

Time-gated luminescence microscopy with responsive nonmetal probes for mapping activity of protein kinases in living cells

Angela Vaasa,^a Kadri Ligi,^a Shabnam Mohandessi,^b Erki Enkvist,^a Asko Uri^{a,*} and Lawrence W. Miller^b

^a *Institute of Chemistry, University of Tartu, 14A Ravila St., 50411 Tartu, Estonia.*

^b *Department of Chemistry, University of Illinois at Chicago, 845 W. Taylor St., Chicago, IL 60607(USA)*

* *Corresponding author. Tel: +372 737 5275; Fax: +372 737 5275; E-mail: asko.uri@ut.ee*

Supplementary information

Design and synthesis of ARC-1185

We have previously shown that a D-alanine after the linker increases selectivity of ARC-type inhibitors towards PKAc and compounds with one linker are slightly more PKAc selective than their analogues with two linkers.¹ Similar tendencies were observed also for luminescence signals of different ARC-Lum probes with various AGC-kinases where compounds with D-alanine and one linker have strongest signal with PKAc (our unpublished data). ARC-1185 was designed according to the previous knowledge with a D-alanine moiety after a single linker to maximize PKAc binding and signal. The synthesis of ARC-Lum probe ARC-1185 was performed according to the protocols disclosed previously for other ARC-Lum probes.² The synthesized compound was purified with reversed-phase high-performance liquid chromatography (RP-HPLC) and the mass spectrum of the product was measured with Thermo Electron LTQ Orbitrap mass spectrometer. ARC-1185 was quantified by UV-vis spectroscopy based on molar extinction coefficient of the incorporated fluorescence dye Texas Red (85,400 M⁻¹cm⁻¹ at 591 nm).

ESI-HRMS analysis data for ARC-1185: calc. monoisotopic mass for C₉₇H₁₄₇N₃₅O₁₇S₂Se 2218.03208, found deconvoluted monoisotopic mass 2218.03002.

Binding assay with luminescence intensity (LUM-assay) or fluorescence anisotropy/polarization (FA-assay) detection

The binding curves were measured according to the protocol described previously.^{2,3} Briefly, all biochemical binding experiments were performed on black low-volume 384-well non-bonding-surface microplates (code 3676, Corning) on a PHERAstar platereader (BMG Labtech) with TRF optical module [ex. 337(50) nm, em. 630(40) nm] using the time-resolved fluorescence (TRF) measurement mode or FP optical module [ex. 540(20) nm, em. 590(20) nm and 590(20) nm] using fluorescence anisotropy/polarization (FP) measurement mode. The microplates were incubated at 30°C for 15 min before each measurement.

To measure luminescence intensities, luminescence lifetimes and dissociation constants of complexes of ARC-1185 with various protein kinases the concentration series of ARC-1185 (3-fold dilutions) was made in the assay buffer (150 mM NaCl, 50 mM Hepes pH=7.5, 5 mM DTT, 0.5 mg/mL BSA) and the fixed concentration of PKAc, ROCKII, MSK1, PKB, PKC δ or Pim1 (final concentration 2 nM) was added to each well.

To characterize the binding of ARC-1185 to negatively charged DNA, the concentration series of salmon sperm DNA or PKAc (3-fold dilutions) was made in the assay buffer and the fixed concentration of luminescent probe ARC-1185 (final concentration 5 nM) was added to each well.

In TRF mode, ARC-1185 probe was excited with a flash of the xenon lamp at 337(50) nm, followed by 50 µs delay time and subsequent acquisition (150 µs) of the luminescence at 630(40) nm.

In FP mode, ARC-1185 was excited with the xenon lamp at 540(20) nm and acquired at 590(20) nm. The data from the assays were fitted with the aid of GraphPad Prism software version 5.0 (GraphPad Software, Inc.) and K_D values were calculated using nonlinear regression analysis:

$$TGL = B + M \frac{[L_t + K_D + kE_0 - \sqrt{(L_t + K_D + kE_0)^2 - 4L_t kE_0}]}{2} \quad (\text{Eq.1})$$

for TGL measurements, and

$$A = A_f + (A_b - A_f) \frac{[L_t + K_D + kE_0 - \sqrt{(L_t + K_D + kE_0)^2 - 4L_t kE_0}]}{2L_t} \quad (\text{Eq.2})$$

for anisotropy measurements,

where B is the background signal; M is the luminescence intensity of the PK/ARC-1185 complex at 1 nM concentration; L_t is the total concentration of ARC-1185; E_0 is the nominal concentration of the kinase; K_D is the dissociation constant between ARC-1185 and PK; k is the fraction of the active kinase; A is measured anisotropy; A_f is the anisotropy of free ARC-1185 and A_b is the anisotropy of ARC-1185 associated with the protein kinase.

