

**Single-cell Reporters for Inflammatory Caspase Activity**

by

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Submitted to the Computational and Systems Biology Program  
in partial fulfillment of the requirements for the degree of

Master of Science in Computational and Systems Biology

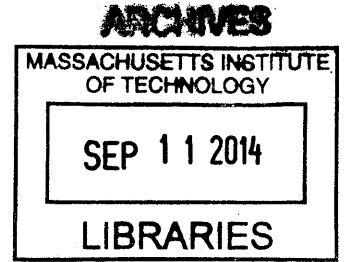
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# Single-cell Studies of Inflammatory Caspase Activity

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## 1. Abstract

Caspases are a 12-member family of human proteases that regulate apoptosis and inflammation. They serve as key effectors downstream of diverse signaling receptors and shape cell fate. Inflammatory caspases mediate the proteolytic processing of inflammatory cytokines and are essential in maintaining immune function, but also lead to disease when deregulated. In order to examine the activity of inflammatory caspases, we generated 2 inflammatory caspase reporters: a fluorescence resonance energy transfer (FRET) inflammatory caspase activity reporter as well as a fluorescent translocation inflammatory caspase reporter. These reporters were then used to study inflammatory caspase activity *in vitro* using recombinant caspases and *in vivo* using a simplified cell culture model. The inflammatory caspase activity reporters have the potential to capture inflammatory caspase activation under a variety of stimuli. They also have several advantages compared to existing methods: they are non-destructive and can be used for live single cell measurements; they do not require the addition of exogenous chemicals or cofactors; and they do not covalently modify the inflammatory caspases. Inflammatory caspase activation is a rapid, asynchronous process, and detecting the activity of the mature inflammatory caspase molecules is made difficult due to the short half-life of the enzyme. The reporters we have developed can fill this need.

## 1: Construction of reporters selective for inflammatory caspase activity.

We generated genetically-encoded reporters based on fluorescence resonance energy transfer (FRET) as well as fluorescent translocation reporters for the inflammatory caspases. The FRET reporter had a design analogous to existing CFP/YFP FRET protease reporters. The translocation reporter contains the same inflammatory caspase-specific substrate sequence, flanked by a nuclear localization signal and a fluorescent protein.

## 2: Validation of reporters.

The FRET reporter was studied *in vitro* using purified recombinant caspases. The translocation reporter was studied *in vivo* using a HeLa reporter cell line that stably expressed the inflammatory caspase translocation reporter. This reporter cell line was then transiently transfected with caspase-1 and the expected change in intracellular distribution of the fluorescent reporter (translocation to the nucleus) was observed. Specificity was studied for both reporters by using recombinant caspase 8 in *in vitro* studies and by stimulating endogenous caspase-8 activation in HeLa cells treated with TNF-related apoptosis-inducing ligand (TRAIL). In both cases, the inflammatory caspase reporters retained selectivity for caspase-1, suggesting that these reporters are selective for inflammatory caspase activity.

## 2. Background and Significance

Proteases were critically important in early biotic environments, enabling the recycling of scarce amino acids, and thus are encoded in every organism and found in every compartment of eukaryotic cells [1]. Proteases and their regulatory genes represent more than 2% of the total human genome [2], comparable to the kinase complement. Caspases are a large, conserved family of aspartate-specific cysteine proteases originally discovered in *C. elegans* that primarily regulate programmed cell death. Caspase-3, -6, -7, -8, and -9 regulate apoptosis, while the inflammatory caspases, caspase-1, -4, -5, and -12, are activated at inflammasomes and process inflammatory pro-cytokines, particularly interleukin-1 $\beta$ , thereby rendering them biologically active. Inflammasomes are large cytoplasmic complexes centered on NOD-like receptors that detect microbial signals as well as nonmicrobial danger signals in the cytosol, and they include adaptor proteins that promote the recruitment and activation of inflammatory caspases [5] (see Figure 1).

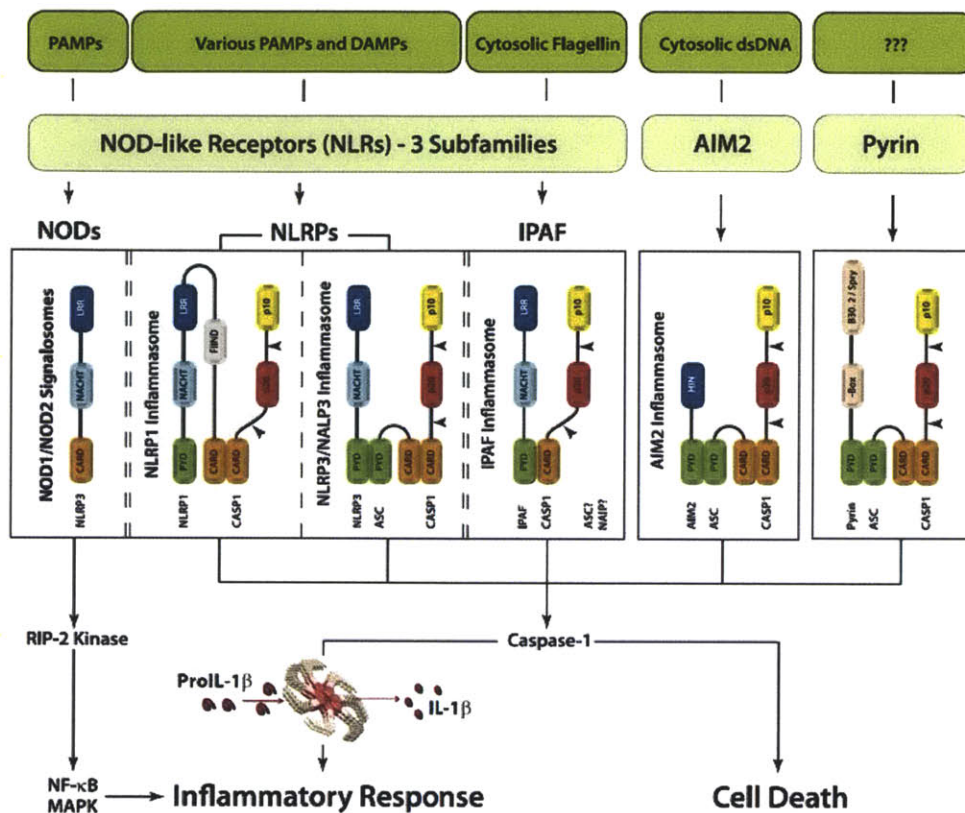


Figure 1: Inflammasome families and known stimuli. Note that most inflammasomes converge on caspase-1 activation. From [5].

