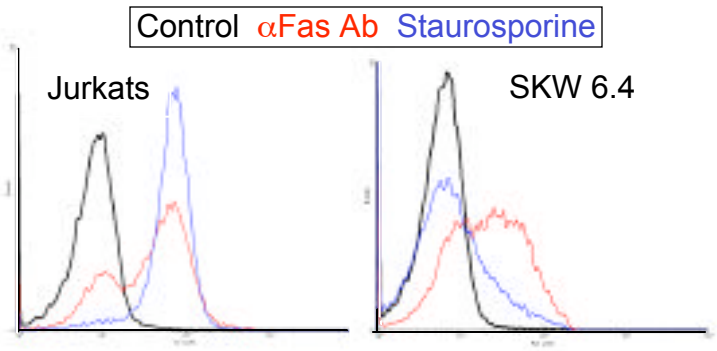
OncoImmunin®, Inc.

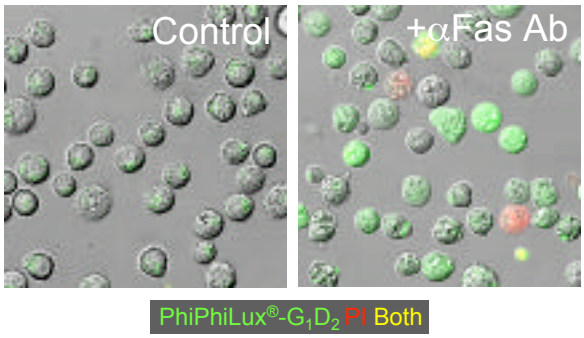
has designed, synthesized, validated, and patented a new class of cell permeable fluorogenic protease substrates. Unique aspects of these probes are: (1) their ability to cross intact cell membranes enables measurement of intracellular protease activities in live cells, (2) incorporation of amino acid sequence information from *both* sides of protease cleavage sites, *i.e.*, P and P' residues, up to a total of ten amino acid residues, provides complete protease recognition sequences, (3) conformations of substrates' peptide moieties assume loop or extended beta sheet conformations resulting in substrates retaining protease *in vivo* specificity and (4) the choice of fluorophores permits simultaneous measurement of multiple proteolytic activities in biologic samples.

PhiPhiLux® is the family of OncoImmunin substrates for the detection of caspase-3 and caspase-3-like activities in live cells. These probes are produced in three colors: G_1D_2 for use with the Ar ion laser 488 nm line, G_2D_2 for mid-500 nm lasers, and R_2D_2 for 633/635 nm lasers.

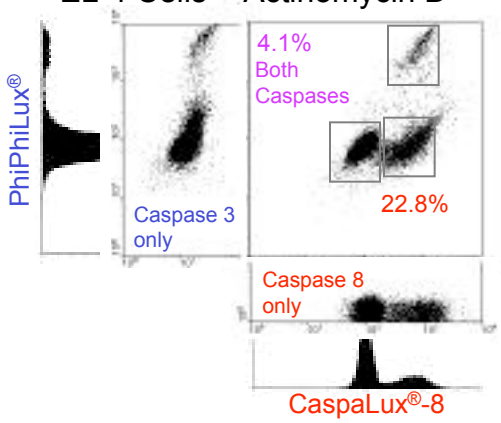
The range in sensitivity between a T-cell leukemia (Jurkat cells) and a Burkitt's lymphoma (SKW 6.4) line to different apoptogens is exemplified by PhiPhiLux®.