Cell culture and time-gated luminescence microscopy

MDCKII cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml of streptomycin at 37°C and 5% CO₂. Cells were passaged using 0.25% trypsin/0.03% EDTA solution. For imaging experiments the cells were trypsinized and reseeded at density of 13,000 cells/well into eight-well chambered slides and incubated at 37°C and 5% CO₂ overnight (20-28 h).

For endpoint imaging the cells were incubated with 10 µM ARC-1185 or 10 µM Texas Red-R9 for 1 h at 37 °C, thereafter the cells were washed three times with PBS supplemented with Ca²⁺ and Mg²⁺ ions and then imaged in 100 µL DMEM (free of phenol-red) at room temperature. The experiment was repeated at least three times.

For real-time (time-lapse) imaging the cells were washed three times with PBS supplemented with Ca²⁺ and Mg²⁺ ions and then imaged in 100 µL of phenol-red free DMEM at room temperature. Thereafter equal volume of ARC-1185 (20 µM in DMEM) solution containing (or not containing) forskolin (FRSK, 50 µM) and 1-methyl-3-isobutylxanthine (IBMX, 200 µM) was added to the cells to obtain the incubation solution containing ARC-1185 (10 µM), FRSK (25 µM or missing) and FRSK (100 µM or missing). The images were taken at the following timepoints: 1, 3, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105 and 120 min after the addition of ARC-1185.

The time-lapse images were analyzed with a ImageJ software version 1.45s (Wayne Rasband, National Institutes of Health, USA). Using ImageJ the luminescence intensity of ARC-1185/kinase complex per area pixel was measured in 9 cells on each image and sets from 3 experiments were averaged (figure 4). Distinction was made between the luminescence intensity change in the cytoplasm and in the nucleus. The background was defined as the area with no cells on the image and it was measured in four different locations on the image. The datasets were then analyzed with GraphPad Prism software version 5.0 (GraphPad Software, Inc.)

The cells were imaged using an epifluorescence microscope (Zeiss Axiovert 200) with a 63X/1.25 N.A. EC Plan Neofluar oil-immersion objective (Carl Zeiss, Inc.).⁴ The experiment was repeated three times.

For time-gated luminescence (TGL) microscopy the microscope was modified with the following components: UV LED emitting at 365 nm (UV-LED-365, Prizmatix); delay generator (DG645, Stanford Research Systems, Inc.); a gated image-intensified CCD camera and camera controller (Mega-10EX, Stanford Photonics, Inc.); and a computer running Piper Control software (v2.4.05, Stanford Photonics, Inc.). Appropriate excitation source (LED, 365 nm), emission filters [TGL (em. 610(75) nm] and dichroics allowed for wavelength selection. The light source and camera timing parameters were as follows: excitation pulse, 100 μ s; delay time, 10 μ s; collection time, 200 μ s.

For steady-state fluorescence microscopy, a 100 W mercury arc lamp for excitation and appropriate filter cube[FL (ex. 545(30) nm, em. 605(15) nm] for wavelength selection was used. The ICCD camera was set to automatic gain level and acquisition time.