Inflammasomes are key regulators of the innate immune response, and chronic activation of these multiprotein complexes has been linked to a variety of chronic diseases, such as atherosclerosis and diabetes [3], while germline mutations in inflammasomes lead to severe autoinflammatory diseases [4]. Similarly, there is genetic evidence for the physiological importance of inflammatory caspases themselves. Deletion of the telomeric portion of chromosome 11, which contains the inflammatory caspases (11q22.3-q25), is strongly correlated with poor prognosis in lung cancer and has also been identified as a contributing factor to metastatic disease in neuroendocrine tumors [6] (see Figure 2).

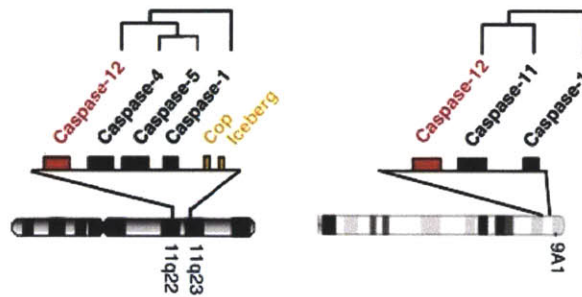


Figure 2: Depiction of inflammatory caspase genes on human chromosome 11 and murine chromosome 9. From [4].

Inflammasome activation leads to the processing of inflammatory pro-caspase-1, generating a mature active caspase-1 that cleaves cytokines which in turn induce inflammation. By a mechanism that remains only partly understood, caspase-1 activation can also induce inflammatory cell death that involves limited apoptotic caspase-7 activation [7]. As key effectors of inflammation, the activation of inflammatory caspase-1 is tightly regulated by a series of feedback mechanisms, including export via microvesicles, proteasomal degradation, and by competitive inhibitors known as Card-only or Pyrin-only proteins. Surprisingly, these inhibitory proteins are evolutionarily recent and are restricted to higher primates (as are caspase-4 and -5 themselves). Several of these inhibitors are located at the same locus as the inflammatory caspases and are therefore likely to have arisen as a result of duplication and divergence events and strong selective pressures [8] (see Figure 2). Bacteria also encode effector proteins that specifically repress the activity of inflammatory caspases directly, implying that preventing inflammatory caspase activation is important in limiting immune function and promoting infection [9].



## 2.1 Initiator vs Effector Caspases: Importance of Order and Activation Cascades

Caspases are synthesized as inactive zymogens and must undergo post-translational activation via proteolytic cleavage. Based on the presence of large prodomains and on the order of their activation, apoptotic caspases have been classified into two families: initiator caspases and effector caspases [10]. In apoptosis, initiator caspases (caspase-8, -9, and -10), also known as “apical caspases,” assemble on upstream signaling receptors and undergo autocatalytic processing and activation. Initiator caspases cleave and activate effector pro-caspases, which then degrade the proteome and kill cells. Activated effector caspases can cleave and activate additional initiator and effector caspase molecules, thereby generating positive feedback that pushes cells irreversibly towards cell death [20]. Structurally, initiator caspases possess extended N-terminal prodomains (90 or more amino acids in length) while effector caspases only have short prodomain sequences that are only 20–30 amino acids in length [10]. By having tightly regulated initiator caspases activate highly active effector caspases, cells generate an irreversible and self-amplifying signal.

## 2.2 Inflammatory Caspases and Order of Activation

Based on their large prodomains, inflammatory caspases are all considered to be members of the initiator caspase family (see Figure 3). Having been discovered first, caspase-1 has received the most attention. However, mounting evidence suggests that caspase-4 and caspase-5 act either upstream or concomitantly with caspase-1. In cell-free extracts, the mature forms of caspase-4 and caspase-5 appear before the mature form of caspase-1, although the order

of appearance in whole cells is unknown [11]. To resolve this uncertainty, methods that provide single cell resolution are required.

Caspase-4 and caspase-5 are ~60% homologous to murine caspase-11 [13]. A widely studied caspase-1 knockout mouse was recently found to actually be a caspase-1/caspase-11 double knockout, suggesting many of the functions attributed to caspase-1 may in fact belong to caspase-11 [7]. Pro-caspase-11 expression appears to be tightly regulated. It is below the detection limit in unstimulated cells, but its expression is strongly increased upon systemic administration of LPS and other stimuli. Caspase-11 knockout mice have similar phenotypes to Casp-1<sup>-/-</sup> mice; they are resistant to LPS-induced endotoxic shock. Casp-11<sup>-/-</sup> embryonic fibroblasts are also resistant to apoptosis induced by ectopic expression of pro-caspase-1, suggesting that caspase-11 is an upstream activator of caspase-1 [15].

It is unclear whether caspase-11 proteolytic cleavage is necessary for its activation. The active p20 and p10 subunits are generally not observed even in cells that have undergone morphological changes and cytokine secretion as a direct result of its activation; this is presumably due to rapid degradation or expulsion from the cell (13). Other long prodomain caspases, such as caspase-9, are also thought to be activated without cleavage, in contrast to caspase-8/-10 which are true zymogens [14]. Thus, attempting to detect mature inflammatory caspase by Western blot is not always a good measure for activation.

Analysis of the inflammatory caspase degradome using mass spectrometry has identified many caspase-1 substrates (over 70) while fewer than 10 substrates were found for caspase-4 and caspase-5. In biochemical assays against synthetic tetrapeptide substrates, caspase-4 and -5 are 100-fold less active than caspase-1. Taken together, these data suggest that the less active caspase-4/-5 could function upstream of caspase-1 [12].

Recent work has demonstrated that human caspase-4 expression is required for activation of caspase-1 via inflammasomes in keratinocytes and in the monocyte-like THP1 cell line, which suggests that caspase-4 has a general role in inflammasome activation [16]. Caspase-4 appears to act upstream of caspase-1 and the inflammasome, akin to murine caspase-11. In cotransfection experiments, casase-4 has been shown to cleave pro-caspase-1, although this result may not have physiological significance given the reliance on overexpression [17].

