction is applied to that channel and image analysis is done on raw uncorrected images.

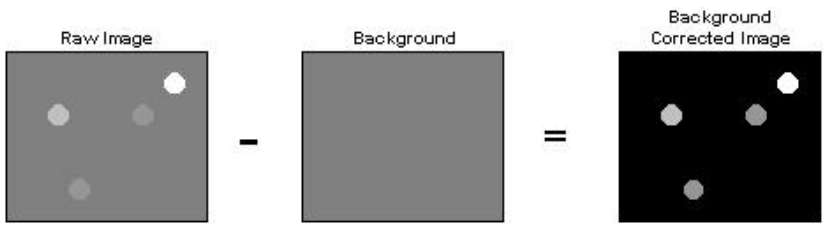


Figure 13: *Image depicting calculation of Background Correction that can be used for each channel*

You can control the creation of the background image by adjusting the **BackgroundCorrectionCh*N*** Assay Parameter. When the Assay Parameter has a positive value, the image is corrected by suppressing high frequency components of the background in the image (low pass filtration). The value refers to the radius of the area that is sampled around each pixel in the image to determine its local background. The value of this Assay Parameter should be larger than the radii of the objects in the image.

If the **BackgroundCorrectionCh*N*** Assay Parameter is given a negative value, a background correction method based on the 3D surface fitting is applied. The absolute value entered corresponds to the radius of an area used to find local minima across the image. These minimum values are used to construct a 3D surface of a background which is then subtracted from the original image. The main advantage of this method is that it minimizes the effect of the background correction (removal) procedure on the intensity values of the analyzed objects. In both cases, the larger the absolute value, the larger the sampled region, and the less subtraction is done. Table 3 gives an overview of the different Background Correction settings that can be used for each channel.

Setting	Background Correction Method
0	No background correction is performed
Positive	Low pass filter method
Negative	3D surface fitting method

Table 3: *Possible Background Correction Methods used in each channel with the Comet BioApplication*

NOTE

Background Correction can be adjusted on each channel separately. In all cases (except where the value = 0), the reported image pixel intensity values will be reduced. The background-corrected image is not stored or shown.

Object Smoothing

Object smoothing is used to blur or smooth the images before objects are identified. This is done sometimes to prevent a jagged object from being fragmented into multiple objects. The Assay Parameters that control comet body and head smoothing are **'CometSmoothFactorCh1', 'Head SmoothFactorCh1'**, and FISH spot smoothing are **'SpotSmoothFactorCh2/Ch3'**.

This parameter corresponds to the radius in pixels of the area used to smooth the image. A small value, such as **3**, means that a region with a radius of 3 pixels is used to smooth the image (region with dimensions 7×7 pixels, or 49 pixels total). Doubling the value to **6**, means that a larger region (13×13 pixels or 169 pixels total) is used to smooth the image, and thus the image will be more smoothed. A value of "**0**" means that smoothing is not done. To get sharper definition of the shapes of the edges of objects (i.e., comets, heads or FISH spots), you may want to keep the appropriate Assay Parameter small. For example, if your nuclear label is not very homogeneous (see Figure 14), the actual object will be erroneously identified as consisting of several smaller sized objects. Then smoothing will result in a homogenizing effect of the nuclear label and will help identify the object with its true bounds.

Smoothing reduces the sharpness of intensity variations by redistributing their relative brightness over the immediate vicinity in the image, connecting fragments of nuclei which can be valuable when measuring morphological changes. Figure 14 shows the effect of smoothing on accurate identification of objects in the nuclear channel (white overlay). Note that insufficient smoothing can result in unwanted object fragmentation (arrows in Panel B) while excessive smoothing can result in poor definition of the object perimeter (D).

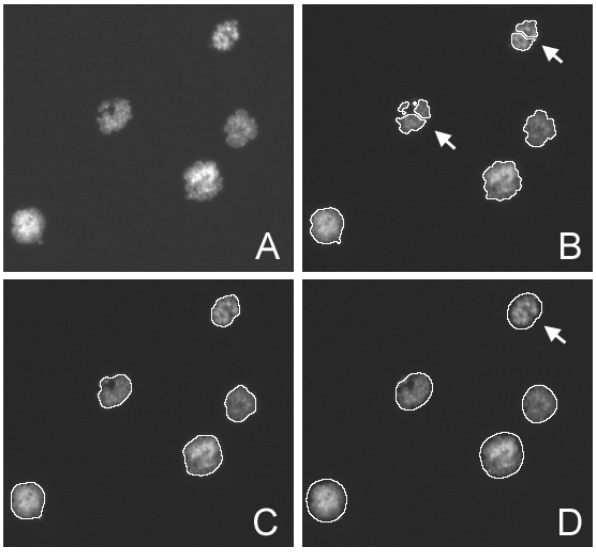


Figure 14: SmoothFactorCh1: *Images show the effect of different degrees of smoothing on the same field of apoptotic cell nuclei.* ***A:*** *Raw Image;* ***B:*** *SmoothFactorCh1 = 0;* ***C:*** *SmoothFactorCh1 = 3;* ***D:*** *SmoothFactorCh1 = 8.*

Spot Detection

The Assay Parameter that controls the detection of spots and spot like objects are **"SpotDetectionCh2/Ch3"**. This parameter is available in channels 2 & 3 only. In the context of the Comet BioApplication, spots are usually entities found within the nucleus/DNA, whose identification is based on their intensity as well as their morphology. Thus spot identification requires specifying the appropriate intensity threshold and morphological ranges and is a two-step process. First, the identification of cellular constituents, which may be potential spots are done. Then the specific differentiation of spots from noise is done and the size range of the spots is specified to establish the valid spots, versus intracellular noise. This parameter can have negative, positive, or "0" values. If the parameter is set to "0", then spot detection is turned off. A positive or negative value determines which method of spot detection is applied to the image to identify the spots. See Table 4 for a brief description of the values and the method used to identify spots.

Setting	Detection Method
0	Spot detection is turned off
Positive	Use morphological method to detect spots
Negative	Use local peak method to detect spots

Table 4: Spot detection methods used by the "**SpotDetectionCh2/Ch3***" Assay Parameters* in the Comet BioApplication

To identify which of the retained pixels with high intensity belong to spots, the change over space within the object is evaluated. First, any remaining background intensity after thresholding is automatically removed and only discrete cellular constituents whose intensity varies rapidly over space remains, as these may be potential spots. When removing slowly varying background intensity for spot identification, the spatial dimension governing which intensity changes are considered slowly varying must be defined. This is set by the Assay Parameter **"SpotDetectionCh2/Ch3"** which is related to the typical radius of typical spots. This Assay Parameter is used as a spatial filter to control the removal of local varying background fluorescence in the object so that individual spots can be identified; the size of this parameter is similar to the size of the spots in pixels that are detected.

For spots, the size of the spot or the particular cellular entity governs the value to be set for the Assay Parameter. For small organelles such as endosomes, a small value would suffice, whereas for larger organelles such as nuclei, the value of this parameter may have to be increased.

Object Segmentation

The Comet BioApplication can resolve individual comets, their heads, and FISH spots successfully. However resolution of comets, heads, or FISH spots into individual entities may not be successful if the comets are lying too close to each other or have multiple heads or if the FISH spots are too close to each other. The Assay Parameters **"CometSegmentationCh1", "HeadSegmentationCh1"** and **"SpotSegmentatioCh2/Ch3"** are useful in resolving these objects that are too close to each other that may not be resolved by other means. The functioning of these parameters are described later in this section.

The segmentation Assay Parameters, can have a positive, negative, or **"0"** value (see table 5 below). If parameter is set to **"0"**, then segmentation is turned off in that channel, for that feature (see below for exception). If the parameter has a positive value, the geometric shape method is used to segment objects, while if the value is negative, then a peak intensity method is used. When a positive value is used, the value should be the approximate radius (in pixels) of the smaller of the two objects being separated. When a negative value is used, the value should be close to the intensity range of the objects that are to be segmented. These methods are illustrated in Figures 15, 16, 17, and 18.

Positive values for these Assay Parameters will select the geometric method, and the value is the approximate radius (in pixels) of the smaller of the objects being separated. The value of this Assay Parameter for the geometric method depends on the magnifying power of the microscope objective and camera setting (pixel binning). This method splits touching objects on the basis of shape, relying on boundary indentations to locate the line of separation. This method works best if the individual objects have smooth boundaries and pronounced indentations at their point of contact. Since the geometric method works on the basis of shape, it can be used to separate objects that are uniform in intensity (i.e., saturated) or that have multiple intensity peaks (i.e., noisy or textured). See Figures 15 and 16 for a description of the working of the geometric method.

A negative value selects the peak intensity method. In this case, the absolute value of the Assay Parameter specifies the minimum relative height of the intensity peak (image contrast) to be used in the segmentation. Making the Assay Parameter more negative reduces the number of objects created by the segmentation. The value of this Assay Parameter for the intensity method does not depend on the power of the microscope objective or camera setting (pixel binning). The intensity method separates touching objects using intensity peaks. Successful segmentation requires a single, dominant intensity peak for each object. This method will not work well if the objects are noisy, textured, or uniform in intensity, in which case they may require object smoothing to reduce over segmentation. The peak intensity method can be used to separate objects that have no boundary indentations. See Figure 15 and 17 for a description of the working of the peak intensity method.

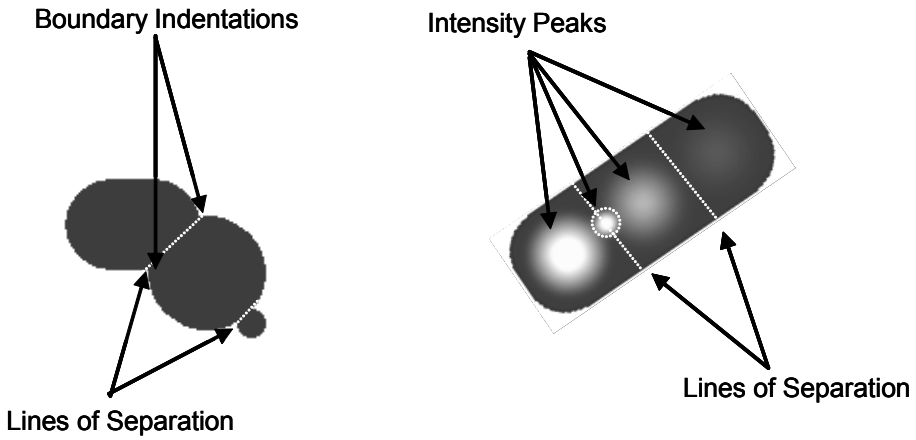


Figure 15: *Positive (geometry method, schematic on left) or Negative (intensity method, schematic on right) values for the object segmentation Assay Parameter*

Parameter Value	Segmentation Method	Value Range
0	0	0
Positive	Geometric method	1 to 255
Negative	Intensity method	-1 to -32767

Table 5: *Options and methods used for object segmentation Assay Parameters*

Geometric Method

Setting the Assay Parameter value equal to the first radius (R1, Figure 16A) produces object separation shown in Figure 16B and setting the Assay Parameter value = R3 results in segmentation shown in Figure 16C. Setting the Assay Parameter value = R2 (the largest radius of all touching objects) produces no segmentation of the smallest object from the larger object.

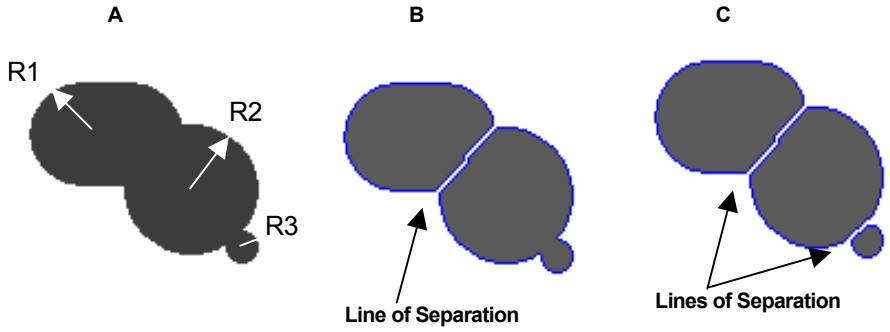


Figure 16: *Current Method for object segmentation using the Geometric Method. A) Radii of touching objects: R1 = 24, R2 = 30, R3 = 7; B) Result of segmentation using* ***"ObjectSegmentationCh**N* **= R1"****; C) Result of segmentation using* ***"ObjectSegmentationCh**N* **= R3"****.*

Intensity Method

Figure 17 shows the intensity profile along the cordial line of an object with four intensity peaks from Figure 15. Relative peak intensity heights measured from the top of a peak to the nearest valley for each of the object are: 1500 (#1), 1250 (#2), 500 (#3) and 200 (#4).

In general terms, these peak intensity heights are nothing but measures of local contrast of an image. The degree of object segmentation can be controlled by setting the Assay Parameter value equal to the lowest relative height of intensity peaks of objects that need to be separated. The lower the value the more objects will be segmented and vice versa. In case of over segmentation of objects, smoothing the objects by setting the Assay Parameter for object smoothing > 0 should alleviate the problem.

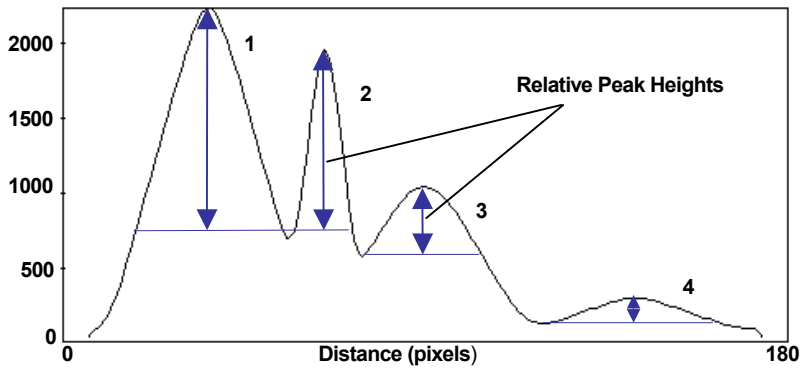


Figure 17: *Intensity profile of an object with four intensity peaks. Peak #1 relative height = 1500, peak #2 relative height = 1250, peak #3 relative height = 500, and peak #4 relative height = 200.*

Only two peaks in Figure 17: #1 and #2 have relative intensity > 1000. Thus, setting the Assay Parameter to the value = -1000 makes only two peaks to be used for segmentation (or marks the two objects that need to be separated) and results in object segmentation as shown in Figure 18A; setting the Assay Parameter to the value = - 400 or -100 marks three or all four objects that need to be separated. The results of segmentation of three and four objects are shown in Figures 18B and 18C.

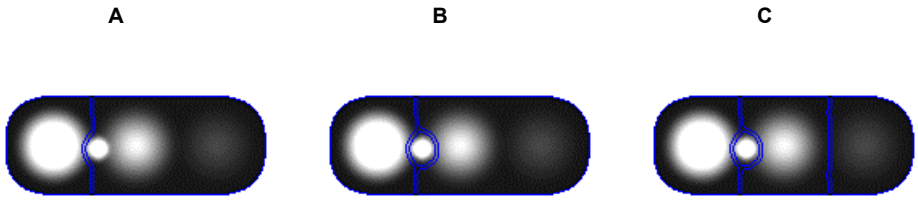


Figure 18: *Current Method for object segmentation using the Intensity Method. A) Result of object segmentation when "***ObjectSegmentationCh*N*** = -1000; B) when "***ObjectSegmentationCh*N*** = -400; C) when "***ObjectSegmentationCh*N*** = -100.*

Reject Border Objects

In the Comet BioApplication, you have the option of not including and analyzing comets that touch the border of the imaged field. This is controlled by the "**RejectBorderCometsCh1**" Assay Parameter. If this parameter has a value of "**1**", objects touching the border are not

analyzed. A value of "**0"** results in all objects in the image field to be eligible for analysis, whether or not they touch the image border.

Basic Assay Parameters

Input Assay Parameters can be found in the Create Protocol View. They can also be accessed from the Protocol Interactive View by clicking on the "Edit Assay Parameters" button. The number of available input parameters will vary depending on the number of Channels chosen for the Assay Protocol and also the mode in which you are using the BioApplication: Basic Mode or Advanced Mode (see the ArrayScan User's Guide for instructions on switching between Basic and Advanced Mode). In the Basic Mode, all of the Advanced Assay Parameters are hidden and none of the Assay Parameters can be altered, irrespective of whether the protocol is write protected or not. In the Advanced Mode, all of the advanced Assay Parameters are hidden by default. But they can be revealed, by unchecking the "Hide Advanced Parameters" box (see the ArrayScan User's Guide for a description). In the Advanced Mode, all of the parameter settings can be adjusted, if the Assay Protocol is not write protected. Table 6 below lists the Basic Assay Parameters for the Comet BioApplication.