Results

Microscopy with Texas Red-R9

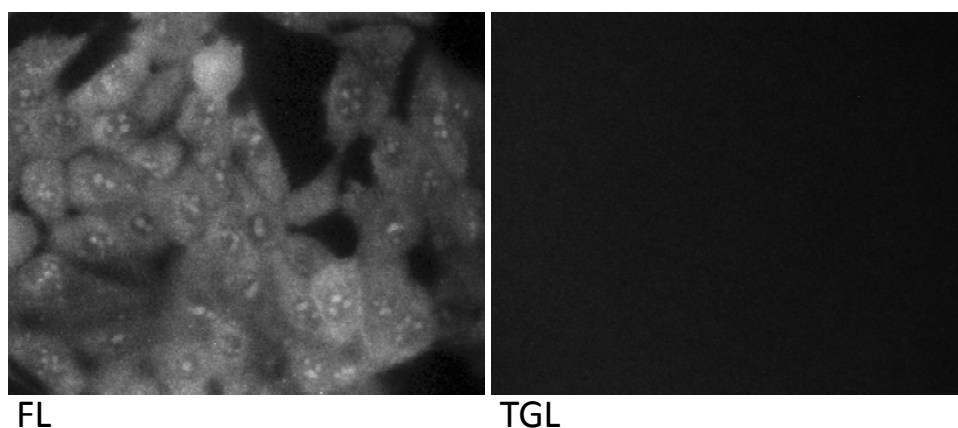


Figure 1. Luminescence imaging of MDCKII cells using Texas Red-R9 (10 μ M, 1 h) with microscopy in steady state fluorescence [FL, λ_{ex} = 545(30) nm, λ_{em} = 605(15) nm) and time-gated luminescence (TGL, λ_{ex} = 365 nm, λ_{em} = 610(75) nm, delay time 10 μ s] modes.