Caspase-5 and caspase-1 have been shown to mutually regulate each other in their protein expression and activation. Knockdown of caspase-5 by shRNA also reduces caspase-1 expression and activation. Additionally, proIL-1 $\beta$  processing occurred most efficiently when both caspase-1 and caspase-5 are coactivated, suggesting that both inflammatory caspases are involved in the generation of active IL-1 $\beta$  [5]. Given that caspase-5 does not cleave IL-1 $\beta$  directly, this is further evidence that it acts upstream of caspase-1 [15]. It has also been shown in hypoxic hepatocytes that caspase-5 can activate caspase-3 and caspase-7, and that knockdown of caspase-5 reduces caspase-3 and -7 activities [18]. This represents a potential link to apoptosis. A recent study of psoriatic skin found that caspase-5 expression was increased 20-fold in lesional psoriatic skin compared with normal skin, while caspase-1 and caspase-4 were upregulated by 1.5 and 1.9 fold respectively [19]. Only mildly elevated levels of the full-length caspase-5 zymogen protein were detected, while the cleaved mature form of caspase-5 was not detected. In similar work with murine caspase-11, the cleaved caspase-11 itself was inconsistently detected, indicating the mature caspase is unstable [13]. Mature human caspase-1 is also known to be unstable, having a half-life of 9 minutes which contrasts sharply with the 8 and 11 hour half-lives of apoptotic caspase-3 and caspase-7, respectively (the latter being a caspase-1 substrate itself) [11].

## 2.3 Inherent Biochemical Challenges of Studying Inflammatory Caspase Activity

Caspase-1 does not appear to be an all-or-nothing switch. Caspase-1 activation primarily results in cytokine maturation and pyroptosis, a form of inflammatory cell death. The use of active site mutants (which are unable to cleave substrates) has demonstrated that caspase-1's enzymatic activity is important for both of these processes. Some investigators have proposed that there are feedback mechanisms that vary across cell types to prevent caspase-1 activation from causing cell death in some cells, such as monocytes (33). Pyroptosis, which commonly results from intracellular infection of macrophages, can be prevented by effector proteins produced by pathogens (such as cowpox-produced CrmA), while endogenous proteins can also directly inhibit caspase-1 by binding to its CARD domain interfering with oligomerization. Such proteins include Iceberg and COP, which are induced by inflammatory stimuli and thus regulated in a signal-dependent fashion [30]. Hence, in cases of cell death, inhibitory mechanisms such as these might act to set a threshold for regulating the deadly effects of caspase-1. The use of active site mutants (which are unable to cleave substrates) has demonstrated that caspase-1's enzymatic activity is important for both of these processes, and thus it is feasible that the outcome of caspase-1 activity is a direct result of the level of caspase-1 activity (which may be a function of the amplitude of caspase-1 activity or the duration of its activation).

Pyroptosis strictly requires caspase-1 and does not require classical apoptotic caspases such as caspase-3 and -8 [7]. Instead, activated caspase-1 activates caspase-7 and an unidentified nuclease that induces DNA cleavage and nuclear condensation without compromising nuclear integrity [8]. An additional problem in our understanding of caspase involvement in inflammasome signaling is that caspase-8 (nominally an apoptotic caspase) is also activated by

the inflammasome and can cleave and activate IL-1 $\beta$  in the absence of caspase-1 [15]. Surprisingly, this activation does not occur downstream of inflammatory caspase activation: caspase-8 directly interacts with inflammasome proteins [16]. Thus, caspase-8 may have an unappreciated role in inflammasome signaling, while caspase-1's role in cytokine maturation may in fact be secondary to its cell death function.

While the apoptotic and inflammatory caspases share certain features, they differ significantly in the kinetics of their activity. Specifically, unlike the apoptotic caspases, the physical presence of mature inflammatory caspases does not correlate with proteolytic activity. Thus, while it is appropriate to look at the accumulation of the mature apoptotic caspases by blotting for the active subunits (usually generated after activating cleavages of the apoptotic caspase proform), measuring the accumulation of the mature inflammatory caspases as a proxy for inflammatory caspase activity can lead to an overestimate of caspase activity due to an apparent spontaneous loss of activity over time as observed in cell-free extracts (see Figure 3).

Besides the issue of caspase activity being a false positive, *in vitro* studies of recombinant inflammatory caspases cleaving synthetic substrates suggests that the inflammatory caspases have a half-life that is an order of magnitude less than the apoptotic caspases (8 minutes vs 8 hours for caspase-1 and caspase-3/-7, respectively) [11]. Thus, to attempt a time series to show activity levels over time must be rapid enough to capture potentially short, intense bursts of activity.