Assay Parameter	Units	Value	Description
UseReferenceWells	Binary	0 or 1	Use Reference Wells to automatically set "LevelHigh" and "LevelLow" parameters 0= No; 1 = Yes
CometMigrationDirection	--	0 to 3	Determines the orientation of the heat and tail of comets in the Protocol Interactive View and Scan View. 0= Top-Bottom (head-tail); 1= Bottom-Top; 2= Left-Right; 3= Right-Left
Comet DataAnalysis	Binary	0 or 1	Determines if all selected comets or only comets designated as normal comets are used in calculation of well features. 0= normal comets only; 1= normal & abnormal Comets
CometType Ch1	Binary	0 or 1	Determines if the comets are bright on dark background or dark on bright background. 0= bright on dark background; 1= dark on bright background
BackgroundCorrectionChN	Pixels	-255 to 255	Radius in pixels used to compute background for background correction in Channels 1-6. 0= no background correction Positive= use low pass filter method Negative= use surface fitting
CometSmoothFactorCh1	Pixels	0 to 255	Degree of image smoothing prior to comet body identification in Channel 1. 0 = do not apply smoothing
CometSegmentationCh1	Pixels	-32,767 to 255	Radius in pixels or intensity range that must be used to segment comet body in Channel 1. 0= do not segment Positive= use shape geometry method Negative= use intensity method

Assay Parameter	Units	Value	Description
RejectBorderCometsCh1	Binary	0 or 1	Includes or rejects comets in Channel 1 that touch image field border from analysis 0= include comets that touch field border 1= reject comets that touch field border
HeadRegionFractionCh1	--	0 to 1	Determines the fractional position along the comet length where the comet is split into head and tail regions. Smaller values mean the split will be closer to the top (or origin) of the comet. A negative sign before the value will display the position of the line of demarcation between the head and tail along with the comet mask.
HeadSmoothFactorCh1	Pixels	0 to 127	Degree of image smoothing prior to comet head identification in Channel 1. 0 = do not apply smoothing
HeadDetectionCh1	Pixels	-32,767 to 255	Radius in pixels or intensity range that must be used to detect the head of the comet. If the head is clearly distinguishable from background then this Assay Parameter can be set to 0, and heads are still identified successfully. 0= do not apply head detection method Positive= use morphological method Negative= use intensity method
HeadIdentificationModfierCh1	--	-1 to 10	Fractional adjustment made to intensity threshold to identify heads in comets. Negative and lower values are used to identify heads that have intensity values closer to rest of the comet body, while higher positive values are used to identify heads that are brighter than the comet body.
HeadSegmentationCh1	Pixels	-32,767 to 255	Radius in pixels or intensity range that must be used to segment heads that are touching each other in Channel 1. 0= do not segment Positive= use shape geometry method Negative= use intensity method
SpotTypeCh2/Ch3	Binary	0 or 1	Determines if the FISH spots in Channel 2 and Channel 3 are bright on dark background or dark on bright background. 0= bright on dark background; 1= dark on bright background
SpotSmoothFactorCh2/Ch3	Pixels	0 to 255	Degree of image smoothing prior to FISH spot identification in Channel 2 or Channel 3. 0 = do not apply smoothing
SpotDetectionCh2/Ch3	Pixels	-32,767 to 127	Radius in pixels or intensity range that must be used to detect the FISH spots. 0= do not apply head detection method Positive= use morphological method Negative= use intensity method

Assay Parameter	Units	Value	Description
SpotSegmentationCh2/Ch3	Pixels	-32,767 to 255	Radius in pixels or intensity range that must be used to segment FISH spots that are touching each other in Channel 2 and Channel 3. 0= do not segment Positive= use shape geometry method Negative= use intensity method
MaskModifierChN	Pixels	-127 to 127	Number of pixels by which the comet mask from Channel 1 is modified in Channels 2-6. Used for gating purposes only. 0= do not modify comet mask from Channel 1 Positive= Expand comet mask by number of pixels Negative= Shrink comet mask by number of pixels

Table 6: *Basic Assay Parameters available for the Comet BioApplication. The Assay Parameters that are shaded in grey are applicable for running Comet-FISH analysis with FISH spots visualized in Channel 2 and/or Channel 3.*

Object Identification Parameters

To identify objects in each of the images from the different channels, an independent intensity threshold must be set for each channel. Object Identification methods are available for Channels 1-3. Channels 4, 5, and 6, which are gating channels do not have any object identification methods. If you create an Assay Protocol with 4-6 channels, then you must choose the method "**None**" as Object identification method for Channels 4, 5, and 6. In the Comet BioApplication, primary objects are identified in Channel 1 for further measurements; only pixels with intensities above this threshold will be considered as belonging to these objects. The proper setting of an intensity threshold is a key early step in identification and configuring the application. In Channels 2 and 3 for comet-FISH analysis, only objects whose intensities are above the set threshold are identified and used for analysis. Thus, it is important to set an appropriate threshold for object identification in each of Channels 1-3. There are three different thresholding options in each of Channels 1-3. For each Channel, you must select both a method and a value. The different options and values available for each channel are summarized in Table 7, and the descriptions of the different methods follow in Table 8.

Intensity Threshold Method	Value Range	Channel Availability				
		Channel 1	Channel 2	Channel 3	Channel 4	Channel 5,6
None	0				✓	✓
Isodata	-0.99 – 9.999	✓	✓	✓		
Triangle	-0.99-9.999	✓	✓	✓		
Fixed	0 - 32767	✓	✓	✓		

Table 7: *Intensity threshold methods available for each Channel in the Comet BioApplication*

NOTE

All Intensity Thresholds are applied to the background-corrected image (when Background Correction is used).

The effective range for object identification is limited to 0-4095 for the following Cellomics HCS Readers: ArrayScan VTI, ArrayScan X.5, and KineticScan® HCS Readers. However, the entire range may be available on images from other sources.

The option "**None"** means that no intensity threshold is applied to images in that channel. This option is available only in Channels 4-6 as these are only gating channels.

The **Fixed Threshold** method sets an intensity threshold independently of the image data. In this case, you select an intensity level between 0 and 32767, and any pixel with an intensity that is equal to or above this intensity is retained for the analysis specific to the channel.

The other two threshold methods (**Isodata**, and **Triangle**) are known as histogram-derived thresholds in that the threshold is chosen from the histogram of pixel intensities in the image (i.e., the image's brightness histogram). The schematics in Figure 19 demonstrate how these histogram-derived threshold values are derived; and their descriptions are in Table 8.

The values entered in the application for the three histogram-derived threshold methods are offsets applied to determine the final threshold which is applied to the image. If the histogram-derived threshold is T, then its relationship to the actual threshold, T_F, which is finally applied to the image is determined from the user-entered offset, o, as:

$$T_F = (1 + o)T$$

For example, suppose for a particular image, an **Isodata Threshold**, *T,* of 1000 is obtained. Then entering an offset, *o,* of 0.9 will result in a final threshold of 1900 being applied to the image, whereas entering an offset -0.9 will result in a final threshold of 100 being applied. The range of possible values for the offset *o* is –0.999 to 9.999. However, note that the resulting applied threshold, T_F, will be restricted to be between the range 1-32767.

The two histogram-derived methods are dependent on the contents of the image, unlike the **Fixed Threshold** method. For example, for a blank image that contains no cellular objects and only has background pixels with a mean intensity value of 500 and standard deviation of 50, it is unlikely that a **Fixed Threshold** of 1000 will cause any pixels to be registered. However, the histogram-derived threshold methods will give thresholds causing pixels in the image to be registered for potential analysis; this is because the histogram is of the pixel intensity distribution of that image, even though there are no cellular objects in the image. Thus, in situations where blank images are expected (e.g., from a loss of signal due to a compound condition, a loss of protein expression, or a lack of label), a histogram-derived threshold method should be avoided; rather a **Fixed Threshold** with a large offset value should be entered.

Threshold Option	Description	Range of Possible Values Entered	Resulting Applied Threshold Range
None	No threshold applied	0	none
Isodata	Adjusts the object identification threshold relative to the Isodata value. The threshold T is chosen so that it is equal to the average of the mean of the pixel intensities to the left of the threshold (m_L) and the mean of the pixel intensities to the right of the threshold (m_R). A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 to 9.999 (offset)	1 to –32,767
Triangle	Adjusts the object identification threshold relative to the Triangle value. The threshold T which gives the maximum distance d A negative value identifies dimmer objects and results in larger object masks. A positive value results in smaller object masks.	-0.999 to 9.999 (offset)	1 to 32,767
Fixed	A fixed pixel intensity value between 0-32767 is applied	0 to 32,767 (true intensity)	0 to 32,767

Table 8: *Intensity threshold descriptions*

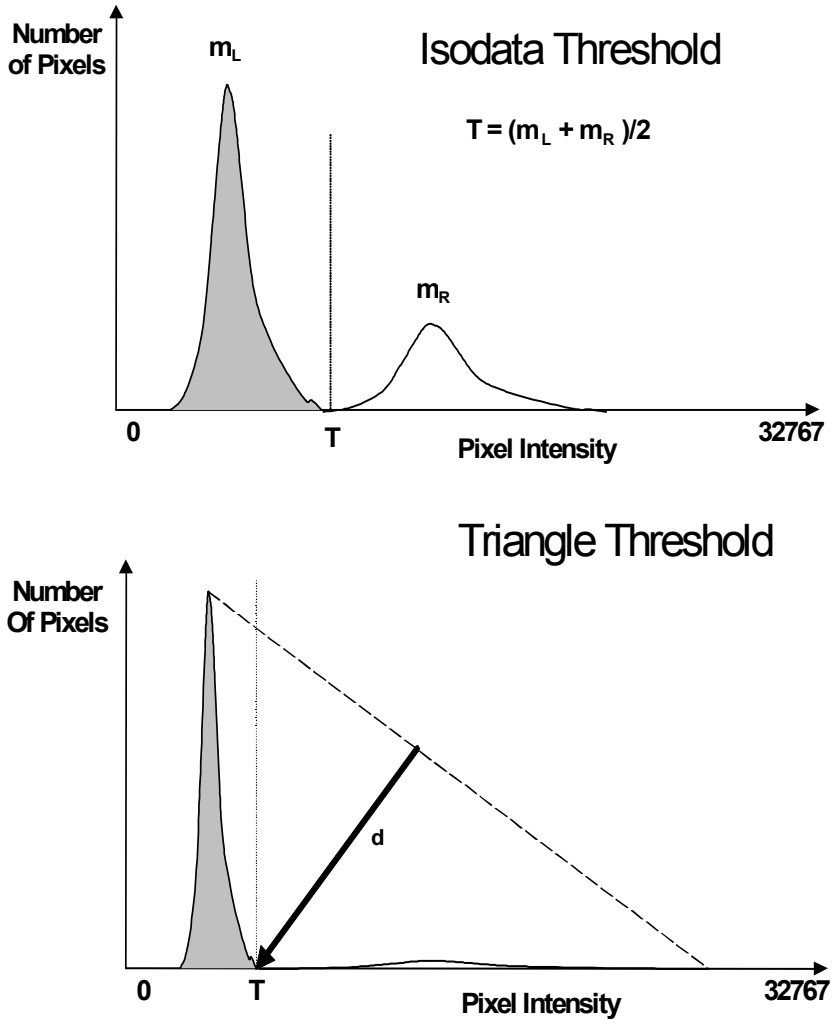


***Figure 19**: Histogram-derived intensity threshold methods. Shown are Isodata (top) and Triangle (bottom) threshold methods. Background peak is shown in gray and object peak is shown in white.*

Object Selection Parameters

Each channel has an associated set of Object Selection Parameters. If an object has all measured features within the ranges specified by the Object Selection Parameters, it is selected for analysis. Rejected objects are removed from further analysis. Object Selection Parameters for all channels are adjustable only in Advanced modes. Table 9 lists all available Object Selection Parameters for this BioApplication.

Parameter	Units	Description
Channel 1 Object Selection Parameters		
CometAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of comets identified in Channel 1
CometShapeP2ACh1	--	Shape measure based on ratio of perimeter squared to 4π*area of comets identified in Channel 1. (CometShapeP2ACh1=1 for circular objects).
CometShapeLWRCh1	--	Shape measure based on ratio of length to width for comet-aligned bounding box of a comet identified in Channel 1
CometAvgIntenCh1	Intensity	Average intensity of all pixels within comets identified in Channel 1
CometVarIntenCh1	Intensity	Standard deviation in intensity of all pixels within comets identified in Channel 1
CometTotalIntenCh1	Intensity	Total intensity of all pixels within a comet identified in Channel 1
HeadAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of comet head identified in Channel 1
HeadShapeP2ACh1	--	Shape measure based on ratio of perimeter squared to 4π*area of comet heads identified in Channel 1. (HeadShapeP2ACh1=1 for circular objects).
HeadShapeLWRCh1	--	Shape measure based on ratio of length to width for comet head aligned bounding box of a comet identified in Channel 1
HeadAvgIntenCh1	Intensity	Average intensity of all pixels within comet heads identified in Channel 1
HeadVarIntenCh1	Intensity	Standard deviation in intensity of all pixels within comet heads identified in Channel 1
HeadTotalIntenCh1	Intensity	Total intensity of all pixels within a comet heads identified in Channel 1
Channel 2 and Channel 3 Object Selection Parameters		
SpotAreaCh2/Ch3	Pixels or μm^2	Area (in pixels or micrometers) of FISH spots identified in Channel 2 or Channel 3
SpotShapeP2ACh2/Ch3	--	Shape measure based on ratio of perimeter squared to 4π*area of FISH spots identified in Channel 2 or Channel 3
SpotShapeLWRCh2/Ch3	--	Shape measure based on ratio of length to width for FISH spot-aligned bounding box of a spot identified in Channel 2 or Channel 3
SpotAvgIntenChCh2/Ch3	Intensity	Average intensity of all pixels within FISH spots identified in Channel 2 or Channel 3
SpotVarIntenCh2/Ch3	Intensity	Standard deviation in intensity of all pixels within FISH spots identified in Channel 2 or Channel 3

Parameter	Units	Description
SpotTotalIntenCh2/Ch3	Intensity	Total intensity of all pixels within a FISH spot identified in Channel 2 or Channel 3
Object Selection Parameters for Channels 2-6		
AvgIntenCh*N*[*]	Intensity	Average intensity of all pixels within modified Channel 1 object mask in Channel *N*
TotalIntenCh*N*[*]	Intensity	Total intensity of all pixels within modified Channel 1 object mask in Channel *N*

Table 9: *Object Selection Parameters available for the Comet BioApplication.* [*] *The object selection parameters "*AvgIntenCh*N" and "* TotalIntenCh*N" are available only for Channels 2-6.*

Gating

The Comet BioApplication, like other Cellomics BioApplications, supports gating on a cell population. This feature provides selective cell processing based on fluorescence intensity. Therefore, in addition to selecting objects for analysis in Channel 1, you can also select or reject objects based on fluorescence intensity in Channels 2-6. Gating allows you to specifically identify a subset based on fluorescence intensity and allows you to limit the analysis to this group of cells. For example, gating may be used to analyze only those cells showing a certain level of expression of a fluorescent reporter such as green fluorescent protein (GFP).

Specifying Mask Modifiers for Gating

The Assay Parameter **"MaskModifierCh*N*"** is used to adjust the size of a primary object mask when it is applied to make measurements in channels 2-6. Changing this Assay Parameter only impacts the region used to determine whether the cell should be gated in or out, as measured by **TotalIntenCh*N*** or **AvgIntenCh*N***. The mask can be dilated or eroded, but will not overlap with other masks from adjacent objects.

Specifying Intensity Ranges for Gating

Intensity ranges for the gating parameters for channels 2-6 can be set in the Create Protocol View by entering upper and lower limits for two intensity Object Selection Parameters, AvgIntenCh*N* and TotalIntenCh*N*. These parameters can also be adjusted interactively in the Protocol Interactive View.

Image Overlays

During a scan, various features can be displayed as color overlays on the channel images. The colors of these overlays can be changed by choosing the color block beside the overlay name in the Create Protocol or Protocol Interactive View (Table 10). All Image Display Option Settings are available when running in Basic Mode or Advanced Mode.