ARC-1185 binding to salmon sperm DNA

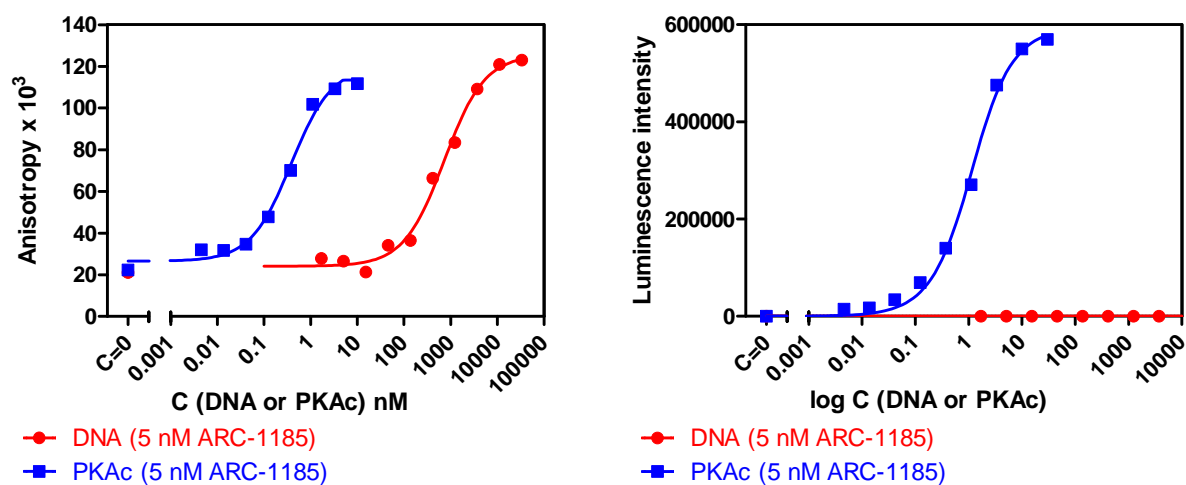
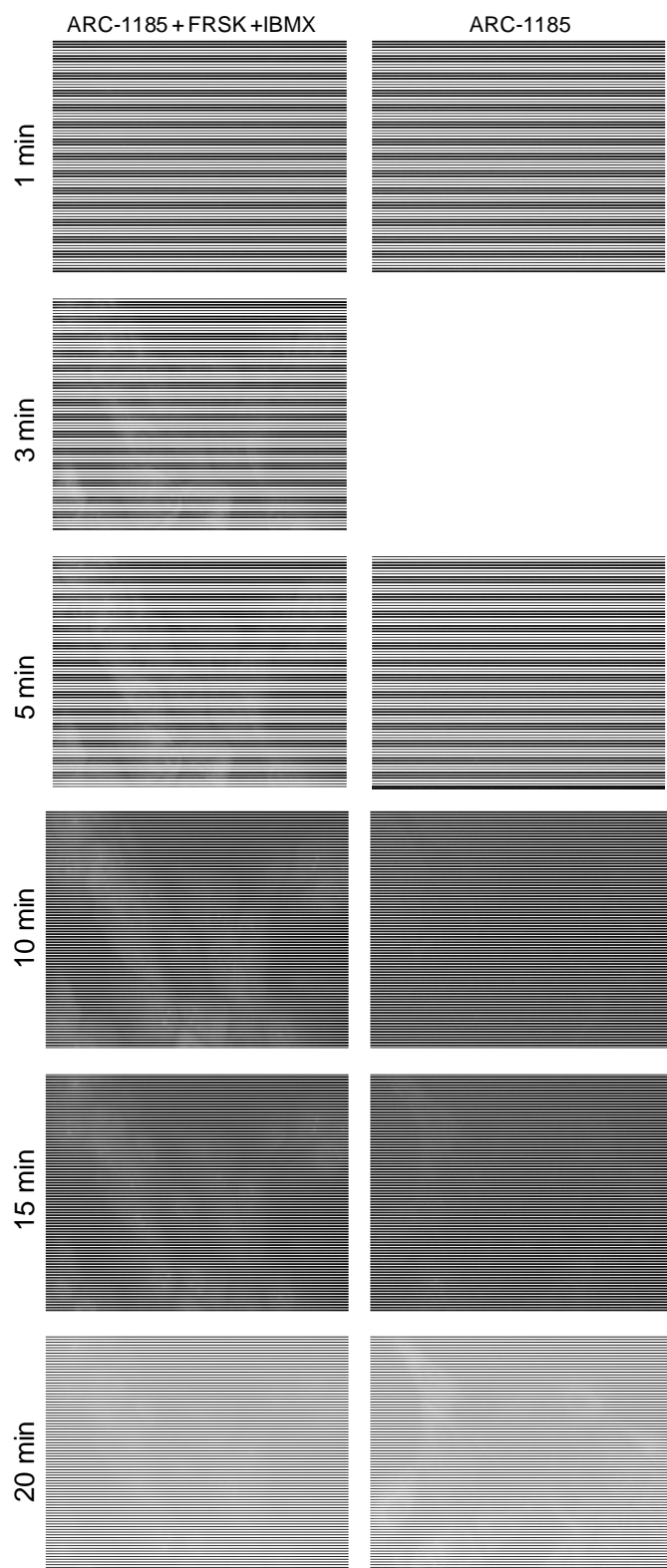
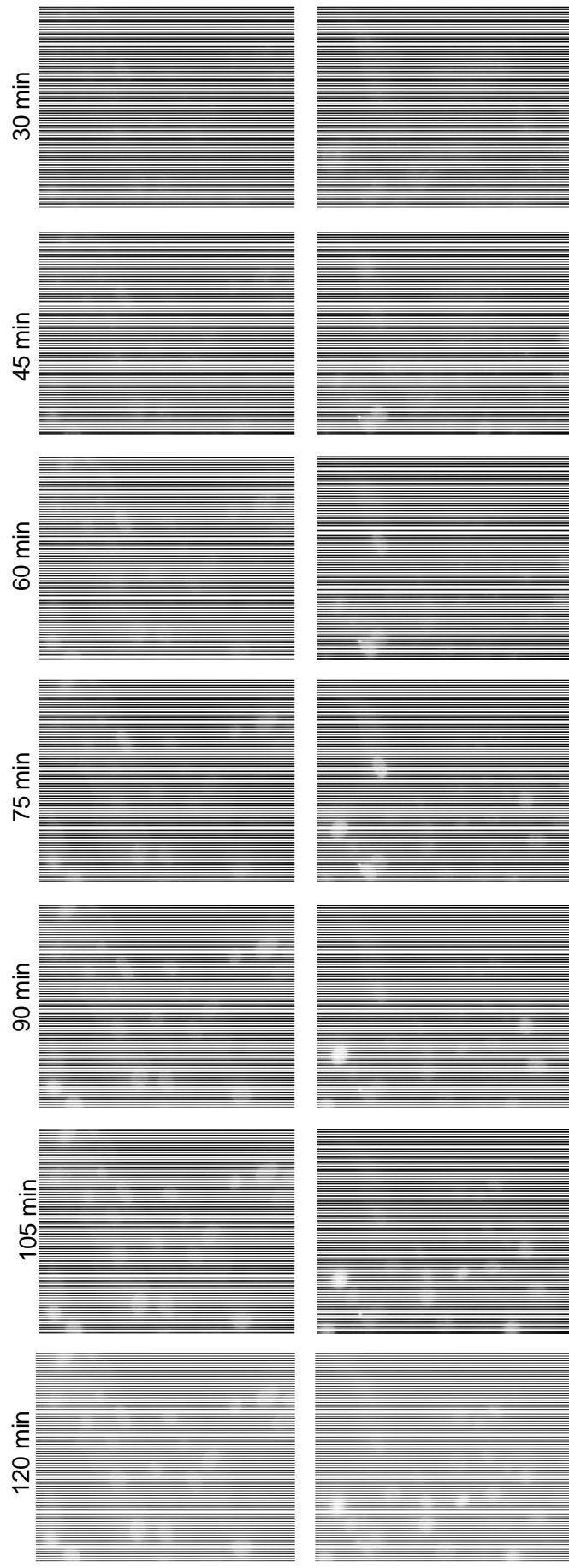


