Topic Introduction

Imaging-Based Methods for Assessing Caspase Activity in Single Cells

Melissa J. Parsons,^{1,2} Markus Rehm,^{3,4} and Lisa Bouchier-Hayes^{1,2,5}

¹Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, Texas 77030; ²Department of Pediatrics-Hematology, Baylor College of Medicine, Houston, Texas 77030; ³Centre for Systems Medicine, Royal College of Surgeons in Ireland, Dublin 2, Ireland; ⁴Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin 2, Ireland

Caspases, a family of proteases that are essential mediators of apoptosis, are divided into two groups: initiator caspases and executioner caspases. Each initiator caspase is activated at the apex of its respective pathway, which generally leads to the cleavage and activation of executioner caspases. Executioner caspases in turn cleave numerous substrates in the cell, leading to its demise. Initiator caspases are activated when inactive monomers undergo induced proximity to form an active caspase. In contrast, executioner caspases are activated by cleavage. Based on this key difference, different imaging techniques have been developed to measure caspase activation and activity on a single-cell basis. Bimolecular fluorescence complementation (BiFC) is used to measure induced proximity of initiator caspases, whereas Förster resonance energy transfer (FRET) permits the investigation of caspase-mediated substrate cleavage in real time. Because many of the events in apoptosis, including caspase activation, are asynchronous in nature, these single-cell imaging techniques have proven to be immensely powerful in ordering and dissecting caspase pathways. When coupled with parallel detection of additional hallmark events of apoptosis, they provide detailed and quantitative kinetic and positional insights into the signal transduction pathways that regulate cell death. Here we provide a brief introduction into BiFC- and FRET-based imaging of caspase activation and activity in single cells.

INTRODUCTION

At the heart of apoptosis lies a family of enzymes called “caspases,” which are cysteine-dependent aspartate-directed proteases. All caspases contain a catalytic cysteine required for their activity, and all cleave their protein substrates at specific aspartate residues (Alnemri et al. 1996). To date, 18 mammalian caspase proteins have been identified in animals and divided into initiator caspases (e.g., caspase-2, -8, and -9) and executioner caspases (caspase-3 and -7). We discuss here methods to study caspase activation and activity using single-cell imaging approaches.

Limitations of Conventional Approaches to Studying Caspase Function

Detecting the activation of specific caspases can be problematic. Because executioner caspases are activated by cleavage, monitoring the disappearance of the full-length protein and the appearance of its cleavage products by western blot is a *bona fide* measure of activation. In contrast, initiator caspases are activated not by cleavage, but rather by dimerization on recruitment to specific large molecular

⁵Correspondence: bouchier@bcm.edu

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mass complexes considered to be activation platforms. Activation platforms, each specific for a particular caspase, rely on the presence of protein–protein interaction domains in the adaptor proteins and in the caspases. In the extrinsic or death-receptor pathway, for example, the ligation of death receptors leads to the formation of an activation platform for the initiator caspase-8. This activation platform is a multimeric signaling complex called the “DISC” (death-inducing signaling complex; Kischkel et al. 1995). Its death effector domains interact with the corresponding domains of caspase-8, shown in Figure 1 (left pathway). Similarly, in the extrinsic or mitochondrial pathway, the multimeric complex known as the “apoptosome” functions as an activation platform for caspase-9 (Riedl and Salvesen 2007). Caspase-9 contains a protein–protein interaction domain, called the caspase-recruitment domain (CARD). When the CARD of caspase-9 binds to the CARD of Apaf-1, the interaction facilitates induced proximity and dimerization of the caspase-9 monomers, shown in Figure 1 (right pathway). Caspase-2 appears also to be activated on an activation platform, in this case termed the “PIDDosome,” which consists of PIDD and the adaptor protein RAIDD (Tinel and Tschopp 2004).

For the initiator caspases-2, -8, and -9, it has been clearly shown that cleavage of the caspase, while it does occur, is not sufficient for caspase activation (Stennicke et al. 1999; Chang et al. 2003; Baliga et al. 2004). Baliga and colleagues showed that only the dimeric species of caspase-2 is active, and that dimerization of a noncleavable mutant of caspase-2 retains only 20% of the activity of the wild-type protein (Baliga et al. 2004). Therefore, the use of western blot analysis to detect protein cleavage does not truly reflect the activation status of the initiator caspase.

Assays using fluorogenic caspase substrates and caspase inhibitors are widely used to determine caspase activities in cell populations and cell extracts. The design of these reagents is based on the predicted preferred substrate peptide motif for each caspase. These motifs are not very specific, however, as caspases substantially overlap in substrate specificities (McStay et al. 2008). Clearly, the results from such population-based analyses must be carefully controlled and interpreted with care. It has been shown that the executioner caspase, caspase-3, can cleave most fluorogenic caspase substrates with high efficiency and therefore bulk activity measurements tend to be dominated by executioner caspase contributions (McStay et al. 2008; for more discussion, see Introduction: **Measuring Apoptosis: Caspase Inhibitors and Activity Assays** [McStay and Green 2014]).