Parameter	Description
Include This Channel In Composite	Determines if the image for this channel is included in the composite image.
NormalComet	Indicates comets that are selected as "Normal" comets by placing a colored ring around the object. Only selected "Normal" comets are included in computation of well averages if the Assay Parameter **"CometDataAnalysis = 0"**. ***Cell level features are computed for all selected comets, whether normal or abnormal.***
AbnormalComet	Indicates comets that are selected as "Abnormal" comets by placing a colored ring around the object. "Abnormal" comets are included in computation of well averages if the Assay Parameter **"CometDataAnalysis = 1"**. ***Cell level features are computed for all selected comets, whether normal or abnormal.***
RejectedComet	Indicates rejected comets by placing a colored ring around such comets.
ValidHead	Indicates a valid comet head by placing a colored ring around heads that are designated as valid.
RejectedHead	Indicates a rejected comet head by placing a colored ring around heads that are designated as rejected.
TailDistance	Indicates the distance between the intensity based center of mass of the comet head to the intensity based center of mass of the tail.
SelectedSpotCh2/Ch3	Indicates FISH spots from Channel 2 or Channel 3 that are selected for analysis.

Table 10: *Image Display Option Settings available when running the Comet BioApplication*

Assay Parameters for Population Characterization

Overview of Population Characterization

The Comet BioApplication provides the ability to characterize individual cells (comets) based on their response compared to a control population. For a particular output feature, a range is determined from a control population (e.g., cells in untreated wells) that has the normal physiological distribution for that feature. The ***Feature*Ch*N*LevelHigh** and ***Feature*Ch*N*LevelLow** Assay Parameters set the upper and lower bounds of this range respectively. The "**Status**" Cell-level feature indicates whether a particular compound treated cell is within or beyond this range (i.e., for a particular Cell-level feature, a cell with Status **0** means that it is within the defined range, a Status value of **1** means that the feature value is greater than the upper limit, and a Status value of **2** means that the feature value is less than the lower limit; Table 11).

Value	Cell Status Definition
0	Cell Feature is less than or equal to defined Level
1	Cell Feature is greater than defined Level
2	Cell Feature is less than defined Level

Table 11: *Numerical Status values for specified Cell Features*

The corresponding Well-level features are the percent of cells that are either greater or less than the limits defining this range. Figure 20 illustrates this concept by showing the distribution of a normal physiological population of cells for a particular Cell-level feature, and the shift in this distribution upon compound treatment (please note that the shift in feature distribution can occur to either above or below the normal physiological distribution).

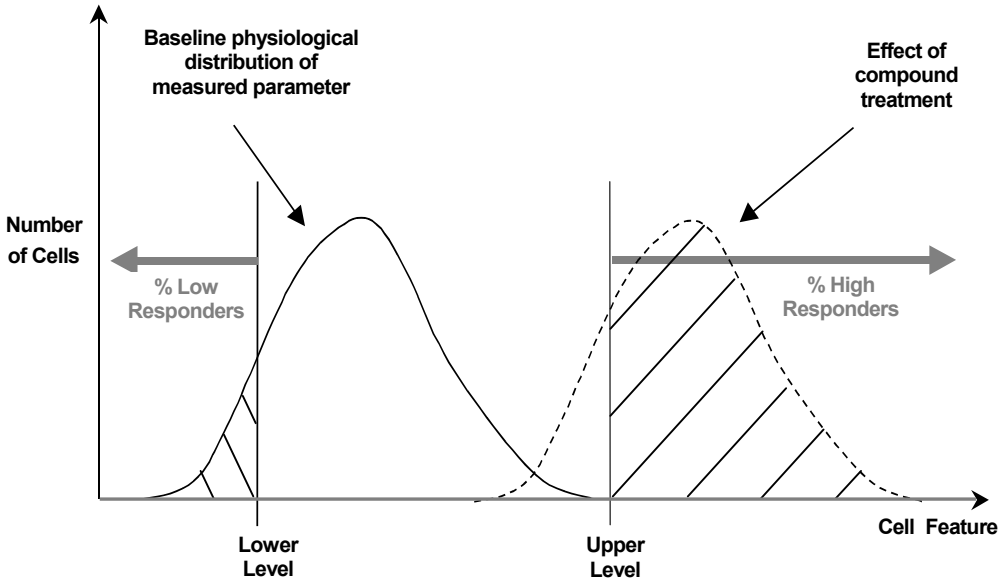


Figure 20: *Schematic of population distribution of a measured feature in a population of untreated and compound treated cells. The upper and lower levels correspond to %Low or %High (or %Responders if combined).*

Setting Cellular Response Levels

The Comet BioApplication offers two ways of setting high and low-response level Assay Parameters. The first is manually entering values for the ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** Assay Parameters in the Protocol Create or Protocol Interactive Views. This requires prior knowledge of typical feature values. The BioApplication then calculates the percentage of cells in each well that are above and below the specified ***Feature*Ch*N*LevelHigh, *Feature*Ch*N*LevelLow** values, respectively.

To automatically determine the ***Feature*Ch*N*LevelHigh**, ***Feature*Ch*N*LevelLow** Assay Parameter values, the BioApplication uses Reference Wells. You designate particular wells on a sample slide or plate as Reference Wells. Reference Wells typically contain an untreated cell population that displays the normal physiological distribution for the feature being measured. These wells are first analyzed and the population distribution for each Cell Feature that can be used for population characterization are determined. The cell population characterization levels

are then set by adding or subtracting from the mean of the distribution its standard deviation multiplied by a user defined coefficient (Correction Coefficient (**_CC**)) found as an Advanced Assay Parameter. The slide or plate is then scanned and the BioApplication calculates percentage of cells that are above and below these levels for each well.

For example, if you want to know the percentage of cells that, with compound treatment, have a response beyond the 95th percentile of the response from a control untreated population of cells, then the coefficient by which to multiply the standard deviation would be two, assuming a normal distribution. The advantage of using Reference Wells to automatically calculate levels is that the levels are determined by a control population of cells and are independent of run-to-run variations when doing the experiment on different days such as different illumination conditions, extent of fluorescent labeling, etc.

Reference Wells Processing Sequence

By setting the **UseReferenceWells** Assay Parameter to **1**, the Reference Wells processing is engaged. Specified fields within the Reference Wells are acquired/analyzed, and Well and Plate Features are computed, following which regular scanning of the slide or plate begins. Plate Feature values are directly used for cell population characterization. Again, if the feature value for **MinRefAvgCometCountPerField** obtained from the Reference Wells is below the value set for that parameter, the BioApplication aborts the use of Reference Wells and processes the plate as if **UseReferenceWells** is set to **0**. The sequence of computation for Reference Wells is as follows:

- Cell Features are computed for every valid comet within a field.
- For each Cell Feature to be used for population characterization, the mean and standard deviation are computed over all comets in the field.
- The automatically calculated level values for each feature are reported as Reference Features having the names **Ref*Feature*Ch*N*LevelHigh, Ref*Feature*Ch*N*LevelLow**.
- Finally, Reference Well and Plate Features are computed as average values for fields in a well, weighted for the number of cells per field, and then as arithmetic average for wells on a plate. Use of a weighted average minimizes the effect of sparse fields.

Identifying Reference Wells and Control Parameters

Reference Wells are specified in the Reference Wells Configuration Window (choose **Change Reference Wells** from the **Tools** menu). Select the wells to be set as Reference Wells and then select the button for the appropriate type of Reference Well (**Known**). Next, enter the Starting Field and Number of Fields. Click the **Apply** button to save the settings. Please refer to your ArrayScan User's Guide for details.

NOTE

The Reference Well settings are not saved as part of the Assay Protocol, rather they are saved in the Scan software application.

TIP: To document the Reference Wells used in a scan, enter them in the Scan Comments box in the Scan Plate View.

Reference Wells are not available for the KineticScan Reader; please use the manual level settings to define subpopulations.

Specifying and Controlling Reference Wells

Two general Assay Parameters controlling the use of Reference Wells are: **UseReferenceWells** and **MinRefAvgCometCountPerField**). **UseReferenceWells** is a binary Assay Parameter that allows you to indicate whether Reference Wells are used to determine the levels necessary to characterize populations.

- **UseReferenceWells = 0** (zero) specifies that Reference Wells are not to be used, resulting in the use of manually entered levels for population characterization.
- **UseReferenceWells = 1** specifies that Reference Wells are to be used.

If Reference Wells are enabled, the Assay Parameter **MinRefAvgCometCountPerField** is used to specify the minimum average number of selected cells per field that you consider acceptable in the Reference Wells. If the measured cell density is less than or equal to this value, the Reference Features will not be used, rather the manually entered levels for population characterization are used.

The Cell Features used to characterize various levels are associated with Assay Parameters in the form:

- FeatureChNLevelHigh
- FeatureChNLevelLow (when Reference Wells are not used)

  -or-

- FeatureChNLevelHigh_CC
- FeatureChNLevelLow_CC (when Reference Wells are used)

where, *Feature* refers to the name of the Cell Feature (such as **CircAvgIntenCh2**) and *N* refers to the specific channel. The ***Feature*ChN LevelHigh, *Feature*ChN LevelLow** Assay Parameter types specify the actual levels and must be manually entered if Reference Wells are not used. The ***Feature*ChN LevelHigh_CC, *Feature*ChN LevelLow_CC** Assay Parameters are correction coefficients (CC) used to derive the ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** values from the mean and standard deviation of the reference well population according to the formula:

- FeatureChNLevelHigh = Mean + FeatureChNLevelHigh_CC $\times$ SD
- FeatureChNLevelLow = Mean - FeatureChNLevelLow_CC $\times$ SD

If the correction coefficient is positive, the level will be greater than the mean, and if it is negative, the level will be less than the mean. A _CC value of "**0"**generates a level that equals the mean. If you are using Reference Wells, it is important to set the values for the correction coefficient Assay Parameters so that the appropriate subpopulations can be identified. Individual cells having feature values above the set or calculated value are identified as responder cells. The number of responder cells, expressed as a percentage of the entire cell population analyzed, is calculated for each assay well.

Advanced Assay Parameters

In Advanced Mode, all basic and advanced input parameters are adjustable, provided the Assay Protocol is not write protected. The **Hide Advanced Parameters** option provides the ability to either view and adjust all the Assay Parameters or view and adjust the subset designated as Basic Parameters. Clear the **Hide Advanced Parameters** checkbox to display all Assay Parameters. Generally, several of the Advanced Assay Parameters are related to definition and reporting of responder cells. They control the analysis of the data resulting from the image analysis (Table 12). In addition there are three Assay Parameters that are related to the Events definition (see next chapter for detailed description of how to set Events, using the Events Wizard).

For each feature reporting population characteristics, there are four advanced Assay Parameters that control its levels: ***Feature*ChN LevelHigh** and ***Feature*ChN LevelLow** that set upper and lower thresholds and the presence of the **_CC** suffix for each which designate that those levels are set using Reference Wells. For example, the Assay Parameters controlling the TailOliveMoment are:

- **TailOliveMomentLevelHigh**
- **TailOliveMomentLevelLow**
- **TailOliveMomentLevelHigh_CC**
- **TailOliveMomentLevelLow_CC**

In the listing of Advanced Parameters in Table 12, rather than listing all level Assay Parameters for each feature, one entry for the feature will be listed giving the four different options, as shown in the following example for the above four features:

- TailOliveMomentLevel ***Low/High, Low/High_CC***

Parameter	Description
MinRefAvgCometCountPerField	Minimum average number of objects per field required for acceptance of reference well results
UseMicrometers	Measure lengths and areas in 0 = Pixels and 1 = Micrometers
Pixel Size	Pixel size in micrometers (depends on objective selection and is not user adjustable)
Type_1_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
Type_2_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
Type_3_EventDefinition	User-defined combination of logic statements involving response feature. ***Can be changed only by using Events Wizard***
CometAreaCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometAreaCh1***

Parameter	Description
CometLengthCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometLengthCh1***
CometWidthCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometWidthCh1***
CometP2ACh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometP2ACh1***
CometTotalIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometTotalIntenCh1***
CometAvgIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometAvgIntenCh1***
CometVarIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometVarIntenCh1***
HeadAreaCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadAreaCh1***
HeadLengthCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadLengthCh1***
HeadWidthCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadWidthCh1***
HeadP2ACh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadP2ACh1***
HeadLWRCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadLWRCh1***
HeadTotalIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadTotalIntenCh1***
HeadAvgIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadAvgIntenCh1***
HeadVarIntenCh1Level ***Low/High, Low/High_CC*** h	Defines population characterization levels for the feature ***HeadVarIntenCh1***
TailAreaCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailAreaCh1***
TailExtentCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailExtentCh1***
TailDistanceCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailDistanceCh1***
TailDistanceExtentRatioCh1Leve ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailDistanceExtentRatioCh1***
TailTotalIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailTotalIntenCh1***
TailAvgIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailAvgIntenCh1***
TailVarIntenCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailVarIntenCh1***
Tail%TotalIntenCh1 ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***Tail%TotalIntenCh1***

Parameter	Description
TailExtentMomentCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailExtentMomentCh1***
TailOliveMomentCh1Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailOliveMomentCh1***
CometSpotCountCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometSpotCountCh2/Ch3***
CometSpotTotalAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometSpotTotalAreaCh2/Ch3***
CometSpotAvgAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometSpotAvgAreaCh2/Ch3***
CometSpotTotalIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometSpotTotalIntenCh2/Ch3***
CometSpotAvgIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***CometSpotAvgIntenCh2/Ch3***
HeadSpotCountCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadSpotCountCh2/Ch3***
Head%SpotsCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***Head%SpotsCh2/Ch3***
HeadSpotTotalAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadSpotTotalAreaCh2/Ch3***
HeadSpotAvgAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadSpotAvgAreaCh2/Ch3***
HeadSpotTotalIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadSpotTotalIntenCh2/Ch3***
HeadSpotAvgIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***HeadSpotAvgIntenCh2/Ch3***
TailSpotCountCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailSpotCountCh2/Ch3***
Tail%SpotsCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***Tail%SpotsCh2/Ch3***
TailSpotTotalAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailSpotTotalAreaCh2/Ch3***
TailSpotAvgAreaCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailSpotAvgAreaCh2/Ch3***
TailSpotTotalIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailSpotTotalIntenCh2/Ch3***
TailSpotAvgIntenCh2/Ch3 Level ***Low/High, Low/High_CC***	Defines population characterization levels for the feature ***TailSpotAvgIntenCh2/Ch3***

Table 12: *Advanced Assay Parameters available for the Comet BioApplication*

Description of Output Features

Output features are the biological measurements produced by the BioApplication. All features are categorized and accessible using the vHCS:View software application. Additionally, a subset of features, the Well Features, is listed in the Scan Plate View and Create Protocol View so that screening results can be viewed concurrently with scanning. The ability to view data during the scanning process allows you to immediately verify success of the run, well by well. All features can also be accessed in the Protocol Interactive View once the algorithm has been applied to the images acquired in that window, irrespective of whether the images are acquired with a plate in the instrument or running a disk based scan using images acquired earlier.

This section describes Cell Features, Field Features, Well Features, and Reference Features that are available as output features from the Comet BioApplication.

Commonly Used Comet BioApplication Features

The Comet BioApplication computes and reports several output features that fall into different categories (see Table 1 in this guide). However, there are some key cell, well, and reference output features that provide a direct measure of the degree or extent of DNA damage by comet formation. These output features are described in this section. The bold type face is the name of the output feature reported by the Comet BioApplication at the cell and well level. A more generic description of the feature name follows each feature name.

- **CometLengthCh1**- this feature reports the total length of the comet
- **TailExtentCh1**- this feature reports the total length of the comet tail
- **Tail%TotalIntenCh1**- this feature reports the % of DNA in the comet tail
- **TailExtentMomentCh1**- this feature reports the tail extent moment
- **TailOliveMomentCh1**- this feature reports the Olive tail moment of the comet

Figure 21 shows a schematic representation of a comet with it's head and tail along with descriptions of some of the features that are computed for each comet. Tables 13, 15, 16, and 17 provide a list of all cell, well, and reference output features respectively, along with a brief description of each of these features.