SKW6.4 ± αFas Ab + PhiPhiLux®-G_1D_2



EL-4 Cells + Actinomycin D



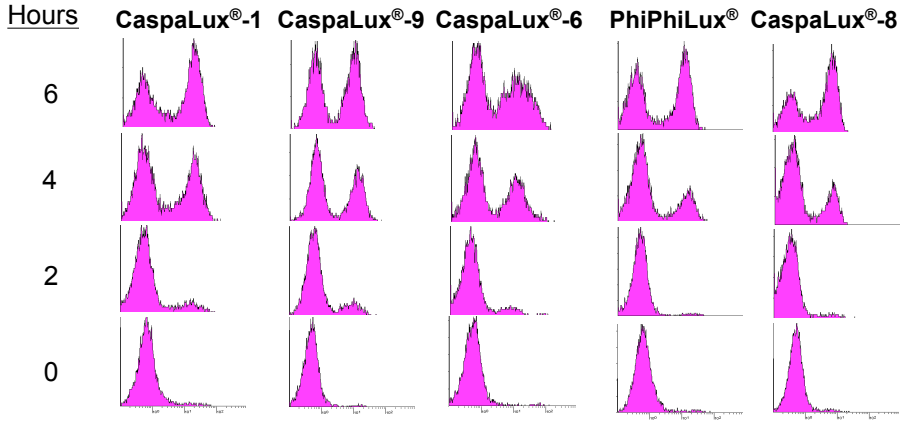
With OncoImmunin's **CaspaLux®** substrates the activities of caspases-1,6,8, and -9 can be measured. Moreover, by combining the 5 different substrates in complementary colors, multiple caspase activities can be determined simultaneously in live cells by both flow cytometry and microscopy. A high throughput assay is also available.

Cell Permeable Fluorogenic Caspase Substrates

PhiPhiLux® (DEVDase substrate)
CaspaLux®-1 (YVHDase substrate)
CaspaLux®-6 (VEIDase substrate)
CaspaLux®-8 (IETDase substrate)
CaspaLux®-9 (LEHDase substrate)

In addition to the caspase substrates presented here OncoImmunin, Inc. has cell permeable fluorogenic substrates for measurement of protease activities associated with cancer metastasis and viral replication.

αFas Ab-Treated Human $CD8^+$ CTL (TIL660)



Mouse Thymocytes + αFas Ab

Cleavage of CaspaLux®-9 PhiPhiLux® Both

selected References:

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Cell Death Diff. 11:175-182 (2004)
Methods Mol. Biol. 263:141 (2004)

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CyToxiLux®, GranToxiLux®, and PanToxiLux™

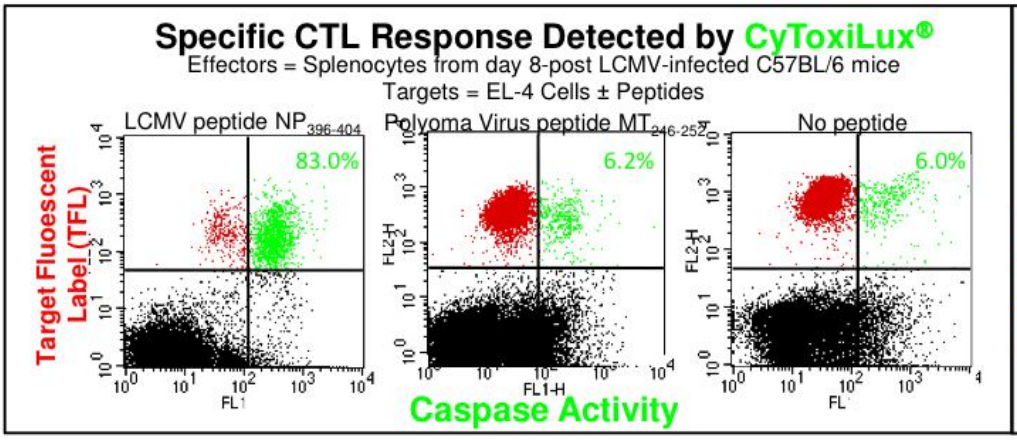
Live, Single Cell-based Fluorogenic Cytotoxicity Assay Kits