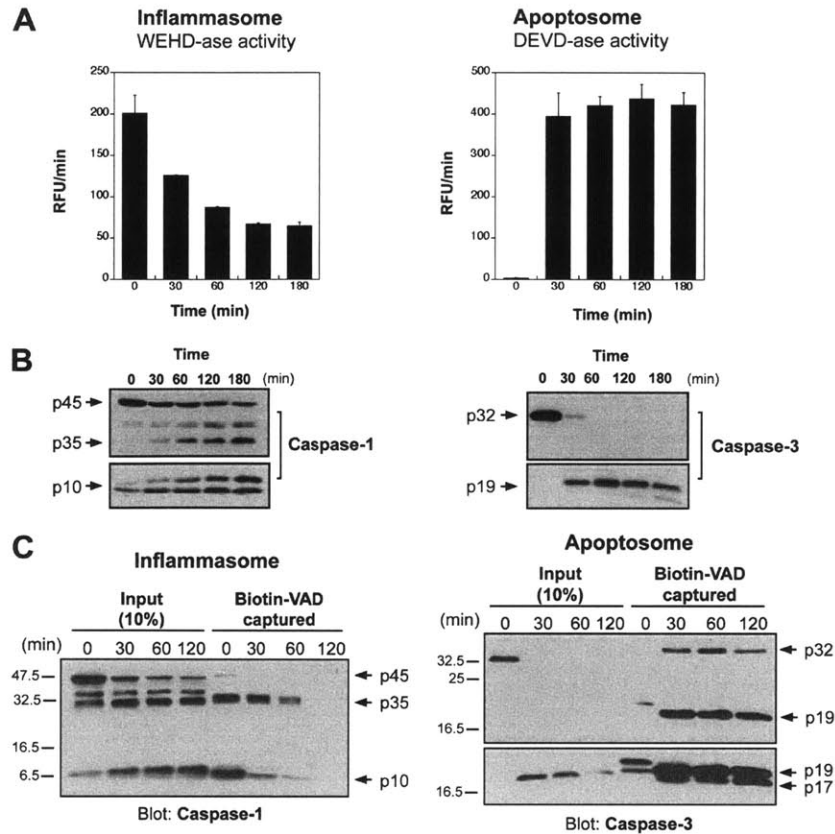


Figure 3: “Time-dependent loss of caspase activity occurs upon activation of inflammasomes, but not apoptosomes, in THP cell-free extracts. A, following activation of inflammasomes (by incubation at 37 °C) or apoptosomes (by addition of 50 µg/ml cytochrome c, 1 mM dATP, 37 °C) in THP-1 cell-free extracts, caspase activity was measured at the indicated time points by hydrolysis of WEHD-AMC or DEVD-AMC. B, using the same approach employed above in A, samples were collected at the indicated times to examine caspase-1 or caspase-3 processing by SDS-PAGE followed by immunoblot analysis. C, following activation of inflammasomes or apoptosomes as described in A, samples (50 µl) were taken at the indicated time points and were incubated for 30 min at 37 °C with biotin-VAD (10 µM) to label active caspases. Biotin-VAD-labeled caspase was then captured using streptavidin beads followed by immunoblotting for the appropriate caspase to determine labeling efficiency.

*Input amounts represent 10% of the total amount of caspase available for capture within the cell-free extracts." Reproduced from [11].*

In summary, there are 3 main biochemical challenges in examining inflammatory caspase activation. Firstly, given the functional overlap with apoptotic caspases, it can be difficult to connect observations to inflammatory caspase activity alone without controlling for the activation of apoptotic caspases. Secondly, the lifetime of the activated inflammatory caspases appears to be short. Thirdly, the accumulation of the mature caspase does not directly reflect the observed proteolytic activity. The disparity in the stability of the the pro-form and active forms further complicates interpretation.

#### 2.4 Existing Methods for Measuring Caspase Activity

Thus, to meaningfully measure the activity of a caspase, one can monitor the cleavage of its substrate. Monitoring the site-specific cleavage of a protein is generally difficult to accomplish in a quantitative manner. The most common method of monitoring protein cleavage is simply size-separation of proteins via gel electrophoresis with subsequent detection (eg a Western blot). However, this is an inherently slow, low-throughput process that measures a population-wide change at a fixed timepoint. Additionally, preparing the samples is destructive, making it impossible to detect protease activity in the same cell through time. Using time-resolved measurement of fluorescence to quantify caspase activity can address some of these shortcomings.

Another method for quantifying caspase activity is bimolecular fluorescence

complementation, wherein split fluorescent protein domains that are individually not fluorescent are fused to interacting proteins (in this case, caspase monomers). When the interacting proteins dimerize, the halves of the fluorescent protein reassemble, generating a fluorescent protein. However, the kinetics of this process are very slow: reassembly and fluorescence takes on the order of 24 hours [21]. Thus, events that occur on the timescale of minutes to a few hours (such as inflammasome-mediated caspase activation) are obscured.

Several groups have created activity based probes that are small chemical molecules, some of which are cell permeable. These fluorescently labeled probes are based on pharmaceutical inhibitors of caspase-1, and thus label active caspase-1 by irreversible covalent binding that destroys caspase activity. Besides the fact that some cannot enter cells and are thus only useful when examining lysed cells, the use of a caspase-1 inhibitor is by definition only useful for an endpoint assay. Simplistic colorimetric reagents, such as the Caspase-Glo assay, also require lysis of cells and lack specificity for caspase-1 and report generally on caspase activation.

Another recent development is luciferase-based biosensors. Although genetically encoded, these reporters require exogenous materials (namely, supplemental luciferin). They provide high signal to background, which makes these reporters good for examining populations of cells but not ideal for single cell studies. Additionally, the signal is not proportional to the level of caspase activity. Furthermore, the timescales not always reflective of the underlying caspase-1 kinetics due to a reliance for the biosensor to go from an unfolded to folded state. This inherent lag in signal generation is clearly problematic for realtime studies.

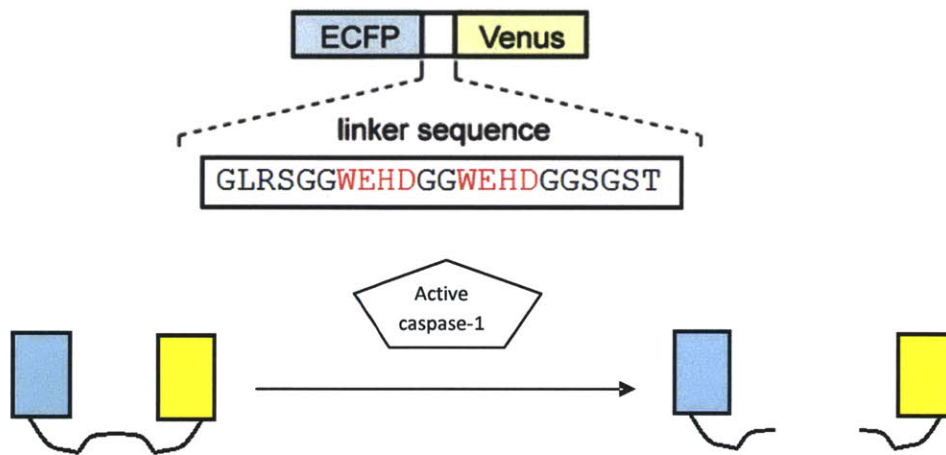
Thus, given the lack of reporters capable of measuring caspase activity temporally in living single cells, as well as capable of comparing multiple caspase activities simultaneously,



we generated both FRET and translocation reporters for inflammatory caspase activity.