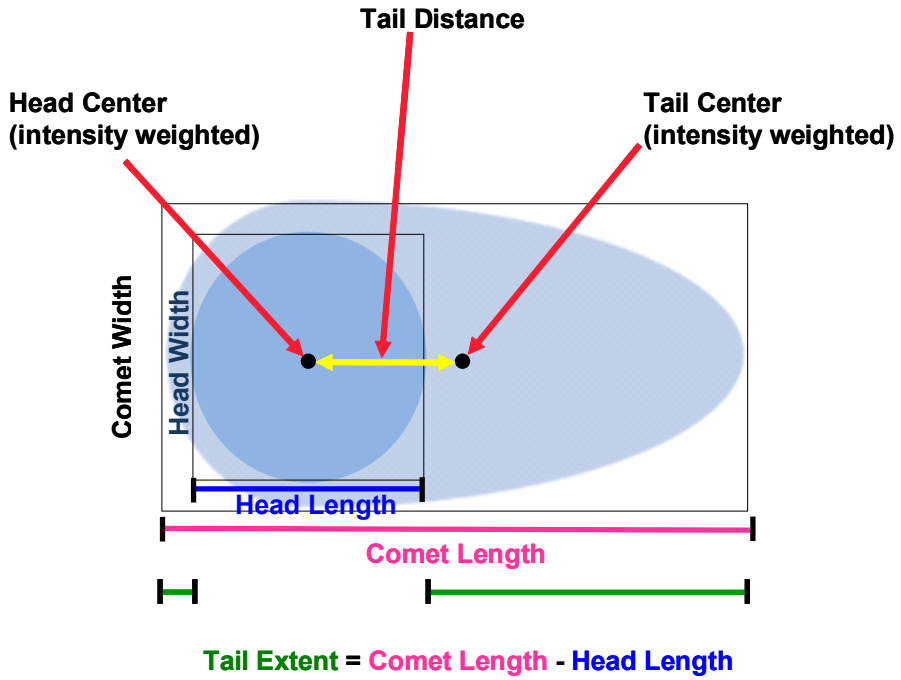


Figure 21: *A schematic representation of a comet with its head and tail defined. The figure also shows how the tail length (**Tail Extent**) is computed.*

CometLengthCh1

This feature, which is both a cell level and a well level feature is the total length of the comet. See Figure 21 for a schematic representation of this feature. The Comet BioApplication determines the comet length from the identified outer edges of the comet near the head to the identified outer edges at the tail of the comet. The value of this feature increases as the damage to the DNA increases and the fragments are separated by electrophoresis.

TailExtentCh1

The feature **TailExtentCh1** is indicative of the tail extent or the tail length. See Figure 21 for a schematic representation of the tail length measurement. Depending on the morphology of the comet and its head, the tail length may also include a small portion of the comet above the comet head. This feature is also directly related to the extent of DNA damage, with increasing lengths indicative of higher DNA damage.

Tail%TotalIntenCh1

This feature indicates the % of total DNA that is in the tail of the comet. DNA content is measured by measuring the total intensity of the fluorescent DNA stain in the comet. Again this feature is also directly related to the extent of DNA damage, with greater DNA damage causing a higher % of the DNA to migrate into the tail region.

TailExtentMomentCh1

This feature is the moment of the tail extent and is also indicative of the extent of DNA damage. Higher tail extent moment usually indicates a greater extent of DNA damage. The tail extent moment is defined as follows:

TailExtentMomentCh1 = (TailExtentCh1) * (Tail%TotalIntenCh1)

As greater DNA damage will increase both tail extent and the % DNA in the tail, the tail extent moment is a sensitive indicator of DNA damage.

TailOliveMomentCh1

This output feature is more commonly known as the Olive tail moment (15) is also a moment and is directly related to the extent of DNA damage. The Olive tail moment is defined as follows:

TailOliveMomentCh1 = (TailDistance) * (Tail%TotalIntenCh1)

where, the tail distance is defined as the distance between the comet center of mass (intensity weighted) and the tail center of mass (intensity weighted). See Figure 21 for a schematic representation of these measurements.

Cell Features

Table 13 shows the output features reported for each selected cell by the Comet BioApplication. These features are accessible in the vHCS: View software application as well as the Cell Feature window displayed in the Protocol Interactive View of the ArrayScan software.

Feature	Unit	Description
Cell#	Number	Unique comet ID
Top	--	Y coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 comet
Left	--	X coordinate (in pixels) of top-left corner of image-aligned bounding box of Ch1 comet
Width	Number	Width (in pixels) of image-aligned bounding box of Ch1 comet
Height	Number	Height (in pixels) of image-aligned bounding box of Ch1 comet
XCentroid	--	X coordinate (in pixels) of center of Ch1 comet
YCentroid	--	Y coordinate (in pixels) of center of Ch1 comet
EventTypeProfile	--	Identifies the types of events that occurred: 1, 2, 3, 12, 23, 13, 123
EventType1Status	--	EventType1 status: 0 = Event did not occur, 1 = Event occurred
EventType2Status	--	EventType2 status: 0 = Event did not occur, 1 = Event occurred
EventType3Status	--	EventType3 status: 0 = Event did not occur, 1 = Event occurred
CometType	Number	Comet migration spread type: 0 = Normal, 1 = Abnormal
CometAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 Comet
CometAreaCh1Status	--	CometAreaCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometLengthCh1	Pixels or μm^2	Length (in pixels or micrometers) of Ch1 Comet
CometLengthCh1Status	--	CometLengthCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometWidthCh1	Pixels or μm^2	Width (in pixels or micrometers) of Ch1 Comet
CometWidthCh1Status	--	CometWidthCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4PI*area of Ch1 Comet (CometP2ACh1 = 1 for circular Comet)
CometP2ACh1Status	--	CometP2ACh1 status: 0 = No response, 1 = High response, 2 = Low response
CometLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 Comet
CometLWRCh1Status	--	CometLWRCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 Comet
CometTotalIntenCh1Status	--	CometTotalIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response

Feature	Unit	Description
CometAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 Comet
CometAvgIntenCh1Status	--	CometAvgIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 Comet
CometVarIntenCh1Status	--	CometVarIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 Head
HeadAreaCh1Status	--	HeadAreaCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadLengthCh1	Pixels or μm^2	Length (in pixels or micrometers) of Ch1 Head
HeadLengthCh1Status	--	HeadLengthCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadWidthCh1	Pixels or μm^2	Width (in pixels or micrometers) of Ch1 Head
HeadWidthCh1Status	--	HeadWidthCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadP2ACh1	Number	Shape measure based on ratio of perimeter squared to 4PI*area of Ch1 Head (HeadP2ACh1 = 1 for circular Head)
HeadP2ACh1Status	--	HeadP2ACh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadLWRCh1	Number	Shape measure based on ratio of length to width of object-aligned bounding box of Ch1 Head
HeadLWRCh1Status	--	HeadLWRCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 Head
HeadTotalIntenCh1Status	--	HeadTotalIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 Head
HeadAvgIntenCh1Status	--	HeadAvgIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
HeadVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 Head
HeadVarIntenCh1Status	--	HeadVarIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailAreaCh1	Pixels or μm^2	Area (in pixels or micrometers) of Ch1 Tail
TailAreaCh1Status	--	TailAreaCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailExtentCh1	Pixels or μm^2	Value (in pixels or micrometers) of CometLengthCh1 - HeadLengthCh1
TailExtentCh1Status	--	TailExtentCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailDistanceCh1	Pixels or μm^2	Distance (in pixels or micrometers) between head and tail intensity centroids of Ch1 comet
TailDistanceCh1Status	--	TailDistanceCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailDistanceExtentRatioCh1	Number	Ratio of TailDistance to TailExtent of Ch1 comet
TailDistanceExtentRatioCh1Status	--	TailDistanceExtentRatioCh1 status: 0 = No response, 1 = High response, 2 = Low response

Feature	Unit	Description
TailTotalIntenCh1	Intensity	Total intensity of all pixels within Ch1 Tail
TailTotalIntenCh1Status	--	TailTotalIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailAvgIntenCh1	Intensity	Average intensity of all pixels within Ch1 Tail
TailAvgIntenCh1Status	--	TailAvgIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailVarIntenCh1	Intensity	Standard deviation of intensity of all pixels within Ch1 Tail
TailVarIntenCh1Status	--	TailVarIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
Tail%TotalIntenCh1	Number	Percentage of total intensity of all pixels within in Ch1 tail relative to total intensity of all pixels within Ch1 comet
Tail%TotalIntenCh1Status	--	Tail%TotalIntenCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailExtentMomentCh1	Pixels or μm^2	Product of tail length (in pixels or micrometers) and percentage of tail total intensity within Ch1 comet
TailExtentMomentCh1Status	--	TailExtentMomentCh1 status: 0 = No response, 1 = High response, 2 = Low response
TailOliveMomentCh1	Pixels or μm^2	Product of tail distance (in pixels or micrometers) and percentage of tail total intensity within Ch1 comet
TailOliveMomentCh1Status	--	TailOliveMomentCh1 status: 0 = No response, 1 = High response, 2 = Low response
CometSpotCountCh2/Ch3	Number	Number of Ch2/Ch3 spots within Ch1 Comet mask
CometSpotCountCh2/Ch3Status	--	CometSpotCountCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
CometSpotTotalAreaCh2/Ch3	Pixels or μm^2	Total area of all Ch2/Ch3 spots within Ch1 Comet mask
CometSpotTotalAreaCh2/Ch3Status	--	CometSpotTotalAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
CometSpotAvgAreaCh2/Ch3	Pixels or μm^2	Average area of all Ch2/Ch3 spots within Ch1 Comet mask
CometSpotAvgAreaCh2/Ch3Status	--	CometSpotAvgAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
CometSpotTotalIntenCh2/Ch3	Intensity	Total intensity of all pixels of all Ch2/Ch3 spots within Ch1 Comet mask
CometSpotTotalIntenCh2/Ch3Status	--	CometSpotTotalIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
CometSpotAvgIntenCh2/Ch3	Intensity	Average intensity of all pixels of all Ch2/Ch3 spots within Ch1 Comet mask
CometSpotAvgIntenCh2/Ch3Status	--	CometSpotAvgIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
HeadSpotCountCh2/Ch3	Number	Number of Ch2/Ch3 spots within Ch1 Head mask
HeadSpotCountCh2/Ch3Status	--	HeadSpotCountCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
Head%SpotsCh2/Ch3	Number	Percentage of Ch2/Ch3 spots within Ch1 Head mask

Feature	Unit	Description
Head%SpotsCh2/Ch3Status	--	Head%SpotsCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
HeadSpotTotalAreaCh2/Ch3	Pixels or μm^2	Total area of all Ch2/Ch3 spots within Ch1 Head mask
HeadSpotTotalAreaCh2/Ch3Status	--	HeadSpotTotalAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
HeadSpotAvgAreaCh2/Ch3	Pixels or μm^2	Average area of all Ch2/Ch3 spots within Ch1 Head mask
HeadSpotAvgAreaCh2/Ch3Status	--	HeadSpotAvgAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
HeadSpotTotalIntenCh2/Ch3	Intensity	Total intensity of all pixels of all Ch2/Ch3 spots within Ch1 Head mask
HeadSpotTotalIntenCh2/Ch3Status	--	HeadSpotTotalIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
HeadSpotAvgIntenCh2/Ch3	Intensity	Average intensity of all pixels of all Ch2/Ch3 spots within Ch1 Head mask
HeadSpotAvgIntenCh2/Ch3Status	--	HeadSpotAvgIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TailSpotCountCh2/Ch3	Number	Number of Ch2/Ch3 spots within Ch1 Tail mask
TailSpotCountCh2/Ch3Status	--	TailSpotCountCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
Tail%SpotsCh2/Ch3	Number	Percentage of Ch2/Ch3 spots within Ch1 Tail mask
Tail%SpotsCh2/Ch3Status	--	Tail%SpotsCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TailSpotTotalAreaCh2/Ch3	Pixels or μm^2	Total area of all Ch2/Ch3 spots within Ch1 Tail mask
TailSpotTotalAreaCh2/Ch3Status	--	TailSpotTotalAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TailSpotAvgAreaCh2/Ch3	Pixels or μm^2	Average area of all Ch2/Ch3 spots within Ch1 Tail mask
TailSpotAvgAreaCh2/Ch3Status	--	TailSpotAvgAreaCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TailSpotTotalIntenCh2/Ch3	Intensity	Total intensity of all pixels of all Ch2/Ch3 spots within Ch1 Tail mask
TailSpotTotalIntenCh2/Ch3Status	--	TailSpotTotalIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TailSpotAvgIntenCh2/Ch3	Intensity	Average intensity of all pixels of all Ch2/Ch3 spots within Ch1 Tail mask
TailSpotAvgIntenCh2/Ch3Status	--	TailSpotAvgIntenCh2/Ch3 status: 0 = No response, 1 = High response, 2 = Low response
TotalIntenCh*N*	Intensity	Total intensity in Ch*N* of all pixels within modified Ch1 comet mask
AvgIntenCh*N*	--	Average intensity in Ch*N* of all pixels within modified Ch1 comet mask

Table 13: *Cell Features available for the Comet BioApplication. For any feature listed as* **FeatureNameCh2/Ch3,** *means that feature is computed for both Channel 2 and Channel 3 provided the assay protocol is a 2 or 3 Channel protocol. For any feature listed as* **FeatureNameCh*N***, *refers to that feature being computed for Channels 2-6, based on the total number of channels in the Assay Protocol.*

Field Features

The Comet BioApplication provides eight "Field Level" output features. These features are essentially quality control features. They provide for a quick assessmentof focus success, effect of compounds that are toxic to the cells, or incorrect object identification. A numerical value for any one of these features in any field that is much different from the values seen in other fields can be a quick indicator of poor focus or near total cell loss, or artifacts being identified and selected as objects due to poor object identification thresholds or non-optimal background correction settings. The table below (Table 14) shows the list of field output features from the Comet BioApplication.

Name	Description
ValidCometCount	Number of valid comets identified in the field (Ch1 comet selection parameters applied)
SelectedCometCount	Number of valid comets selected for analysis in the field (Ch2-6 object selection parameters applied)
%SelectedComets	Percentage of valid comets selected for analysis in the field
NormalCometCount	Number of normal comets of all comets selected for analysis in the field
%NormalComets	Percentage of normal comets of all comets selected for analysis in the field
AbnormalCometCount	Number of abnormal comets of all comets selected for analysis in the field
%AbnormalComets	Percentage of abnormal comets of all comets selected for analysis in the field
AnalyzedCometCount	Number of selected comets used for statistical data analysis (only normal comets or both normal and abnormal comets) in the field

Table 14: *Field Features available for the Comet BioApplication*

Well Features

Many Well Features are derived from the Cell Features. Such features are identified by a prefix, as listed below, to the corresponding Cell Feature name (Table 13).

Feature Prefix	Well Feature Definition	Units
MEAN_	The arithmetic mean (average) of the Cell Feature value for all selected cells in the well	Same as Cell Feature
SD_	The standard deviation of the Cell Feature value for all selected cells in the well	None
SE_	The standard error of the mean of the Cell Feature value for all selected cells in the well	None
CV_	The coefficient of variation of the Cell Feature value for all selected cells in the well	Percent
%HIGH	The percentage of cells in the well whose feature value is **above** the value specified in the **FeatureChNLevelHigh** Assay Parameters or as calculated by the Reference Wells (i.e., % of cells with status = 1).	Percent
%LOW	The percentage of cells in the well whose feature value is **below** the value specified in the **FeatureChNLevelLow** Assay Parameters or as calculated by the Reference Wells (i.e., % of cells with status = 2).	Percent

Table 15: *General Well Feature definitions for Comet BioApplication*

The Comet BioApplication also reports the following Well Features in the Well Detail window of the vHCS: View software application and in the Protocol Interactive View and Scan Plate View of the ArrayScan software. (Table 16).

Well Feature	Description
ValidCometCount	Number of valid comets identified in the well (Ch1 object selection parameters applied)
SelectedCometCount	Number of valid comets selected for analysis in the well (Ch2-6 object selection parameters applied)
%SelectedComets	Percentage of valid comets selected for analysis in the well
NormalCometCount	Number of normal comets of all comets selected for analysis in the well
%NormalComets	Percentage of normal comets of all comets selected for analysis in the well
AbnormalCometCount	Number of abnormal comets of all comets selected for analysis in the well
%AbnormalComets	Percentage of abnormal comets of all comets selected for analysis in the well
ValidFieldCount	Number of fields in which comets were selected for analysis in the well
SelectedCometCountPerValidField	Average number of comets selected for analysis per valid field in the well
AnalyzedCometCount	Number of selected comets used for statistical data analysis in the well (only normal comets or both normal and abnormal comets)
EventType1CometCount	Number of comets selected for analysis in the well in which EventType1 occurred

Well Feature	Description
%EventType1Comets	Percentage of comets selected for analysis in the well in which EventType1 occurred
EventType2CometCount	Number of comets selected for analysis in the well in which EventType2 occurred
%EventType2Comets	Percentage of comets selected for analysis in the well in which EventType2 occurred
EventType3CometCount	Number of comets selected for analysis in the well in which EventType3 occurred
%EventType3Comets	Percentage of comets selected for analysis in the well in which EventType3 occurred

Table 16: *Additional Well Features available for the Comet BioApplication*

Reference Features

The Comet BioApplication reports the following Reference Features in the Scan Plate View of the Scan software application as well as the Plate Detail window of the vHCS: View software application (Table 17). These features are computed and reported only when the Assay Parameter "**UseReferenceWells = 1**".