Furthermore, the onset times of caspase activation differ considerably among cells in clonal populations, so that bulk measurements of caspase activation do not reflect intracellular signaling kinetics (Rehm et al. 2002). However, imaging-based approaches for measuring caspase activation and activity can address these shortcomings by describing apoptosis signaling with higher specificity inside single living cells in space and time.

Advantages of Imaging for Monitoring Apoptotic Events

Single-cell imaging techniques have proved immensely powerful in ordering and dissecting events during apoptosis, many of which are asynchronous in nature. One of the main advantages of single-cell imaging over more conventional methods of studying cell death pathways is its ability to precisely measure these asynchronous events directly within the complex environment of the living cell. For example, in studies of the intrinsic (mitochondrial) pathway, live cell imaging was uniquely able to show that release of cytochrome *c* from mitochondria proceeds in individual cells at different times in response to a cellular stress, but, in each individual cell, the release was complete and kinetically invariant (Goldstein et al. 2000). Further analysis confirmed that mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release preceded executioner caspase activation in a manner that described the kinetics of these processes and their relative timing in great detail (Rehm et al. 2003). The availability of more sophisticated microscope systems that provide improved resolution, coupled with the development of enhanced fluorescent proteins and superior molecular tools, has enabled us to uncover even more and surprising details about these pathways. For example, higher resolution imaging of the release of the proteins Smac and Omi (other activators of caspases) from mitochondria that—unlike cytochrome *c*—are subject to proteasomal degradation on release, revealed that some mitochondria in fact remain intact. These intact mitochondria are essential for

FIGURE 1. Caspase activation pathways: schematic representation of caspase activation in the extrinsic pathway and the intrinsic pathway. In the death receptor or extrinsic pathway (shown on the *left*), ligands such as FasL, TNF, and TRAIL initiate apoptosis. Ligation of death receptors on the cell surface result in assembly of the death-inducing signaling complex (DISC), the activation platform for caspase-8. DISC assembly is mediated by specific protein-protein interactions. As shown the death domain (DD) in the receptor Fas binds to the DD in the adaptor protein FADD. FADD in turn binds to caspase-8 via death effector domains (DEDs) present in both sequences. Recruitment of caspase-8 to the activation platform leads to dimerization and activation of caspase-8. In the intrinsic or mitochondrial pathway (shown on the *right*), mitochondrial outer membrane permeabilization (MOMP) initiates cytochrome *c* release from the mitochondria. Cytochrome *c* promotes the assembly of the Apaf-1 apoptosome, which is the activation platform for caspase-9. Apaf-1 binds to caspase-9 via caspase recruitment domains (CARDs) present in both sequences. This binding promotes dimerization and activation of caspase-9. Caspases-8 and -9 directly activate executioner caspases, including caspase-3. Caspase-8 also induces cleavage of the protein Bid. Cleaved Bid can then induce MOMP leading to indirect activation of downstream caspases.

recovery from death signals when caspase activation is blocked (Tait et al. 2010). Such discoveries would have been impossible without recent advances in imaging techniques.

Adapting imaging techniques for direct measurement of caspases in single cells has been a little less straightforward. The main challenge has been to distinguish activation and activity associated with one specific caspase from general caspase activity in the cell. This concern is further confounded by the differences in how initiator and executioner caspases are activated. New techniques such as caspase bimolecular fluorescence complementation (BiFC) and improvements to more established tools such as Förster resonance energy transfer (FRET)-based caspase substrates have provided superior means to study activation and activity associated with specific caspases and to measure exactly when and where an individual caspase is activated in the cell. We summarize these imaging-based approaches below and describe them in detail in the accompanying protocols (see Protocol: **Measuring Initiator Caspase Activation by Bimolecular Fluorescence Complementation** [Parsons and Bouchier-Hayes 2015] and Protocol: **Measuring Caspase Activity by Förster Resonance Energy Transfer** [Rehm et al. 2015]).

CASPASE BiFC FOR MEASURING THE INDUCED PROXIMITY OF INITIATOR CASPASES

Caspase BiFC is an imaging-based technique that measures the proximal step in initiator caspase activation: the induced proximity of caspase monomers on recruitment to activation platforms. We describe caspase BiFC in detail in an accompanying protocol (see Protocol: **Measuring Initiator Caspase Activation by Bimolecular Fluorescence Complementation** [Parsons and Bouchier-Hayes 2015]). This strategy can be used to visualize the assembly of initiator caspase activation platforms in single cells and assess the conditions under which they form, their location in cells and their kinetic relationship to other steps in apoptosis such as MOMP. This method was developed to investigate the caspase-2 activation pathway; however, the same technique can be adapted to other initiator caspases (Bouchier-Hayes et al. 2009).