## 2.5 Design of Novel Inflammatory Caspase Reporters

### A. FRET reporter



### B. Translocation reporter

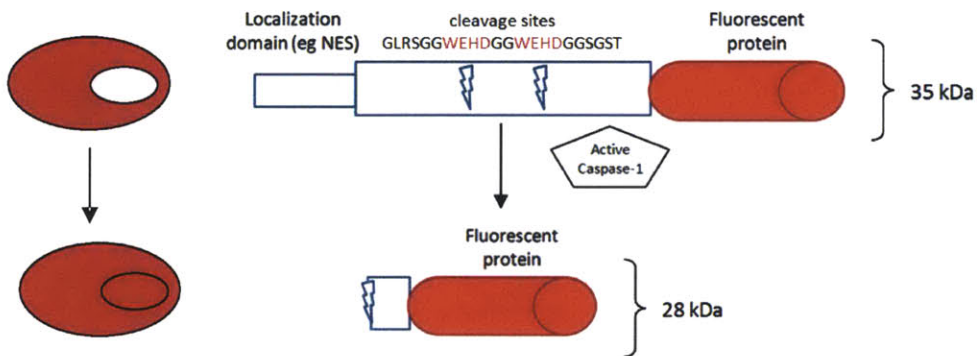


Figure 4: These schematics portray the design of the FRET inflammatory caspase activity reporter (A) and inflammatory caspase translocation reporter (B). Cleavage by active inflammatory caspase-1 causes a clear change in these biosensors, which provides a single live cell realtime signal.

The choice of an appropriate target sequence is essential for ensuring adequate caspase specificity. Positional scanning peptide substrate libraries were tested against recombinant caspases in order to establish the substrate specificities of all caspases, and the inflammatory caspases clearly differed from the apoptotic caspases in terms of optimal substrate [19, 20]. Thus, we used a tandem repeat of the optimal peptide substrate sequence WEHD to generate our inflammatory caspase activity reporters (Figure 4). The use of the proper target sequence makes it possible to attain sufficient specificity and time resolution using either the FRET or translocation reporter.

An established approach to detecting caspase activity is to measure the loss of Förster resonance energy transfer (FRET) between peptide-linked fluorescent proteins flanking a caspase target site; cleavage of the target site leads to a loss of FRET signal, indicating caspase activity [20]. Thus, to investigate inflammatory caspase signaling, we generated a CFP-YFP FRET cleavage reporter for Caspase-1. The choice of an appropriate target sequence is essential for ensuring adequate caspase specificity. Accordingly, we selected the experimentally-derived optimal cleavage sequences for inflammatory caspases based on the literature [12]. The recombinant reporter was constructed and tested *in vitro* as described below and demonstrated good specificity for inflammatory caspase-1 compared to apoptotic caspase-8 (see Figure 6).

However, in order to enable the study of the order of caspase activation the ability to detect the activity of multiple caspases in realtime is required. This can be achieved by multiplexing the caspase activity reporters. We have found that FRET reporters are not suitable for multiplexing because each reporter's donor-acceptor pair requires 2 often overlapping channels out of the 3 total channels available for live cell experiments.

The overall architecture of the translocation reporters is a localization domain and a fluorescent protein flanking a central caspase cleavage sequence. When the central target sequence is cleaved by an active caspase, the free fluorophore will translocate into the nucleus rapidly [18]. When combined with a nuclear counterstain, this permits automatic image segmentation. Given that the timescale of pyroptosis *in vivo* is 30 minutes, the translocation step (which takes 1-2 minutes) will be fast enough to give meaningful data. Apoptosis is a much slower process that takes an hour or more, so it will be straightforward to monitor that process as well.

By using fluorescent caspase-cleavage translocation reporters to generate single cell time courses of simultaneous caspase activities, we can begin to explore fundamental questions about caspase activation. As in apoptotic caspase signaling, using inflammatory caspase reporters will allow us to infer the order of inflammatory caspase activation, as well as study the activation of inflammatory caspases upstream of apoptotic caspases.

The use of single cell techniques is essential for ordering the inflammatory caspases because their activation is asynchronous and rapid; it cannot be resolved using bulk measurements (see Figure 5). In addition, the apparent instability of the mature caspases precludes endpoint assays. With the realtime data from these reporters, we can determine the order of caspase activation downstream of the inflammasome, and how quickly they are activated. By studying these processes *in vivo* in stem cell-derived macrophages, we can study caspase activation in the context of inflammatory signalling.

## Single cell vs bulk

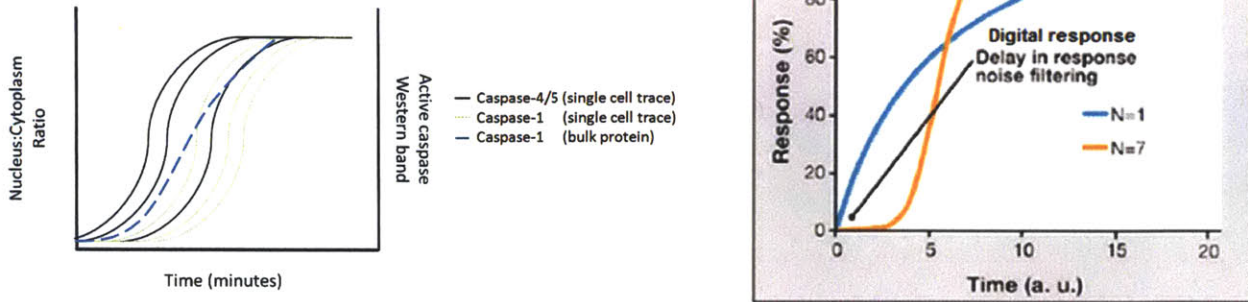


Figure 5: A) Bulk measurements provide aggregate levels of caspase expression or activity, but fail to capture important cell-to-cell variation. The use of single cell reporters thus provides important resolution, which can drive insight into how inflammatory caspase activity maps to cell fate. Single-cell realtime reporters can also reveal important characteristics of inflammatory caspase activity, such as time delays.