Feature	Description
RefAvgCometCountPerField	Average number of comets per field in reference wells
RefCometAreaCh1LevelHigh	High-response level for CometAreaCh1 computed from reference well results
RefCometAreaCh1LevelLow	Low-response level for CometAreaCh1 computed from reference well results
RefCometLengthCh1LevelHigh	High-response level for CometLengthCh1 computed from reference well results
RefCometLengthCh1LevelLow	Low-response level for CometLengthCh1 computed from reference well results
RefCometWidthCh1LevelHigh	High-response level for CometWidthCh1 computed from reference well results
RefCometWidthCh1LevelLow	Low-response level for CometWidthCh1 computed from reference well results
RefCometP2ACh1LevelHigh	High-response level for CometP2ACh1 computed from reference well results
RefCometP2ACh1LevelLow	Low-response level for CometP2ACh1 computed from reference well results
RefCometLWRCh1LevelHigh	High-response level for CometLWRCh1 computed from reference well results
RefCometLWRCh1LevelLow	Low-response level for CometLWRCh1 computed from reference well results
RefCometTotalIntenCh1LevelHigh	High-response level for CometTotalIntenCh1 computed from reference well results
RefCometTotalIntenCh1LevelLow	Low-response level for CometTotalIntenCh1 computed from reference well results
RefCometAvgIntenCh1LevelHigh	High-response level for CometAvgIntenCh1 computed from reference well results
RefCometAvgIntenCh1LevelLow	Low-response level for CometAvgIntenCh1 computed from reference well results

Feature	Description
RefCometVarIntenCh1LevelHigh	High-response level for CometVarIntenCh1 computed from reference well results
RefCometVarIntenCh1LevelLow	Low-response level for CometVarIntenCh1 computed from reference well results
RefHeadAreaCh1LevelHigh	High-response level for HeadAreaCh1 computed from reference well results
RefHeadAreaCh1LevelLow	Low-response level for HeadAreaCh1 computed from reference well results
RefHeadLengthCh1LevelHigh	High-response level for HeadLengthCh1 computed from reference well results
RefHeadLengthCh1LevelLow	Low-response level for HeadLengthCh1 computed from reference well results
RefHeadWidthCh1LevelHigh	High-response level for HeadWidthCh1 computed from reference well results
RefHeadWidthCh1LevelLow	Low-response level for HeadWidthCh1 computed from reference well results
RefHeadP2ACh1LevelHigh	High-response level for HeadP2ACh1 computed from reference well results
RefHeadP2ACh1LevelLow	Low-response level for HeadP2ACh1 computed from reference well results
RefHeadLWRCh1LevelHigh	High-response level for HeadLWRCh1 computed from reference well results
RefHeadLWRCh1LevelLow	Low-response level for HeadLWRCh1 computed from reference well results
RefHeadTotalIntenCh1LevelHigh	High-response level for HeadTotalIntenCh1 computed from reference well results
RefHeadTotalIntenCh1LevelLow	Low-response level for HeadTotalIntenCh1 computed from reference well results
RefHeadAvgIntenCh1LevelHigh	High-response level for HeadAvgIntenCh1 computed from reference well results
RefHeadAvgIntenCh1LevelLow	Low-response level for HeadAvgIntenCh1 computed from reference well results
RefHeadVarIntenCh1LevelHigh	High-response level for HeadVarIntenCh1 computed from reference well results
RefHeadVarIntenCh1LevelLow	Low-response level for HeadVarIntenCh1 computed from reference well results
RefTailAreaCh1LevelHigh	High-response level for TailAreaCh1 computed from reference well results
RefTailAreaCh1LevelLow	Low-response level for TailAreaCh1 computed from reference well results
RefTailExtentCh1LevelHigh	High-response level for TailExtentCh1 computed from reference well results
RefTailExtentCh1LevelLow	Low-response level for TailExtentCh1 computed from reference well results
RefTailDistanceCh1LevelHigh	High-response level for TailDistanceCh1 computed from reference well results
RefTailDistanceCh1LevelLow	Low-response level for TailDistanceCh1 computed from reference well results
RefTailDistanceExtentRatioCh1LevelHigh	High-response level for TailDistanceExtentRatioCh1 computed from reference well results
RefTailDistanceExtentRatioCh1LevelLow	Low-response level for TailDistanceExtentRatioCh1 computed from reference well results

Feature	Description
RefTailTotalIntenCh1LevelHigh	High-response level for TailTotalIntenCh1 computed from reference well results
RefTailTotalIntenCh1LevelLow	Low-response level for TailTotalIntenCh1 computed from reference well results
RefTailAvgIntenCh1LevelHigh	High-response level for TailAvgIntenCh1 computed from reference well results
RefTailAvgIntenCh1LevelLow	Low-response level for TailAvgIntenCh1 computed from reference well results
RefTailVarIntenCh1LevelHigh	High-response level for TailVarIntenCh1 computed from reference well results
RefTailVarIntenCh1LevelLow	Low-response level for TailVarIntenCh1 computed from reference well results
RefTail%TotalIntenCh1LevelHigh	High-response level for Tail%TotalIntenCh1 computed from reference well results
RefTail%TotalIntenCh1LevelLow	Low-response level for Tail%TotalIntenCh1 computed from reference well results
RefTailExtentMomentCh1LevelHigh	High-response level for TailExtentMomentCh1 computed from reference well results
RefTailExtentMomentCh1LevelLow	Low-response level for TailExtentMomentCh1 computed from reference well results
RefTailOliveMomentCh1LevelHigh	High-response level for TailOliveMomentCh1 computed from reference well results
RefTailOliveMomentCh1LevelLow	Low-response level for TailOliveMomentCh1 computed from reference well results
RefCometSpotCountCh2/Ch3LevelHigh	High-response level for CometSpotCountCh2/Ch3 computed from reference well results
RefCometSpotCountCh2/Ch3LevelLow	Low-response level for CometSpotCountCh2/Ch3 computed from reference well results
RefCometSpotTotalAreaCh2/Ch3LevelHigh	High-response level for CometSpotTotalAreaCh2/Ch3 computed from reference well results
RefCometSpotTotalAreaCh2/Ch3LevelLow	Low-response level for CometSpotTotalAreaCh2/Ch3 computed from reference well results
RefCometSpotAvgAreaCh2/Ch3LevelHigh	High-response level for CometSpotAvgAreaCh2/Ch3 computed from reference well results
RefCometSpotAvgAreaCh2/Ch3LevelLow	Low-response level for CometSpotAvgAreaCh2/Ch3 computed from reference well results
RefCometSpotTotalIntenCh2/Ch3LevelHigh	High-response level for CometSpotTotalIntenCh2/Ch3 computed from reference well results
RefCometSpotTotalIntenCh2/Ch3LevelLow	Low-response level for CometSpotTotalIntenCh2/Ch3 computed from reference well results
RefCometSpotAvgIntenCh2/Ch3LevelHigh	High-response level for CometSpotAvgIntenCh2/Ch3 computed from reference well results
RefCometSpotAvgIntenCh2/Ch3LevelLow	Low-response level for CometSpotAvgIntenCh2/Ch3 computed from reference well results
RefHeadSpotCountCh2/Ch3LevelHigh	High-response level for HeadSpotCountCh2/Ch3 computed from reference well results
RefHeadSpotCountCh2/Ch3LevelLow	Low-response level for HeadSpotCountCh2/Ch3 computed from reference well results
RefHead%SpotsCh2/Ch3LevelHigh	High-response level for Head%SpotsCh2/Ch3 computed from reference well results
RefHead%SpotsCh2/Ch3LevelLow	Low-response level for Head%SpotsCh2/Ch3 computed from reference well results

Feature	Description
RefHeadSpotTotalAreaCh2/Ch3LevelHigh	High-response level for HeadSpotTotalAreaCh2/Ch3 computed from reference well results
RefHeadSpotTotalAreaCh2/Ch3LevelLow	Low-response level for HeadSpotTotalAreaCh2/Ch3 computed from reference well results
RefHeadSpotAvgAreaCh2/Ch3LevelHigh	High-response level for HeadSpotAvgAreaCh2/Ch3 computed from reference well results
RefHeadSpotAvgAreaCh2/Ch3LevelLow	Low-response level for HeadSpotAvgAreaCh2/Ch3 computed from reference well results
RefHeadSpotTotalIntenCh2/Ch3LevelHigh	High-response level for HeadSpotTotalIntenCh2/Ch3 computed from reference well results
RefHeadSpotTotalIntenCh2/Ch3LevelLow	Low-response level for HeadSpotTotalIntenCh2/Ch3 computed from reference well results
RefHeadSpotAvgIntenCh2/Ch3LevelHigh	High-response level for HeadSpotAvgIntenCh2/Ch3 computed from reference well results
RefHeadSpotAvgIntenCh2/Ch3LevelLow	Low-response level for HeadSpotAvgIntenCh2/Ch3 computed from reference well results
RefTailSpotCountCh2/Ch3LevelHigh	High-response level for TailSpotCountCh2/Ch3 computed from reference well results
RefTailSpotCountCh2/Ch3LevelLow	Low-response level for TailSpotCountCh2/Ch3 computed from reference well results
RefTail%SpotsCh2/Ch3LevelHigh	High-response level for Tail%SpotsCh2/Ch3 computed from reference well results
RefTail%SpotsCh2/Ch3LevelLow	Low-response level for Tail%SpotsCh2/Ch3 computed from reference well results
RefTailSpotTotalAreaCh2/Ch3LevelHigh	High-response level for TailSpotTotalAreaCh2/Ch3 computed from reference well results
RefTailSpotTotalAreaCh2/Ch3LevelLow	Low-response level for TailSpotTotalAreaCh2/Ch3 computed from reference well results
RefTailSpotAvgAreaCh2/Ch3LevelHigh	High-response level for TailSpotAvgAreaCh2/Ch3 computed from reference well results
RefTailSpotAvgAreaCh2/Ch3LevelLow	Low-response level for TailSpotAvgAreaCh2/Ch3 computed from reference well results
RefTailSpotTotalIntenCh2/Ch3LevelHigh	High-response level for TailSpotTotalIntenCh2/Ch3 computed from reference well results
RefTailSpotTotalIntenCh2/Ch3LevelLow	Low-response level for TailSpotTotalIntenCh2/Ch3 computed from reference well results
RefTailSpotAvgIntenCh2/Ch3LevelHigh	High-response level for TailSpotAvgIntenCh2/Ch3 computed from reference well results
RefTailSpotAvgIntenCh2/Ch3LevelLow	Low-response level for TailSpotAvgIntenCh2/Ch3 computed from reference well results

Table 17: *Reference Features available for the Comet BioApplication. These features are computed and reported by the Comet BioApplication only if the Assay Parameter* ***"UseReferenceWells=1"****. For any reference feature listed as* ***Ref*****FeatureNameCh2/Ch3,** *that feature is computed for both Channel 2 and Channel 3 provided the Assay Protocol is a 2 or 3 Channel protocol.*

Chapter

3

Using the Comet BioApplication

The Comet BioApplication can be used to quantitate DNA damage caused by DNA damaging compounds in a variety of cell types. On the ArrayScan VTI HCS Reader, the BioApplication can be used with a 10X or a 20X objective. Typically the recommended objectives are 10X and 20X as the resolution provided by the 5X objective may not be sufficient to analyze DNA damage by the Comet BioApplication. Any cell type that you typically use for comet analysis can be used with the Comet BioApplication. In this section, we will provide you with recommended protocol setting for some main Assay Parameters with the 10X and 20X objectives for use with CHO-K1 cells.

The protocols supplied with the BioApplication are example protocols. We encourage you to modify and optimize the protocols for your own particular biological situation.

This chapter also describes the use of the BioApplication Events Wizard to describe "Events" and quantify cellular responses. The BioApplication Event Wizard is a software tool that is designed to allow entry, reading, and modifying Event Definitions as logical statements. Event Definitions are stored in the Assay Protocol as Assay Parameters of the type **Type_X_EventDefinition,** where X can be 1-3, allowing definition of up to three distinct Events.

Using the Comet BioApplication with 10X and 20X Objectives for analysis of DNA Damage in CHO-K1 cells

Assay Protocol Configuration

This section only provides a description of some of the protocol settings that are relevant to measuring comet formation in CHO-K1 cells using the 10X objective on the ArrayScan HCS Reader.

Assay Parameter	Value	Reason
CometMigrationDirection	0	Head-to-tail orientation was top to bottom
CometDataAnalysis	0	Compute and report well level output features only from comets that are characterized as “normal”
CometTypeCh1	0	Bright comets on a dark background
BackgroundCorrectionCh1	-64	Apply surface fitting method of background correction
CometSmoothFactorCh1	6	Applying a smoothing factor of 6 to better define the comet over the background
CometSegmentationCh1	0	No segmentation applied as most of the comets seemed to be well separated from each other
RejectBorderCometsCh1	1	Any comet touching the field edge was excluded from analysis
HeadRegionFractionCh1	0.5	The region to separate the head and tail regions was set at 50% of the length of each comet
HeadSmoothFactorCh1	2	Applying a smoothing factor of 2 to better define the head of the comet
HeadDetectionCh1	0	Set to 0, because the head was well defined in most of the comets
HeadIdentificationModifierCh1	0.65	To identify only the bright region of the head as compared to the background
HeadSegmentationCh1	0	No segmentation of the head was desired

Table 18: *Settings for major Assay Parameters in the Comet BioApplication, used to quantify comet formation in CHO-K1 cells with the 10x objective*.

Assay Parameter	Value	Reason
CometMigrationDirection	0	Head-to-tail orientation was top to bottom
CometDataAnalysis	0	Compute and report well level output features only from comets that are characterized as “normal”
CometTypeCh1	0	Bright comets on a dark background
BackgroundCorrectionCh1	-128	Apply surface fitting method of background correction
CometSmoothFactorCh1	6	Applying a smoothing factor of 6 to better define the comet over the background
CometSegmentationCh1	0	No segmentation applied as most of the comets seemed to be well separated from each other
RejectBorderCometsCh1	1	Any comet touching the field edge was excluded from analysis
HeadRegionFractionCh1	0.5	The region to separate the head and tail regions was set at 50% of the length of each comet
HeadSmoothFactorCh1	8	Applying a smoothing factor of 8 to better define the head of the comet
HeadDetectionCh1	8	Set to 8, so head could be better identified over the comet background
HeadIdentificationModifierCh1	-0.55	To identify only the bright region of the head as compared to the background
HeadSegmentationCh1	0	No segmentation of the head was desired

Table 19: *Settings for major Assay Parameters in the Comet BioApplication, used to quantify comet formation in CHO-K1 cells with the 20x objective*.

Event Definition Using the BioApplication Event Wizard

The BioApplication Event Wizard is a software tool that is designed to allow entry, reading, and modifying Event Definitions as logical statements. Event Definitions are stored in the Assay Protocol as Assay Parameters of the type **Type_X_EventDefinition,** where X can be 1-3, allowing definition of up to three distinct Events.

The Wizard can be used to enter and edit Event Definitions as values for the **Type_X_EventDefinition** Assay Parameter in the Assay Protocol for the Comet BioApplication. This section provides a detailed description of the operation of the BioApplication Event Wizard. The Wizard should only be used after the Comet BioApplication has been installed on your computer.

NOTE

A **maximum of 3 Cell Features** can be combined using logical operators to describe an Event.

Note that the Event Definition Assay Parameters **must not** be modified via the Scan software application. Events can only be defined and edited via the BioApplication Event Wizard. Altering the values within the protocol (through the Create Protocol or Protocol Interactive Views) may cause your protocol to become inoperable.

Definition of Events requires that the following steps be followed, in the order listed. It is strongly recommended that you **do not run the Scan software application and the BioApplication Event Wizard at the same time**. Operation of the BioApplication Event Wizard must be consistent with the steps described.

STEP I

1. Create a protocol using the Scan software application without defining Events. Set optimized parameter values (upper and lower limits) for Cell Features to be used for Event Definition.
2. Save protocol.
3. Close the Scan software application.

STEP II

1. Open the BioApplication Event Wizard.
2. Open the protocol saved in Step I using the BioApplication Event Wizard and define up to 3 events at the level of individual cells.
3. Save updated Assay Protocol.
4. Close the BioApplication Event Wizard.