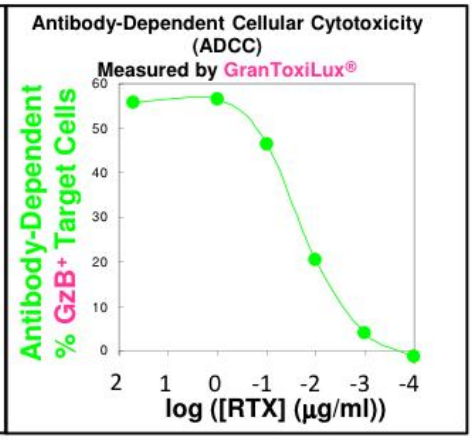
Principle of assays:

Following the successful delivery of a lethal hit by cytotoxic lymphocytes, protease activities leading to cell death in individual target cells can be measured by flow cytometry or fluorescence microscopy. In each kit cleavage of a cell permeable fluorogenic substrate reports the following activities :

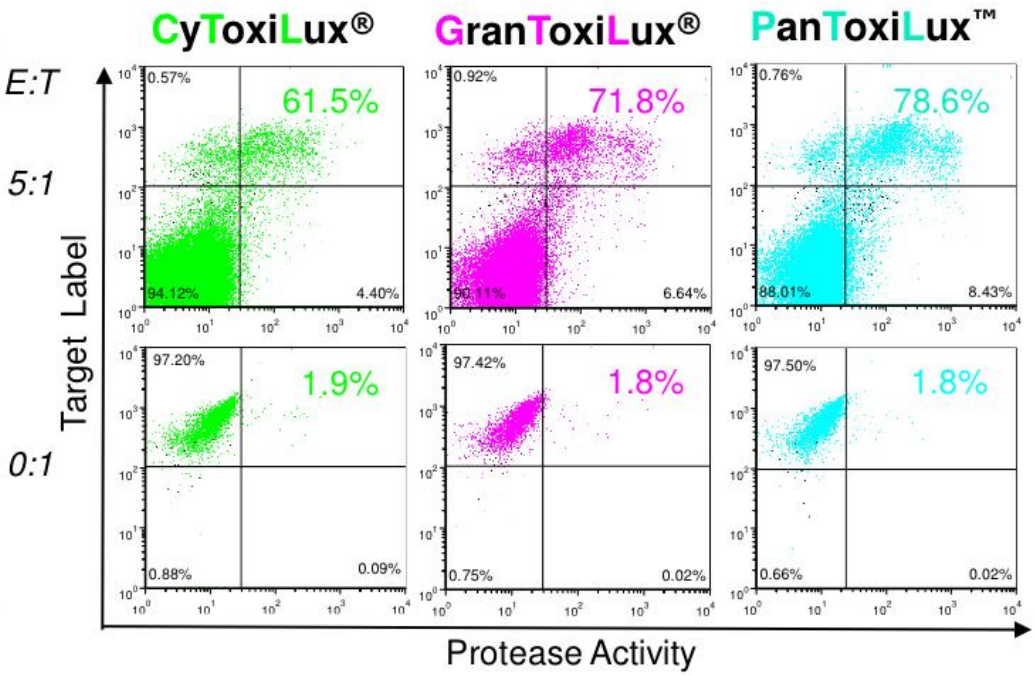
CyToxiLux® : downstream caspase
GranToxiLux®: granzyme B
PanToxiLux™: granzyme B and upstream caspase

Measurements with CyToxiLux®, GranToxiLux®, and PanToxiLux™ are superior to bulk assays, e.g., LDH and ^{51}Cr release, in terms of both time (0.3-2.0 hr. *vs.* 4 hr.) and sensitivity (relatively weak CTL responses against subdominant epitopes are detectable).





Comparison of 3 cytotoxicity kits with Jurkat cells as targets (T) and NK92 as effectors (E). The most sensitive kit, PanToxiLux™, provides the earliest and most intense signal in target cells. Measure-ments with all probes showed less than 2% background, *i.e.,* in targets alone..