### 3. Research Design & Methods

#### 3.1 Fluorescence Resonance Energy Transfer Reporter for Inflammatory Caspase Activity

##### Restriction Enzyme Cloning

The inflammatory caspase FRET reporter was constructed from IC-RP, an apoptotic caspase reporter [20]. The plasmid was constructed from pECFP-C1 with a Venus YFP between BamHI and XbaI restriction sites. A linker encoding the WEHDGGWEHD tandem inflammatory caspase cleavage sequences as a BspEI-BamHI fragment was added between the CFP/YFP fluorescent protein FRET pair. As with the existing reporter, serine and glycine residues were added next to the caspase cleavage sequence to ensure a flexible linker.

## **Recombinant Protein Production**

The inflammatory caspase FRET reporter was subcloned in a pET-28 vector as an AgeI and XbaI fragment in order to allow for recombinant protein production in a bulk E Coli liquid culture. This IPTG inducible plasmid allowed for high expression levels of FRET reporter construct. IC-RP, the apoptotic initiator caspase reporter, was also subcloned into pET-28. After a standard bacterial transformation onto a kanamycin containing agar plate, single colonies for both reporters were used to inoculate 10 mL liquid cultures overnight at 37°C. These starter cultures were then added to 250mL liquid cultures and incubated at 37°C for 3-4 hours until the A550 was between 0.5-0.7. 1mM IPTG was then added to induce protein expression and liquid cultures were incubated at 37°C for an additional 3-4 hours. Liquid culture was spun down using centrifugation, and cells were lysed in order to perform immunoprecipitation with GFP-Trap beads, which enable the purification of proteins containing GFP or its derivatives (in this case, CFP and YFP).

## ***in vitro* Caspase Digestion Assay**

The immunoprecipitated FRET report protein was then dialyzed into a caspase activity buffer [as in 32]. A Bradford assay was performed to normalize the concentrations of the inflammatory and apoptotic caspase reporters by adding caspase activity buffer. Recombinant caspase-1, -4, and -8 were purchased from Enzo Biosciences. The enzymatic digestion reaction was incubated for 3 hours at 37 °C. 3 units of Caspase-1 and Caspase-8 were added to cuvettes with the IC-RP and inflammatory caspase reporter proteins both present in substantial excess (0.5 mM). The CFP-YFP fluorescence resonance energy transfer was monitored using a fluorescent plate reader.

## Western Blotting

Digests were performed as above using 0.25 mM of the substrate proteins and 3 U of recombinant caspase-1, -4, and -8. After a 90 minute incubation at 37°C, the fractions were quenched with 4x SDS sample buffer. Samples were then added to a 12% polyacrylimide gel for electrophoretic separation. The gel was then transferred to a PVDF membrane overnight and blotted with a GFP antibody that recognizes CFP / YFP.

### 3.2 Fluorescent Translocation Reporter for Inflammatory Caspase Activity

We constructed a translocation reporter via Gibson assembly with a cytoplasmic localization domain (a nuclear exclusion signal) and an mCherry fluorescent protein (as in [22]) flanking a central inflammatory caspase cleavage sequence (WEHD; see Figure 4). When the central target sequence is cleaved by an active caspase, the fluorescent protein can translocate into the nucleus. A blue fluorescent protein-tagged histone can be included to enable effective segmentation of the nucleus and cytoplasm. Reporter levels are then determined by measuring the intensity of the same segmented regions in both channels [18]. Quantitative caspase activity data can then be extracted from live-cell movies using an analysis pipeline already in place in the lab for apoptotic caspases. Other investigators have generated data for caspase-8 and caspase-10 simultaneously using translocation reporters. The translocation step only takes 1-2 minutes, which is fast enough to give meaningful data [22].

### **Stable cell line generation**

Stable 293T and HeLa expressing the translocation reporter were produced by transfecting wild-type cells with the reporter plasmid and performing a chemical selection using puromycin. Single chemically selected cells were subsequently plated to 96 wells plates using flow cytometry in order to generate clonal stable reporter cells.

### **Imaging assay**

The reporter cells were transiently transfected with a caspase-1 expression plasmid to generate active caspase-1 as in [31], alongside a separate plasmid encoding GFP to serve as a technical control for transfection. After 16 hours of incubation post-transfection, cells were fixed using paraformaldehyde and imaged using the Perkin-Elmer Operetta High Content Imaging System.

## 4. Results

### 4.1 *in vitro* Results for Inflammatory Caspase FRET Reporter

The FRET reporter demonstrated good selectivity for caspase-1, and caspase-8 did not significantly cleave the inflammatory caspase FRET reporter. However, the recombinant caspase-1 did cleave the caspase-8 reporter. This may not be an issue *in vivo* since endogenous caspase-1 is an unstable enzyme and its activity has a short half-life. The recombinant caspase-1 used for this *in vitro* study was modified to make it more stable and retain consistent activity.