STEP III

1. Restart the Scan software application and open the protocol saved in Step II (Event Definitions will appear as numeric strings in the Assay Parameter window)
2. Copy Event Definitions from the Protocol Comments field in Create Protocol View into the Scan Comments field in Scan Plate View.
3. Scan the assay plate.
4. Close the Scan software application.

STEP IV (Optional)

1. Open the saved protocol at a later time using BioApplication Event Wizard.

2. Evaluate and/or modify saved Event Definitions.
3. Close BioApplication Event Wizard and start the Scan software application to scan a new assay plate or analyze previously acquired images using modified Event Definitions.

Assay Protocols edited with the BioApplication Events Wizard are not saved as new versions of the protocol.

Features

The following features are included in the BioApplication Event Wizard:

- Enables use of standard Boolean operators (NOT, AND, ANDNOT, OR, ORNOT, XOR, NAND, and NOR)
- Read-Only protocol protection
- Displays previously entered Event Definitions in saved Assay Protocols
- Ability to clear each Event Definition individually
- Rapid construction of logical statements using Cell Features and logical operators
- Automated validation of each Event Definition when loading or saving protocols
- Ability to save updated protocol
- Addition of Event Definitions to the Protocol Comments field in the Assay Protocol

Steps for Running the Event Wizard with the Comet BioApplication

1. Create a protocol using the Comet BioApplication without defining Events. Set optimized parameter values (LevelHigh and LevelLow) for detection of responder cells associated with Cell Features to be used for Event Definition.
2. Save protocol.
3. Close the Scan software application (suggested).

Starting the BioApplication Event Wizard

To start the BioApplication Event Wizard,
Double-click the **BioApplication Event Wizard** desktop icon

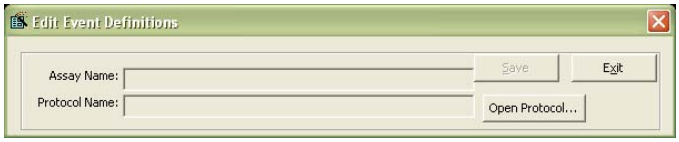
-or-

For Cellomics HCS Readers: From the Windows **Start** menu, select **Programs** > **Cellomics** > **BioAppEventWizard**.

For vHCS Discovery Toolbox computers: From the Windows **Start** menu, select **Programs** > **Cellomics > vHCS Scan > BioAppEventWizard**.

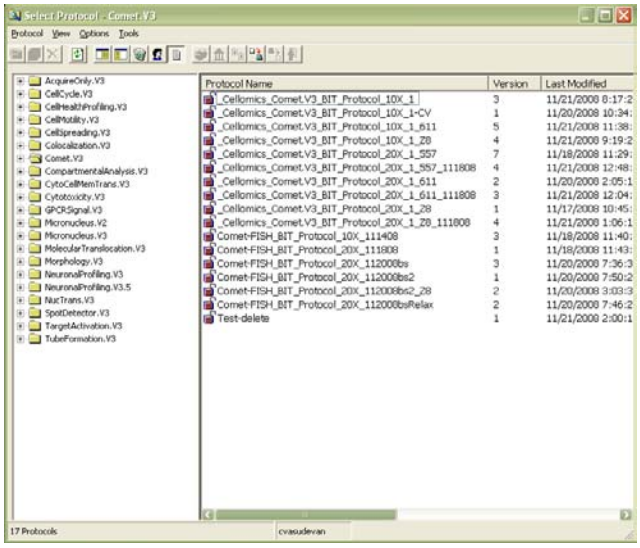
Protocol Selection and Loading

The first window that is displayed (see below) allows selection of any Comet Assay Protocol. Note that Event Definitions can only be specified for pre-existing Assay Protocols.

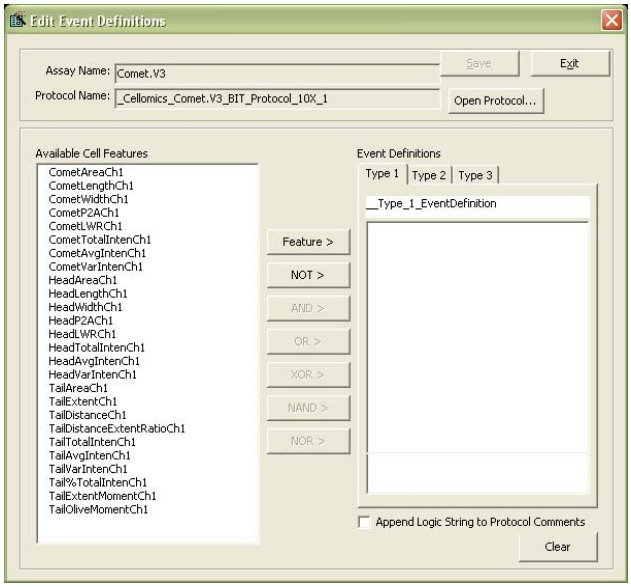


To select an Assay Protocol,

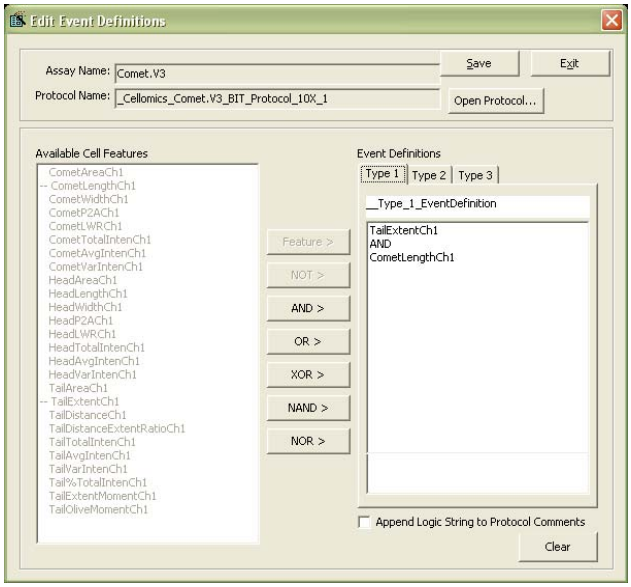
1) Click on the **Open Protocol** button. The Protocol Manager will open as shown below.



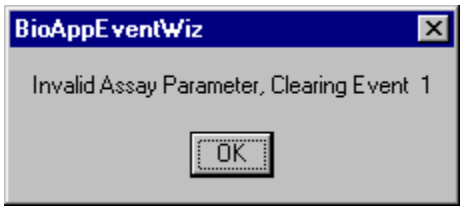
2) Open the Comet.v4 folder and select the desired Assay Protocol from the list of available protocols. Double-click the protocol for which you want to define Events. Once a protocol is selected, the window expands to its full extent as shown in the following figure. Cell Features available for Event Definition are listed on the left in the Available Cell Features section.



3) Define **Event Type X**, by selecting the appropriate tab. Select the desired cell features from the Available Cell Features list and the type of Boolean operator to define the desired event. The following figure shows Event Type 1 defined as **"TailExtentCh1" AND "CometLengthCh1"**.



Once the protocol is loaded, the **Type_X_EventDefinition** Assay Parameters (1-3) are automatically validated. If Event Definition is invalid due to unintentional editing from within the Scan software application, the following message will be displayed, and the Event Definition will be cleared.



If the Assay Protocol is Read-Only, you will only be able to view the Event Definitions and a message above the **Save** button will indicate that the protocol cannot be edited.

4) Once you have completed the Event Definitions, click the **Save** button and then click the **Exit** button. The window will close and you can then open the Scan software application.

Defining Events

This section describes the steps involved in defining individual Events in a Comet BioApplication Assay Protocol. Note that logical statements used to define Events can include up to three Cell Features and three logical operators.

Once the protocol has loaded in the Events Wizard, each Event Definition can be viewed by clicking on the appropriate tab (Type 1-3).

The procedure to be followed when constructing Event Definitions is described in the sequence of screenshots below. Cell Features are combined with Boolean operators to produce Event Definitions. Any Cell Feature can be selected by clicking on the feature name in the Available Cell Features list and then clicking the **Feature** > button. Boolean operators, are selected by clicking on the Operator buttons (**NOT** >, **AND** >, **OR** >, etc.). Figure 22 shows a schematic description of the Boolean operators. The operator buttons are disabled whenever they cannot be used. Click the appropriate buttons in sequence to build the Event Definition, as shown below.

Addition of a Cell Feature to the Events means that the cell must be a responder (Status =1 or Status = 2) to meet the definition of the event.

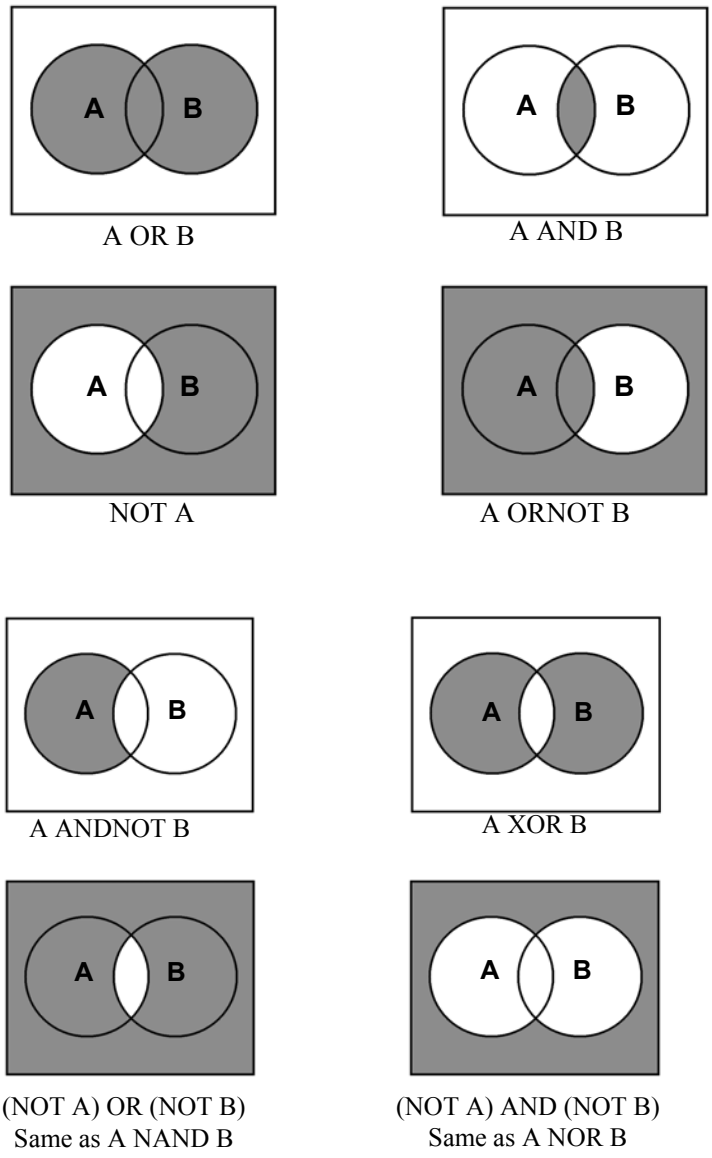


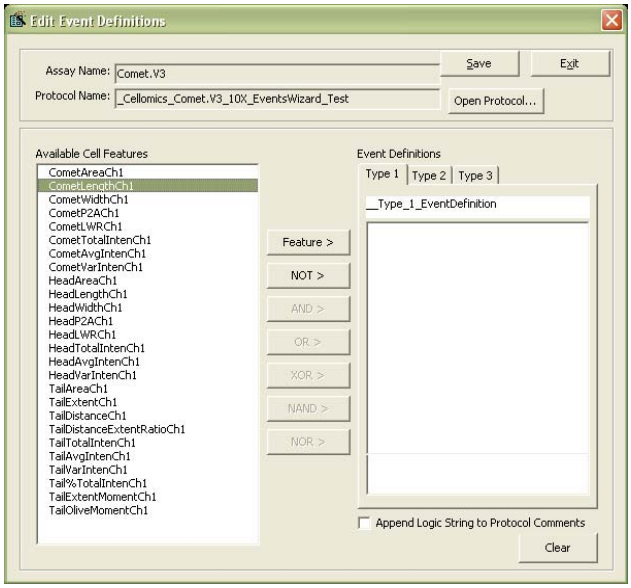
Figure 22: *Schematic definition of available Boolean Operators assuming two Cell Features of interest, A & B*

To build an Event Definition,

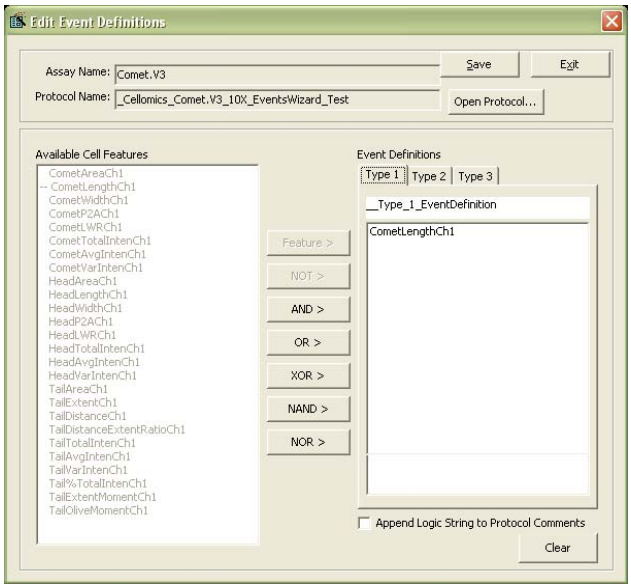
1) Select the Event Definition that you want to specify or edit by clicking on the **Type 1**, **Type 2**, or **Type 3** tab.

2) If you wish to edit a pre-existing Event Definition, click the **Clear** button.

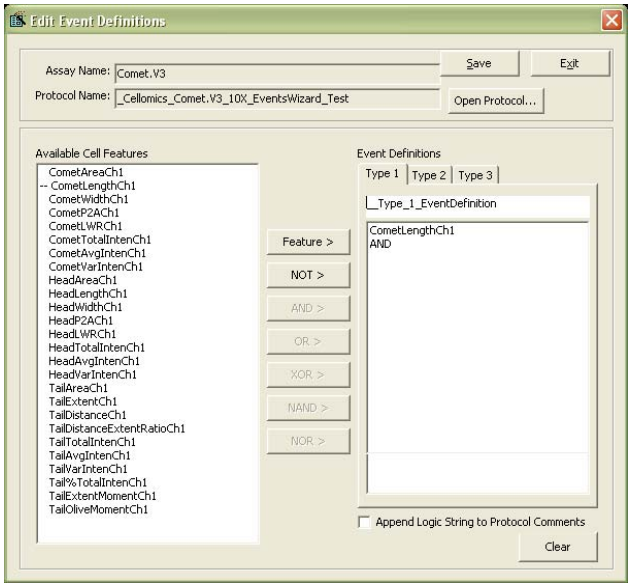
3) Select the desired Cell Feature by clicking on the feature name from the Available Cell Features list. You may also choose to select **NOT>** first (before the Feature) to indicate that you want to include only non-responders (Status = 0) in the definition of the event. In the following example, the feature Event Type 1 will be defined as **"CometTotalIntenCh1_AND_TailToatlIntenCh1" .**



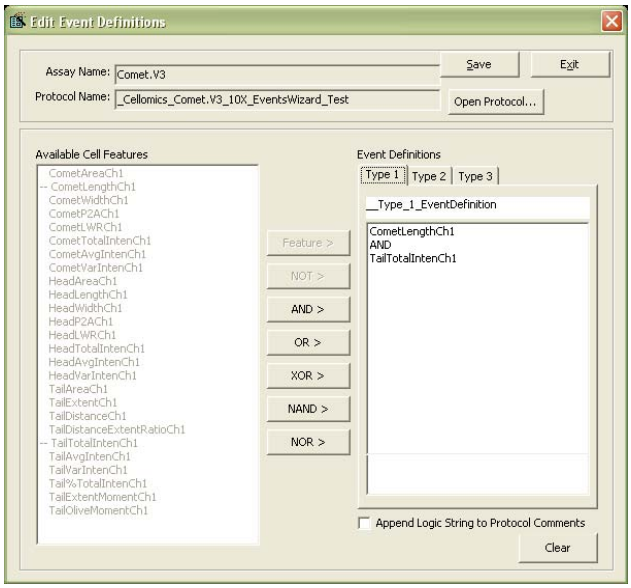
4) Click the **Feature>** button to transfer the Cell Feature into the Event Definition (see below).



5) Click on the desired Operator button to transfer it into the Event Definition. In this example, the **AND** operator is selected (see below).