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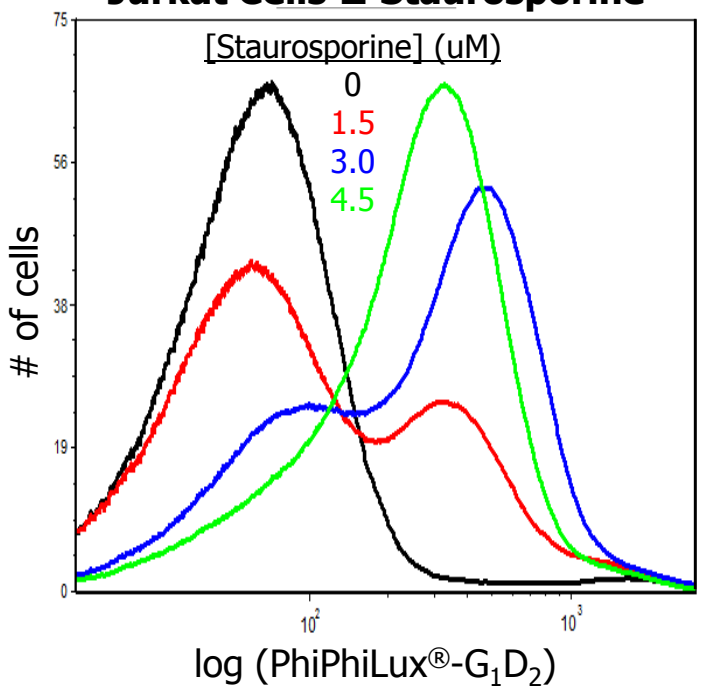
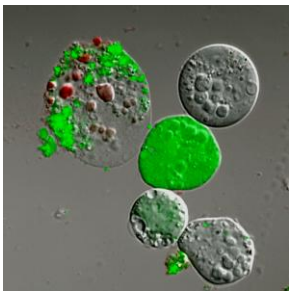
Cell Permeable Fluorogenic Protease Substrates for the Detection of Apoptosis and Autophagy in Live Cells

OncoImmunin, Inc.'s cell permeable, fluorogenic protease substrates enter *live* cells by crossing all membranes by passive diffusion, thus enabling measurement of protease activities in their physiologic environments. Incorporation of amino acid sequences from both sides of protease cleavage sites, *i.e.*, $P_{1\text{-}n}$ and $P'_{1\text{-}n}$ residues, imparts physiologic conformations resulting in extremely high specificity. Once a substrate is recognized and cleaved by its cognate protease, cleaved fragments are trapped in the protease's physiologic microenvironment allowing real-time imaging as well as quantitation by flow cytometry.

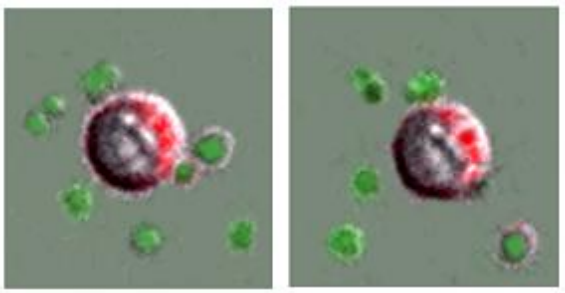
Jurkat Cells ± Staurosporine





PhiPhiLux® detects caspase 3 activity in the cytosol of apoptotic HL-60 cells with late apoptotic cells allowing entry of PI.

Caspase 8 activity is localized in blebs while caspase 6 activity is in cytosol of CTLs induced by αFas Ab (2 time points shown)



Autophagy in Live Cells

LC3-G_1D_2 detects the appearance of Atg4B activity with substrate containing the microtubule-associated protein 1A/1B-light chain 3 (LC3) sequence

5 hours in minimal medium (EBSS) induces LC3-G_1D_2 cleavage in live (PI^-) EL-4 Cells