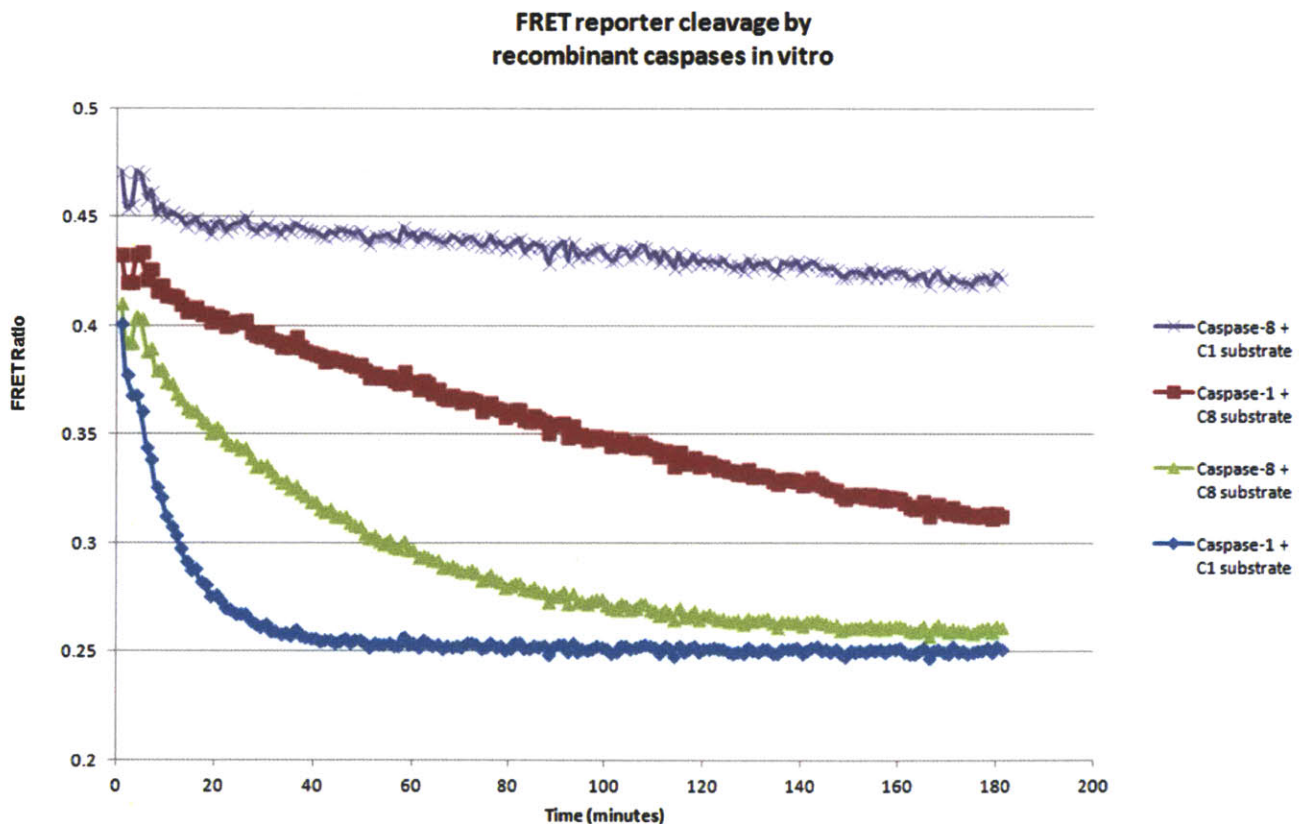




Figure 6: *In vitro* characterization of FRET caspase cleavage reporter. Blue and green represent caspase-1 and caspase-8 with matched substrates, red and purple are controls to show off-target cleavage by caspase-1 and -8 respectively. Caspase-8 is clearly specific and does not significantly cleave the caspase-1 substrate, but caspase-1 does cleave the caspase-8 substrate (albeit not nearly as well as caspase-8 itself).

A Western blot of *in vitro* digestion with recombinant caspase-1, -4, and -8 shows that caspase-1 and caspase-4 cleave the inflammatory caspase FRET reporter as expected. However, caspase-4 (which was used in equal amount to caspase-1) appears to be a less effective protease, as indicated by the presence of uncleaved reporter.

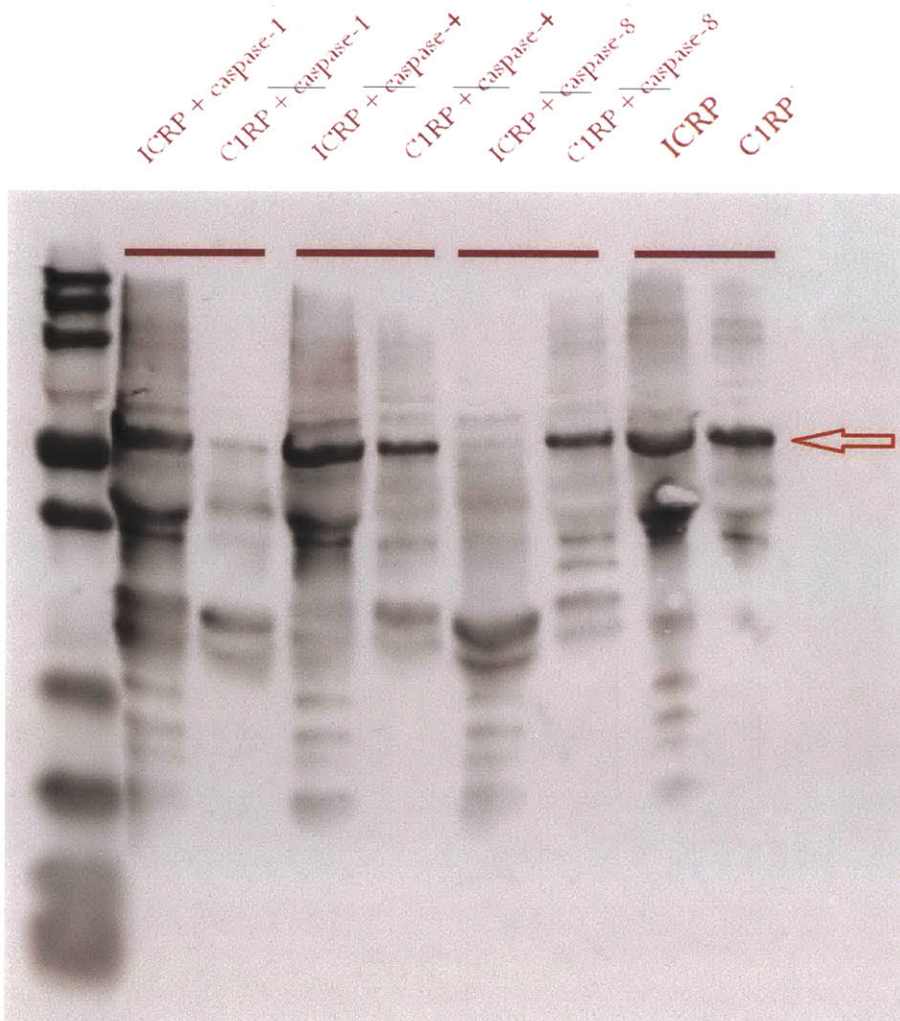
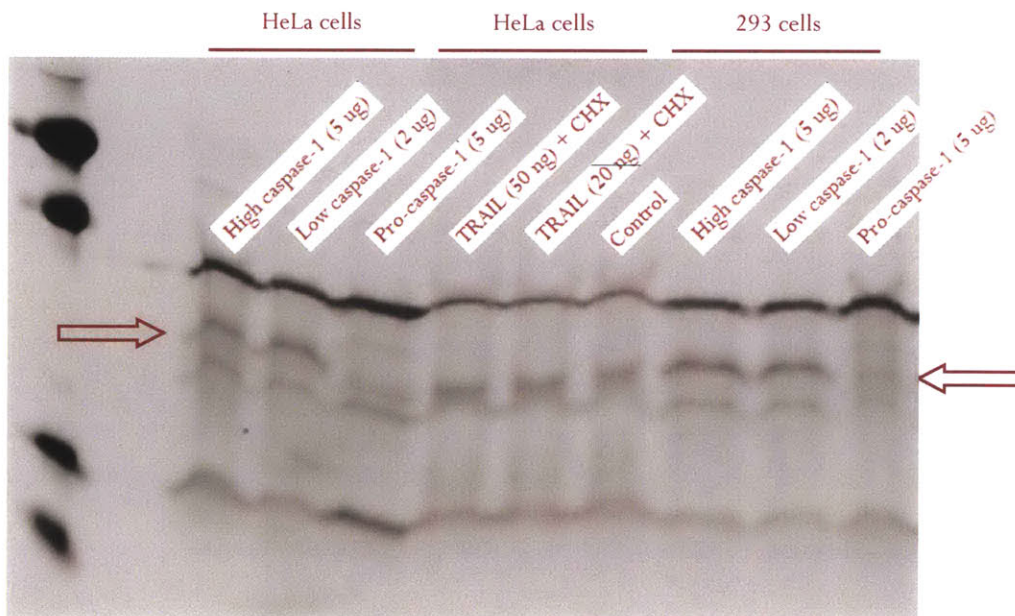