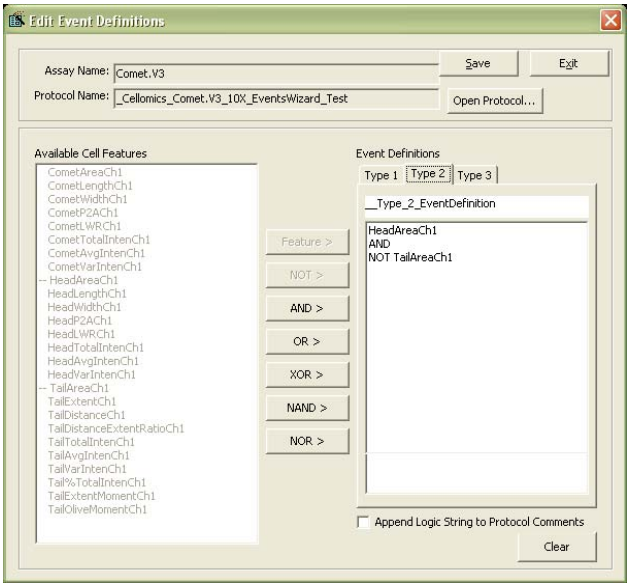
6) Repeat the cycle for adding another Cell Feature to the Event Definition. In this case, the Cell Feature "**TailTotalIntenCh1**" has been selected. By clicking the **Feature** > button, this feature is transferred into the Event Definition.



Type_1_EventDefinition in the previous case is:

"CometLengthCh1_AND_TailTotalIntenCh1". In other words, cells meeting the Event 1 criteria must be responders for both features.

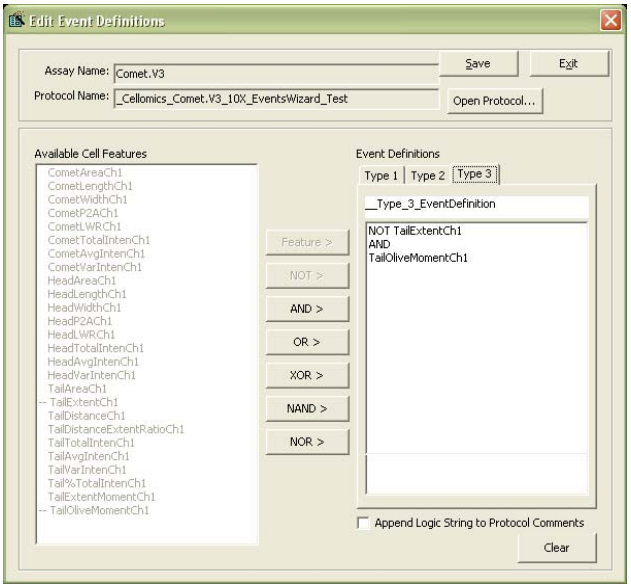
Entry of a logical operator may be followed by entry of another Cell Feature. Alternatively, the operators **AND** and **OR** can be inverted by adding the **NOT** operator by clicking the **NOT >** button prior to selecting the Cell Feature, as shown below.



Type_2_EventDefinition in the above example is:

"HeadAreaCh1_AND_NOT_TailAreaCh1". Thus for Type 2 Event, cells must be a responder for HeadAreaCh1 and not a responder for TailAreaCh1.

Typically, logical statements used to define Events begin with a Cell Feature. Alternatively, a logical statement may begin with the logical operator NOT as shown in the following example.



Type_3_EventDefinition in the above example is:

"NOT_TailExtentCh1_AND_TailOliveMomentCh1". Thus cells meeting criteria for Event 3 are only those cells that are non-responders for TailExtentCh1 and responders for TailOliveMomentCh1.

To clear an Event Definition,

- Once a protocol is loaded, click the **Clear** button to clear any Event Definition. This feature can be used to redefine any event.

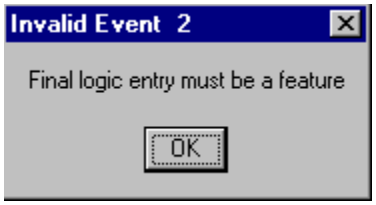
To save the updated Assay Protocol,

- Click the **Save** button. Note that an Assay Protocol cannot be renamed using the BioApplication Event Wizard. Renaming of protocols can only be done through the Scan software application.

NOTE

The BioApplication Event Wizard will only make changes to pre-existing Assay Protocols by adding or modifying Event Definitions. If you wish to create a new protocol, use the **Save Assay Protocol As** option under the **File** menu within the Scan software application prior to starting the Event Wizard.

The BioApplication Event Wizard has the capability to automatically validate Event Definitions. If Event Definition validation fails, the protocol will not be saved. For example, Event Definitions are considered invalid if they do not end with a Cell Feature. In this case, clicking on the **Save** button will display the following message.



To exit the BioApplication Event Wizard,

- Click the **Exit** button at any time to exit the Wizard without saving any changes made to Event Definitions.

Once the updated Assay Protocol is saved, Event Definitions are viewable in the **Protocol Comments** field in the **Create Protocol View** upon opening the saved Assay Protocol using the Scan software application.

Chapter

4

iDev Software Workflow

This chapter describes the tasks in the workflow for Protocol optimization of the Comet V4 BioApplication using the iDev software. More detailed technical descriptions of each Assay Parameter can be found in Chapter 2 of this guide.

iDev Protocol Optimization Tasks

The iDev Assay Development software provides guided workflows for Assay Protocol optimization. Assay Parameters which are user adjustable input parameters that control image analysis are organized in a task list. Each task contains only the Assay Parameters necessary for optimization of that task and has been designed to walk you through a defined workflow for Protocol optimization. Below are basic descriptions of each task and a summary of the Assay Parameters contained within that task. Chapter 2 describes the technical feature of each of the Assay Parameters in greater detail.

Image Preprocessing

Image Preprocessing is the processing of images prior to object identification. The task comprises background removal for all selected channels. In this task, you can set background removal methods and values for each of the channels in the Protocol.

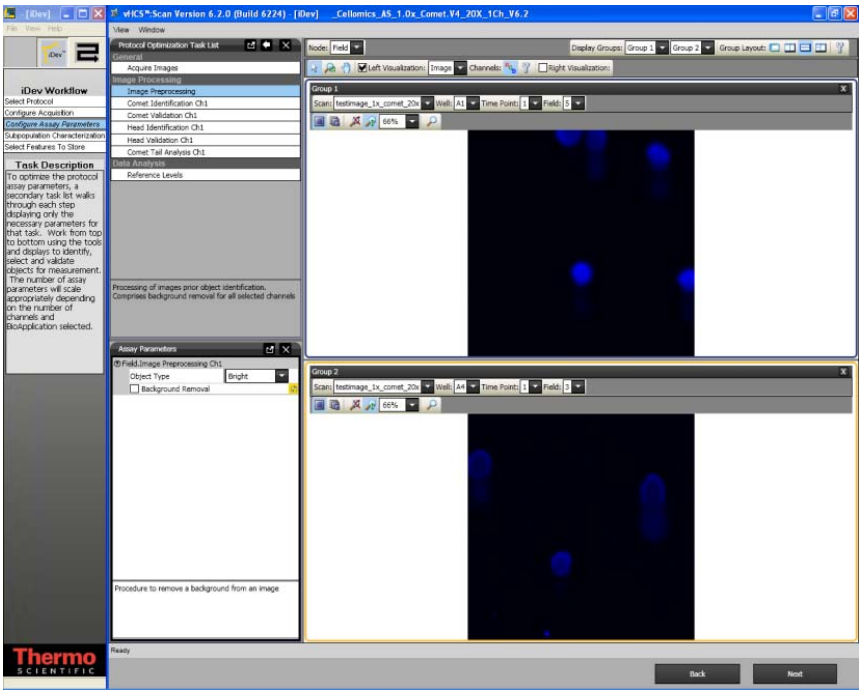


Figure 23. *Protocol Optimization Task – Image Preprocessing*

Comet Identification Ch1

Comet Identification is the identification of comets in the Channel 1 image. This task involves setting up methods and values for primary object identification, object smoothing, and object segmentation for comets in Channel 1.

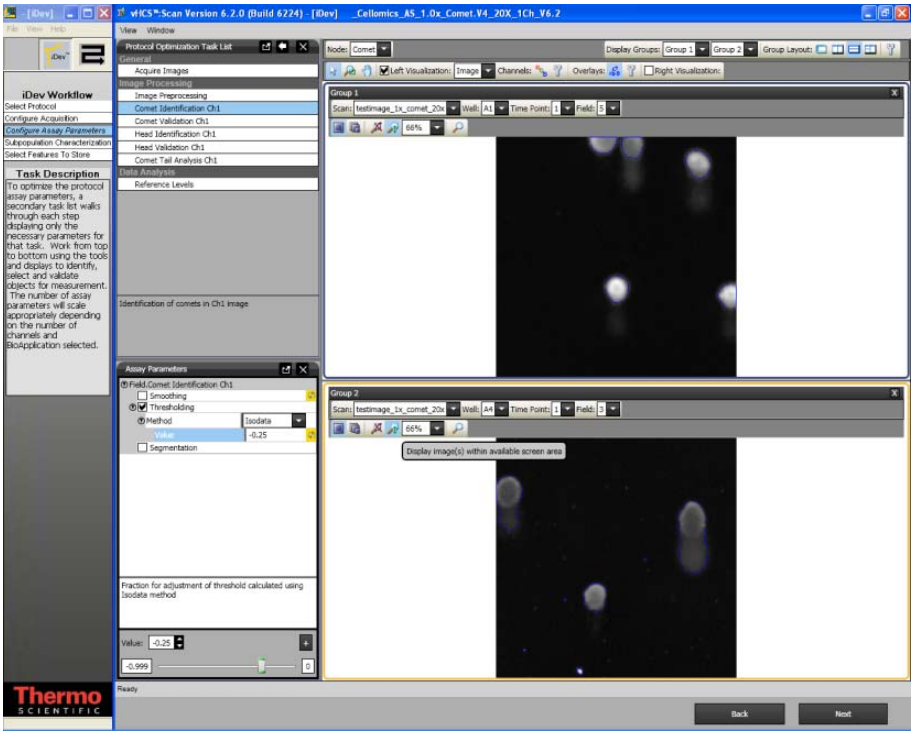


Figure 24. *Protocol Optimization Task – Comet Identification Ch1*

Comet Validation Ch1

In this task, you set the Assay Parameters that determine the direction of comet migration in the image field and the fraction of the comet's length that demarcates the head region. You can also set selection/rejection of the comets identified in Channel 1 based on area, shape, and intensity features calculated in Channel 1of comets in Channel 1 based on selection/rejection features. In this task you can also determine if objects that are touching the edges of the field are included or rejeceted from analysis by checking/clearing the **Comet.BorderObjectsCh1** task.

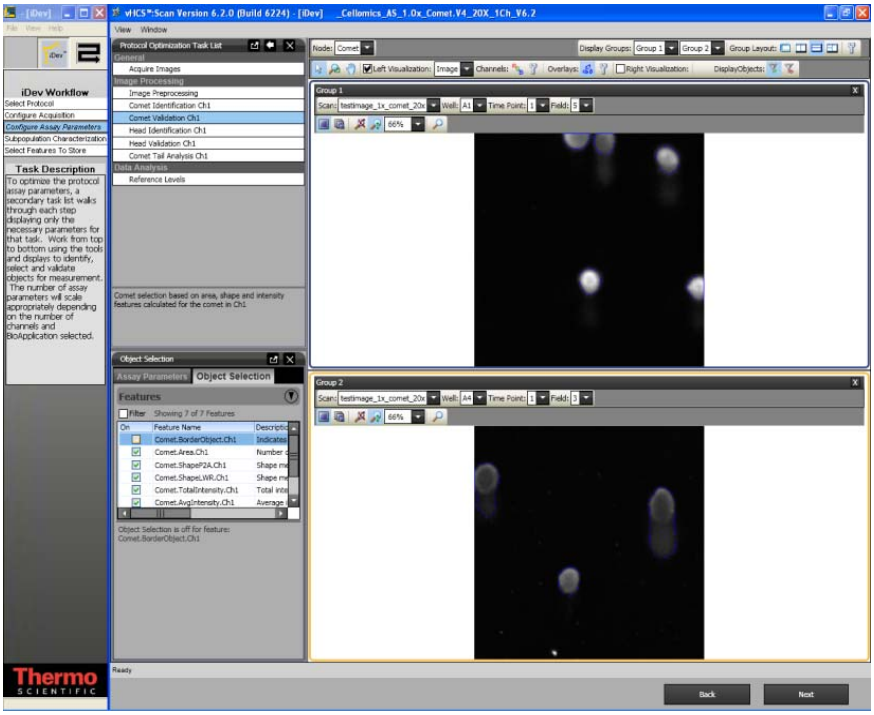


Figure 25. *Protocol Optimization Task – Comet Validation Ch1*

Comet Selection Ch2 through ChN

This Comet Selection task is comet selection based on intensity features computed in Channels 2 through Channel N under the primary object mask derived from Channel 1. In this task, you will set selection / rejection of the primary object based on intensity measurements in Channel 2 through Channel N under a modified primary object mask. This task is similar to setting the Assay Parameter, **MaskModifierChN,** in the ArrayScan Classic software. The primary object mask can be dilated (> 0), or eroded (< 0) if the **ROI Mask Creation** box is checked.

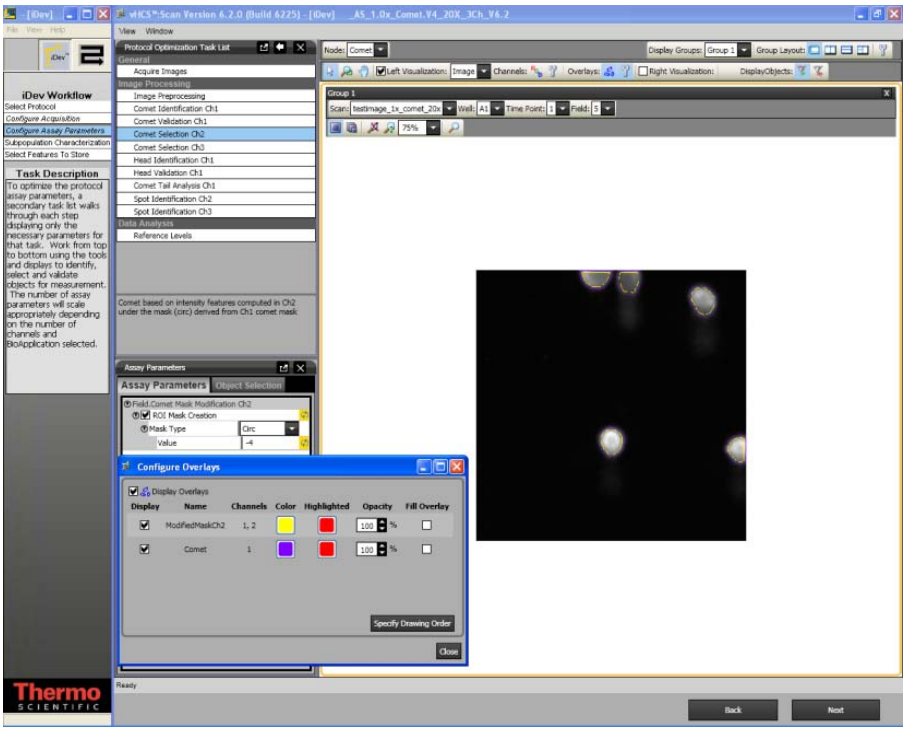


Figure 26. *Protocol Optimization Task – Comet Selection Ch2 through ChN*

Head Identification Ch1

In this task, you set the Assay Parameters to detect the head region of the comet from images in Channel 1. This includes setting the method and value for smoothing, head detection, thresholding, and segmentation.