Figure 2. Titration of ARC-1185 (5 nM) with salmon sperm DNA (●) and PKAc (■). Binding measurements were performed on a PHERAstar platereader (BMG Labtech) using fluorescence anisotropy (ex. 540 nm, em. 590 nm and 590 nm)³ (A) and time-gated luminescence [ex. 337(50) nm, em. 630(40) nm; delay time 50 μ s, integration time 150 μ s] readouts² (B).

Time-lapse microscopy with ARC-1185

Figure 3. TGL signal intensity of MDCKII cells while loaded with ARC-1185 (10 μ M), non-activated (right column) or PKA-activated (left column) with forskolin (25 μ M) and IBMX (100 μ M) as monitored with TGL microscopy [λ_{ex} = 365 nm, λ_{em} = 610(75) nm, delay time = 10 μ s]. All images are rendered at identical contrast levels. Representative of experiments repeated three times with similar results.





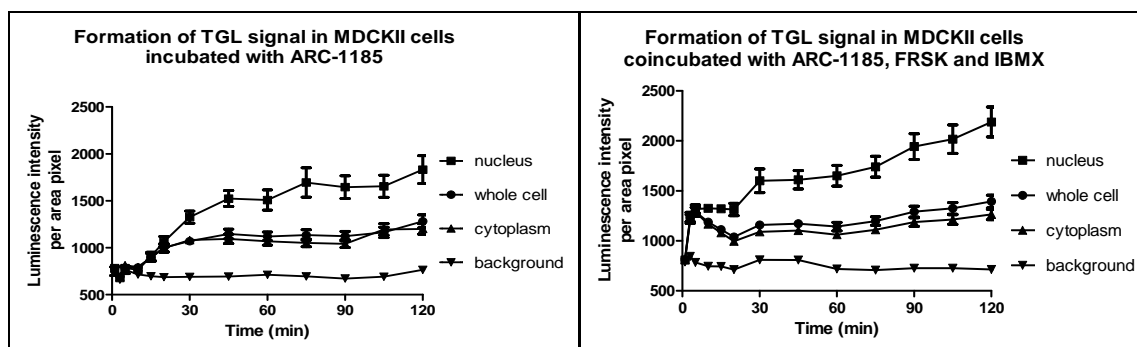


Figure 4. Comparison of the intensity of the TGL signal of ARC-1185/kinase complex in MDCKII cells activated or not activated with FRSK and IBMX. Each point on the graph represents TGL signal averaged over 3x9 cells imaged on three different days. The error bars represent the standard error of mean.

References

- 1 D. Lavogina, M. Lust, I. Viil, N. König, G. Raidaru, J. Rogozina, E. Enkvist, A. Uri, D. Bossemeyer, *J. Med. Chem.*, 2009, **52**, 308.
- 2 E. Enkvist, A. Vaasa, M. Kasari, M. Kriisa, T. Ivan, K. Ligi, G. Raidaru and A. Uri, *ACS Chem. Biol.*, 2011, **6**, 1052.
- 3 A. Vaasa, I. Viil, E. Enkvist, K. Viht, G. Raidaru, D. Lavõgina and A. Uri, *Anal. Biochem.*, 2009, **385**, 85.
- 4 N. Gahlaut and L.W. Miller, *Cytometry A.*, 2010, **77**, 1113.