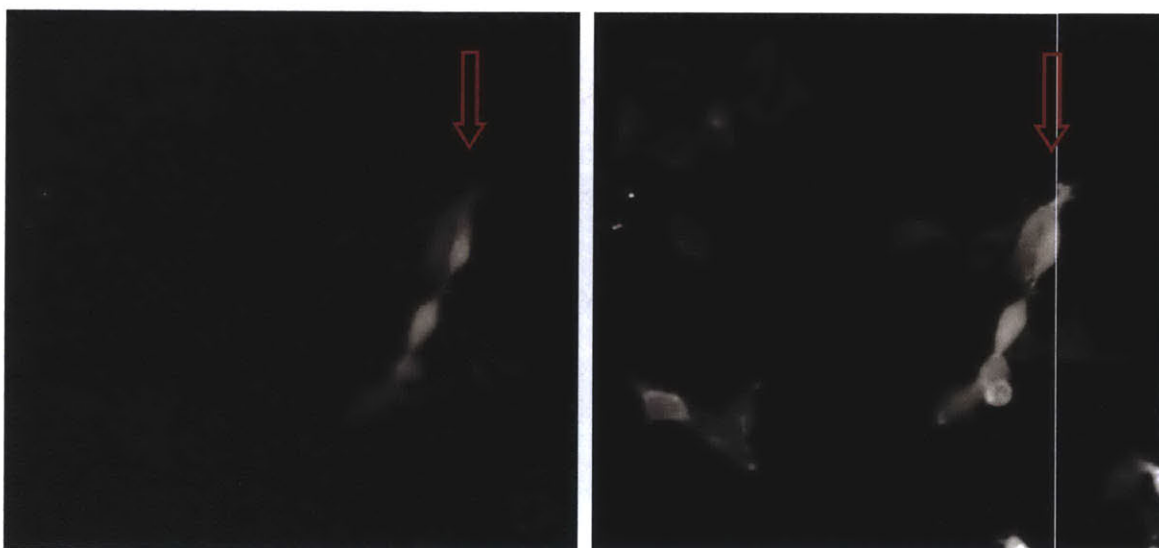
Figure 7: Western blot analysis of IC-RP (apoptotic caspase FRET reporter) and inflammatory caspase reporter (CIRP) digested *in vitro* by caspase-1, -4, and -8 for 90 minutes. Main band represents uncleaved CFP-YFP protein.

#### 4.2 *in vivo* Results for Inflammatory Caspase Translocation Reporter

The inflammatory caspase translocation reporter was cleaved by transiently transfected caspase-1 in the HeLa and 293 reporter cells, but was not cleaved by endogenously produced caspase-8 when stimulated with TRAIL (Figure 8). In the imaging assay, many of the cells that were transiently transfected (left panel of Figure 9) had an even distribution of the translocation reporter in the cytoplasm and nucleus, confirming that the reporter can capture inflammatory caspase activation in single cells.



*Figure 8 (above): Western blot of lysates from HeLa and 293T reporter cell lines that were transiently transfected with caspase-1 for 24 hours or treated with varying doses of TRAIL and cyclohexamide for 24 hours stained for mCherry. Band at 35 kDa represents uncleaved translocation reporter, and band at 28kDa represents translocation reporter cleavage product.*



*Figure 9: Image of cells transiently transfected with caspase-1 after 16 hours. Panel A: GFP was co-transfected as a control. Panel B: mCherry translocation reporter shows even distribution in transfected cells while remaining in the cytoplasm in untransfected cells.*

## 5. Concluding remarks

By creating inflammatory caspase activity reporters, I was able to detect the activity of inflammatory caspases in live single cells. In the appropriate cellular context, these reporters will be report on inflammatory caspase activation in live single cells in real time. Thus, these reporters should prove to be valuable tools in studying inflammatory cell death. Moreover, the translocation reporters have the potential to be multiplexed with existing apoptotic caspase reporters and thus allow for detailed study of the crosstalk between apoptotic and inflammatory caspases, which will conclusively establish the individual roles of caspases in determining cell fate. By monitoring caspase activity in a specific, temporally resolved manner we can understand the interplay between apoptotic and inflammatory caspase activity and begin to delineate the pathways that drive inflammatory disease pathology. A key advantage of our reporters is that they do not appear to be sensitive to caspase-8 activity, which makes them better than existing reporters based on the canonical IL-1B sequence which is known to be cleaved by caspase-8 [31]. Additionally, our translocation reporter also uses a single fluorescent channel, enabling multiplexing. This is important in order to enable the monitoring of multiple reporters simultaneously, especially in light of the potential for caspase-8 involvement in inflammatory cytokine maturation. To truly test these reporters will require the generation of transgenic mouse or differentiation of a stem cell precursor, as the mature immune cells that express caspase-1 endogenously are resistant to genetic modification.

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