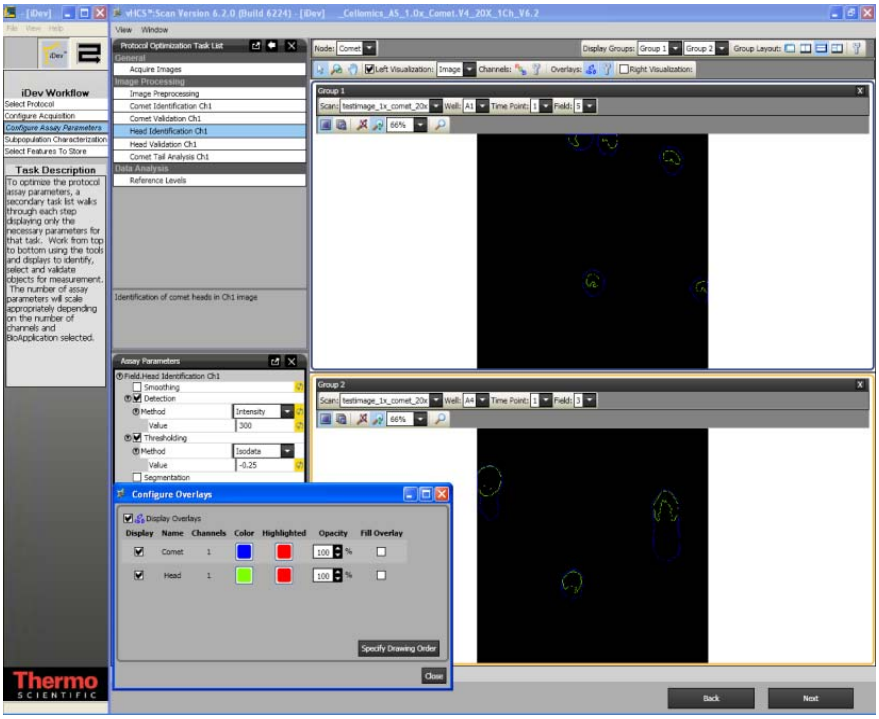


Figure 27. *Protocol Optimization Task – Head Identification Ch1*

Head Validation Ch1

In this task, you set selection/rejection parameters for comet head based on area, shape, and intensity features for the comet images in Channel 1.

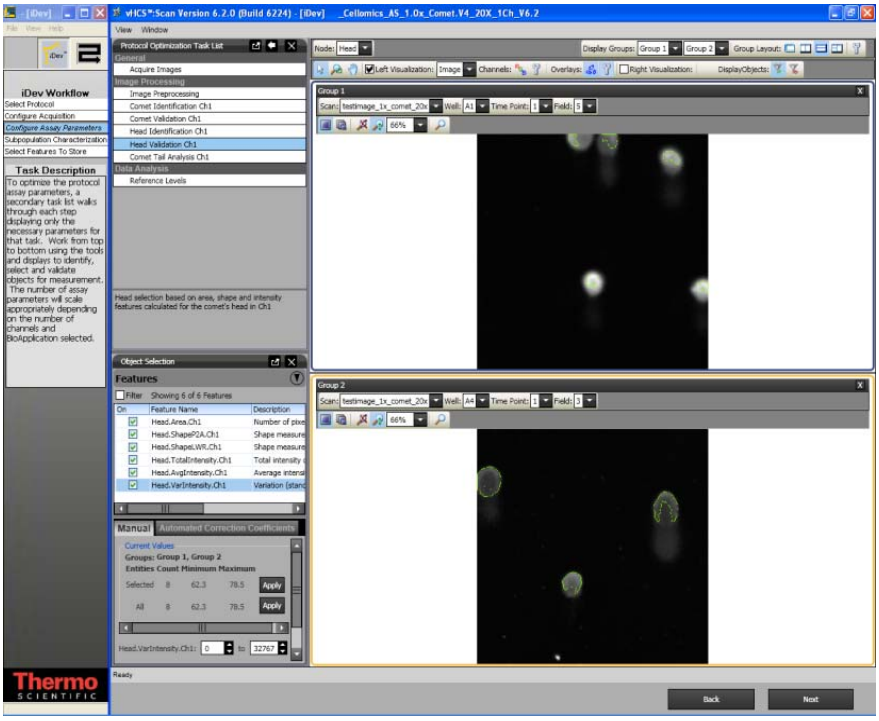


Figure 28. *Protocol Optimization Task – Head Validation Ch1*

Comet Tail Analysis Ch1

In this task, tail analysis of images from Channel 1 are performed. You also set the overlays for tail distance in this task.

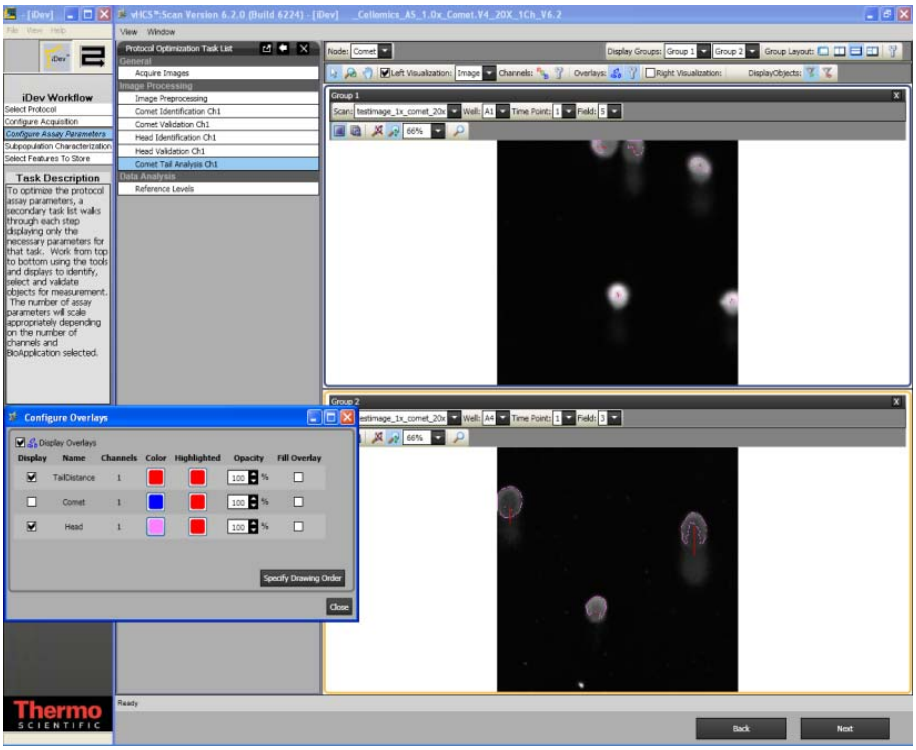


Figure 29. *Protocol Optimization Task – Comet Tail Analysis Ch1*

Spot Identification Ch2 and Ch3

In this task, you can set methods and values for identifying spots in Channels 2 and 3. This includes setting the method and value for smoothing, detection, thresholding, and segmentation.

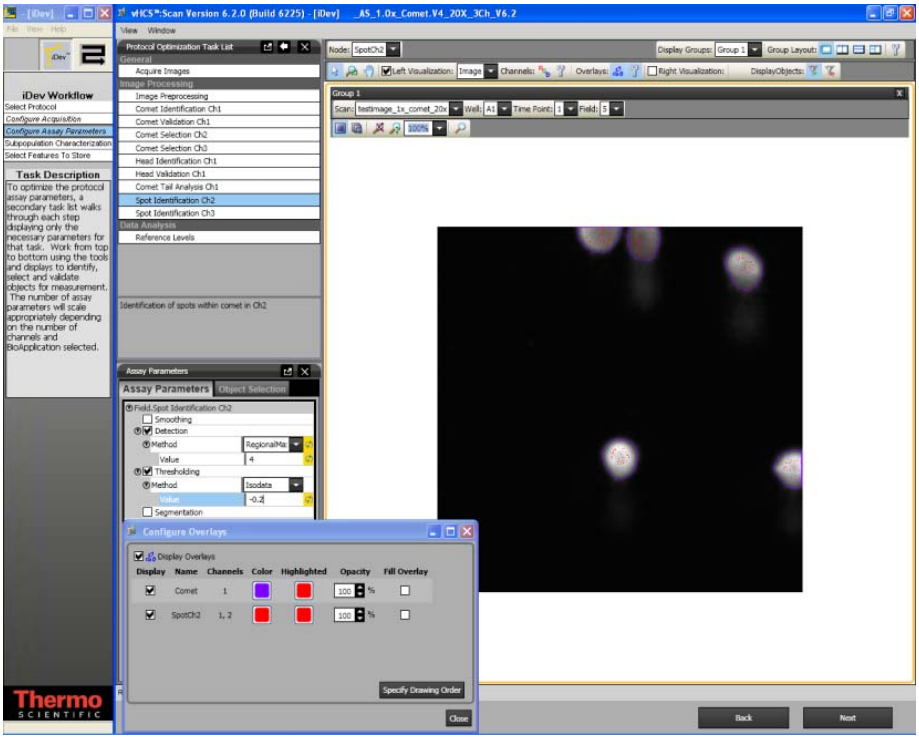


Figure 30. *Protocol Optimization Task – Spot Identification Ch2 and Ch3*

Spot Validation Ch2 and Ch3

In this task, you set the selection/rejection of the spots identified in Channels 2 and 3 based on area, shape, and intensity features.

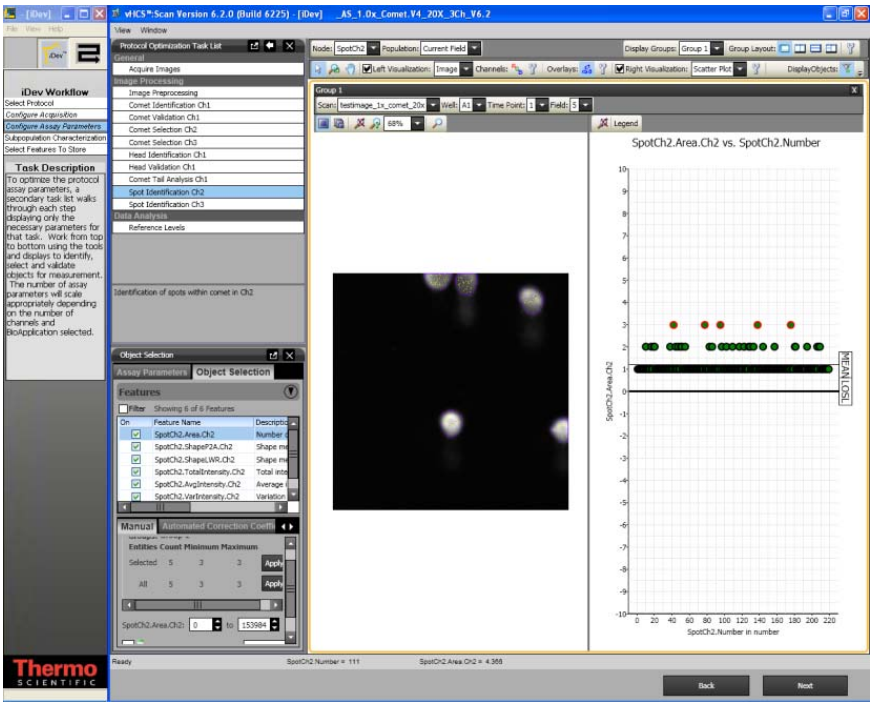


Figure 31. *Protocol Optimization Task – Spot Validation Ch2 and Ch3*

Reference Levels

In this task, you can set level high and level low for computing % responders and/or %High and %Low based on several calculated Reference Level features. Reference Levels can be manually or automatically set by adjusting the level value or correction coefficient under the Reference Level task and by checking the **Use Reference Wells** option in the Subpopulation Characterization task.

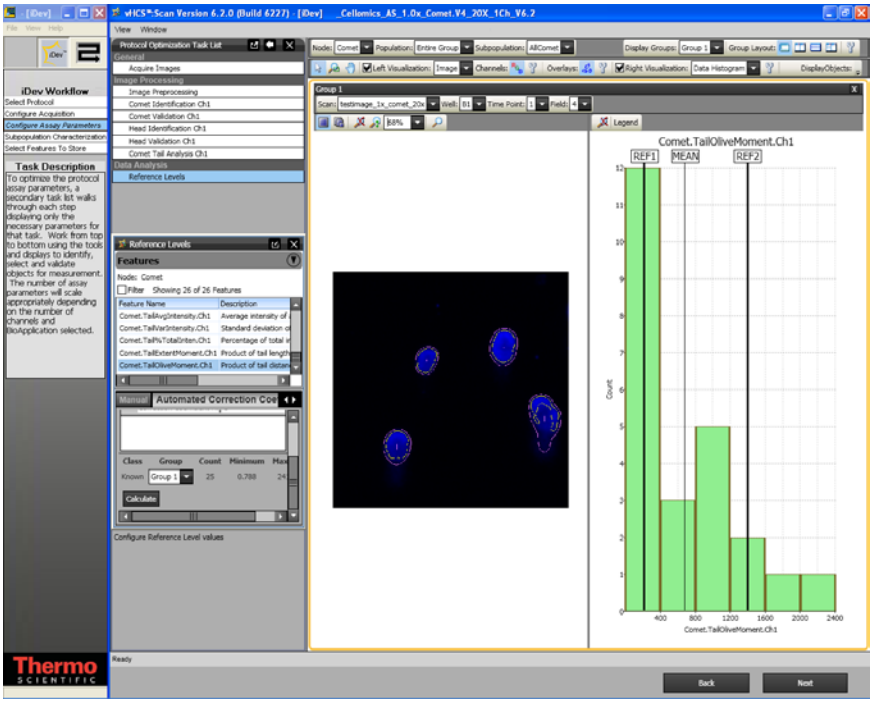


Figure 32. *Protocol Optimization Task – Reference Levels*

Setting Events in the iDev Assay Development Workflow

The process for setting Events in the iDev Assay Development Workflow is identical to that outlined in Chapter 3 of this guide. However, because of the integrated workflow in the iDev product, Events can be set in the Subpopulation Characterization task rather than the BioApplication Event Wizard. Events are based on Reference Levels set in the Protocol optimization task of the Configure Assay Parameters step in the iDev workflow.

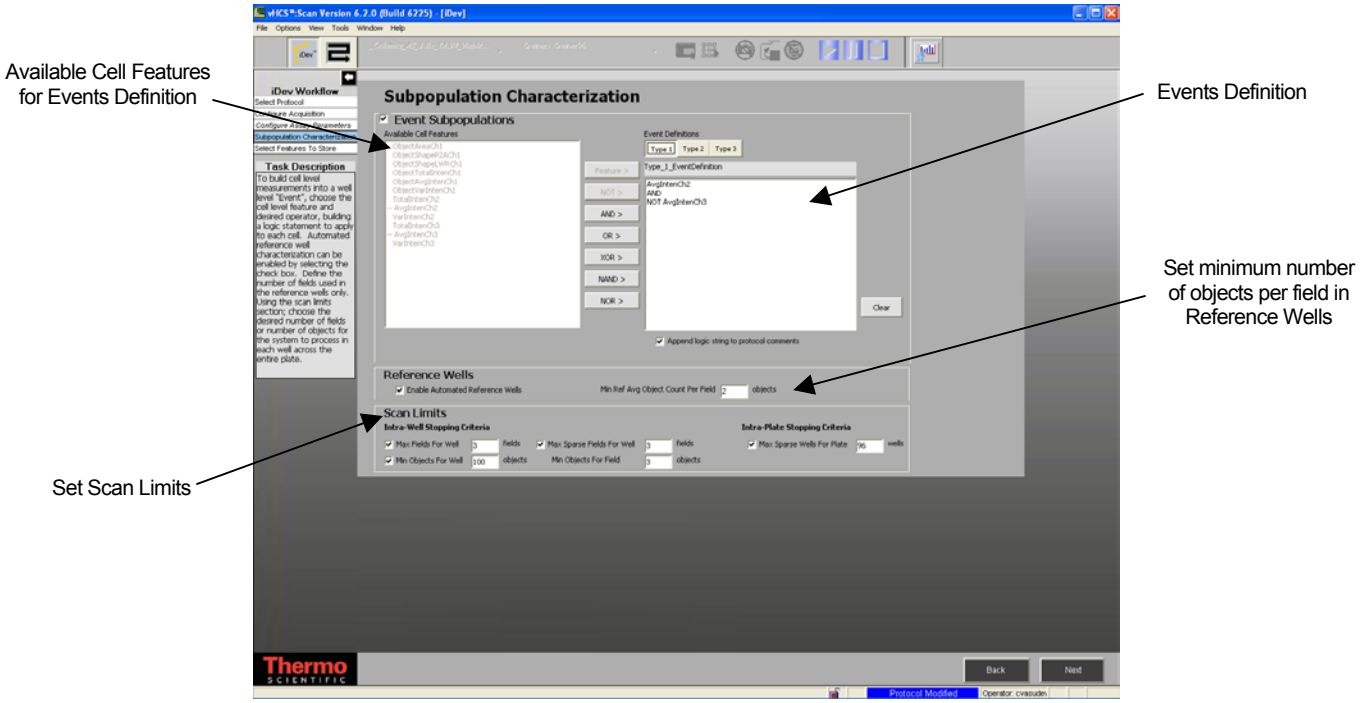


Figure 33. *Subpopulation Characterization Task*

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