In BiFC, “split” fluorescent proteins are used to measure protein–protein interactions in cells (Shyu et al. 2006). The fluorescent protein Venus, a brighter and more photostable version of yellow fluorescent protein (YFP), is often used in these studies. In this approach, the fluorescent protein is separated into two fragments; of note, each fragment on its own is not fluorescent. When each fragment is fused to one partner of an interacting protein pair, these nonfluorescent protein fragments can associate, on interaction of the partners, to reform the fluorescent molecule. Caspase BiFC adapts the technique slightly—instead of measuring direct protein–protein interactions, it detects recruitment of the caspase to its activation platform. The caspase or portion of the caspase that binds to the activation platform (the interacting prodomain that contains a CARD or DED) is fused independently to both fragments of Venus. In untreated cells, caspase monomers fused to the Venus fragments remain separate from each other. On treatment with an activating stimulus (e.g., heat shock for caspase-2), the caspase fusion proteins are recruited to the activation platform via their protein–protein interaction domains. Once aligned at the activation platform, the induced proximity of caspase monomers results in association of the Venus fragments, thereby restoring its fluorescence (Bouchier-Hayes et al. 2009; see also Fig. 2A). This fluorescence readout can be used as a measure of caspase activation, allowing us to determine when and where caspase-2 activation occurs on a cell-by-cell basis.

Split Venus is particularly suitable for real-time imaging applications of caspase BiFC because the reassociation occurs relatively quickly (it has been detected in as little as 5 min [Schmidt et al. 2003]) and occurs under physiological conditions. In contrast, a split version of mCherry (a red-shifted fluorescent protein) requires incubation at 30°C for 30 min for complementation to occur (Fan et al. 2008); mCherry is therefore not suitable for real time imaging of caspase BiFC.

Caspase BiFC experiments must be carefully controlled for specificity of the interaction between each initiator caspase with its respective activation platform, for example, by disrupting the interaction between the caspase and its adaptor protein. One main advantage of this technique is that the

FIGURE 2. Imaging-based caspase activation assays. (A) Caspase BiFC. The model for measurement of caspase-2-induced proximity by the PIDDosome is shown. (B) FRET-based caspase substrate. (C) Cell-permeable caspase substrate. TcapQ647 is shown as an example.

molecular components of the activation platform do not need to be fully characterized to obtain meaningful data. Indeed, this technique can even be used to determine the relative requirements of each component of an activation platform. For example, RAIDD, the direct adaptor for caspase-2, induces robust caspase-2 BiFC. In contrast, PIDD (which recruits caspase-2 via RAIDD binding) can match the level of caspase-2 BiFC induced by RAIDD only if a small amount of RAIDD is also present, thus recapitulating the binding properties of the endogenous proteins. In the same way, the subcellular localization of initiator caspase induced proximity on recruitment to distinct activation platforms can be directly tested using this strategy.

FRET FOR MEASURING CASPASE ACTIVITY

FRET-based caspase substrates represent a second set of useful imaging tools for dissecting caspase activation pathways in real time. Unlike caspase BiFC, FRET probes can be used to measure the activity of executioner caspases as well as initiator caspase activation (Rehm et al. 2002; Albeck et al. 2008; Hellwig et al. 2008). We describe this approach in detail in an accompanying protocol (see Protocol: **Measuring Caspase Activity by Förster Resonance Energy Transfer** [Rehm et al. 2015]). Each FRET probe comprises a pair of fluorescent proteins joined by a peptide (also called the flexible linker sequence) corresponding to a caspase cleavage site (Rehm et al. 2002). The most popular FRET

pair consists of the cyan fluorescent protein and yellow fluorescent protein (CFP-YFP); here, CFP is the “donor molecule” and YFP is the “acceptor molecule.”

When the caspases are inactive, the CFP and YFP moieties are in close proximity and the CFP donor molecule directly transfers its excited state energy to the YFP acceptor molecule. Therefore, excitation of the donor CFP results in emission from the acceptor YFP, and the fraction of absorbed energy that is transferred to the acceptor is interpreted as the FRET efficiency. This technique is also referred to as sensitized emission-based FRET. At the same time, the transfer of energy to the acceptor reduces the CFP fluorescence quantum yield. Quantum yield, defined as the number of photons emitted per photons absorbed, is a measure of the efficiency of the fluorescence process. Caspase-mediated cleavage of the substrate peptide linking the two proteins causes their dissociation and results in a disruption of energy transfer, measured as reduced YFP emission and increased CFP emission on donor excitation (Fig. 2B). These fluorescence changes can be measured over time, with the onset of FRET disruption reflecting the time of caspase activation. Furthermore, the rate of substrate cleavage reflects the amounts of intracellular caspase activity (Rehm et al. 2002, 2006). Therefore, changes in FRET can determine both the timing and efficiency of caspase substrate cleavage, making this a very useful tool for interrogating caspase pathways in live cells.

Since the generation of the first caspase-cleavable FRET substrates, a number of further improvements have been made. CFP and YFP variants with higher quantum yields, such as Cerulean and Venus, have been developed; and the length of the flexible linker sequence between donor and acceptor has been optimized, yielding variants with 10-fold higher FRET efficiencies (Nagai and Miyawaki 2004). Traditional FRET measurements of protein–protein interactions can be prone to artifacts due to even small differences in the expression level of each fluorescent protein. In contrast, caspase FRET substrates ensure that donor and acceptor fluorophores are present at equimolar amounts, thereby significantly simplifying the analysis. Furthermore, the ratiometric analysis of fluorescence emission in sensitized emission-based FRET experiments is highly sensitive and can detect as little as 5% substrate cleavage using conventional wide-field fluorescence microscopes (Hellwig et al. 2010). The ratiometric analysis also corrects for unspecific noise that may arise from changes in cellular morphology, cell volume, and instrument focus drifts. As an alternative to ratiometric FRET analysis, FRET measurements of caspase-cleavable FRET substrates can also be conducted by fluorescence lifetime imaging microscopy (FLIM). This technique is sensitive to the lifetime of the excited state of the donor, which is shorter in the presence of the FRET acceptor. FLIM requires additional instrumentation, but, when adjusted properly, it can be more sensitive than ratiometric FRET measurements in the presence of sufficiently high fluorophore concentrations. However, FLIM measurements are less favorable for prolonged time-lapse imaging due to higher levels of phototoxicity.

The considerable overlap in the substrate specificities of each caspase means that the choice of substrate must be carefully considered when designing FRET-based caspase probes. It has been shown that changing the amino acid sequence of the linker from the caspase-3 preferred substrate, DEVD to DEVDR increased the selectivity for caspase-3 relative to caspase-8 by 20-fold (Albeck et al. 2008). Such modifications have dramatically improved the specificity of these probes. In addition, carefully designed control experiments, in which individual caspases have been depleted or post-MOMP caspase activation has been prevented, have provided the opportunity to determine the relative contribution of initiator and executioner caspases to FRET substrate cleavage in the same pathway (Rehm et al. 2002; Hellwig et al. 2008).

ADDITIONAL APPROACHES

Other approaches to measure caspase activity by live cell imaging include the use of cell-permeable fluorogenic caspase substrates and localized GFP-labeled caspase substrates that redistribute following cleavage. Several probes of these types have been developed and many are commercially or freely available. TcapQ647 is one example (Bullok and Piwnica-Worms 2005; Maxwell et al. 2009), comprising a Tat permeation peptide sequence, the caspase-3-preferred substrate DEVD, and an activat-

able dye pair consisting of the quencher QSY and the far-red emitting fluorophore Alexa Fluor 647 (Fig. 2C). When TcapQ647 is cleaved by caspases, the fluorophore is released from the quencher, enabling detection of caspase activity. The commonly used, commercially available probe FLICA (fluorescently labeled inhibitor of caspases) is available from Immunochemistry Technologies. FLICA probes consist of a preferred caspase substrate sequence and a fluoromethyl ketone (FMK) moiety that allows the probe to bind irreversibly to the active caspase conjugated to a fluorescent tag. Therefore, the probe is retained in cells only when bound to an active caspase. One advantage of this type of probe is that it can be added directly to the cells, in contrast to BiFC and FRET approaches that require the probe to be introduced into the cell by transfection. However, often the delay in uptake of the probe into the cytosol, or the timing required for wash steps, can make precise kinetic measurements difficult.

Localized caspase substrates that redistribute on cleavage typically are constructed by fusing GFP with a caspase-cleavable linker to membrane anchors or nuclear export sequences. Caspase activity is then detected and measured by the subcellular redistribution of GFP. Such probes and their use have been described for the measurement of initiator and executioner caspases (Henderson et al. 2005; Beaudouin et al. 2013). Although their sensitivity may be lower than that of FRET-based caspase substrates, their cleavage kinetics closely correlate (Joel Beaudouin, personal communication and unpublished data). These constructs can therefore provide important insights into the subcellular activation sites of caspases.

SUMMARY

Direct imaging of caspase activation and activity in real time can be a highly informative way of investigating caspase pathways, especially when combined with complementary biochemical approaches (see Introduction: **Measuring Apoptosis: Caspase Inhibitors and Activity Assays** [McStay and Green 2014]) and genetic models such as knockout and transgenic animals. Many challenges still remain, but the ongoing development of new probes and more user-friendly instrumentation will continue to improve these techniques, giving us superior tools to accurately dissect cellular events in caspase pathways in real time.

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