

Rapid kinetics procedures

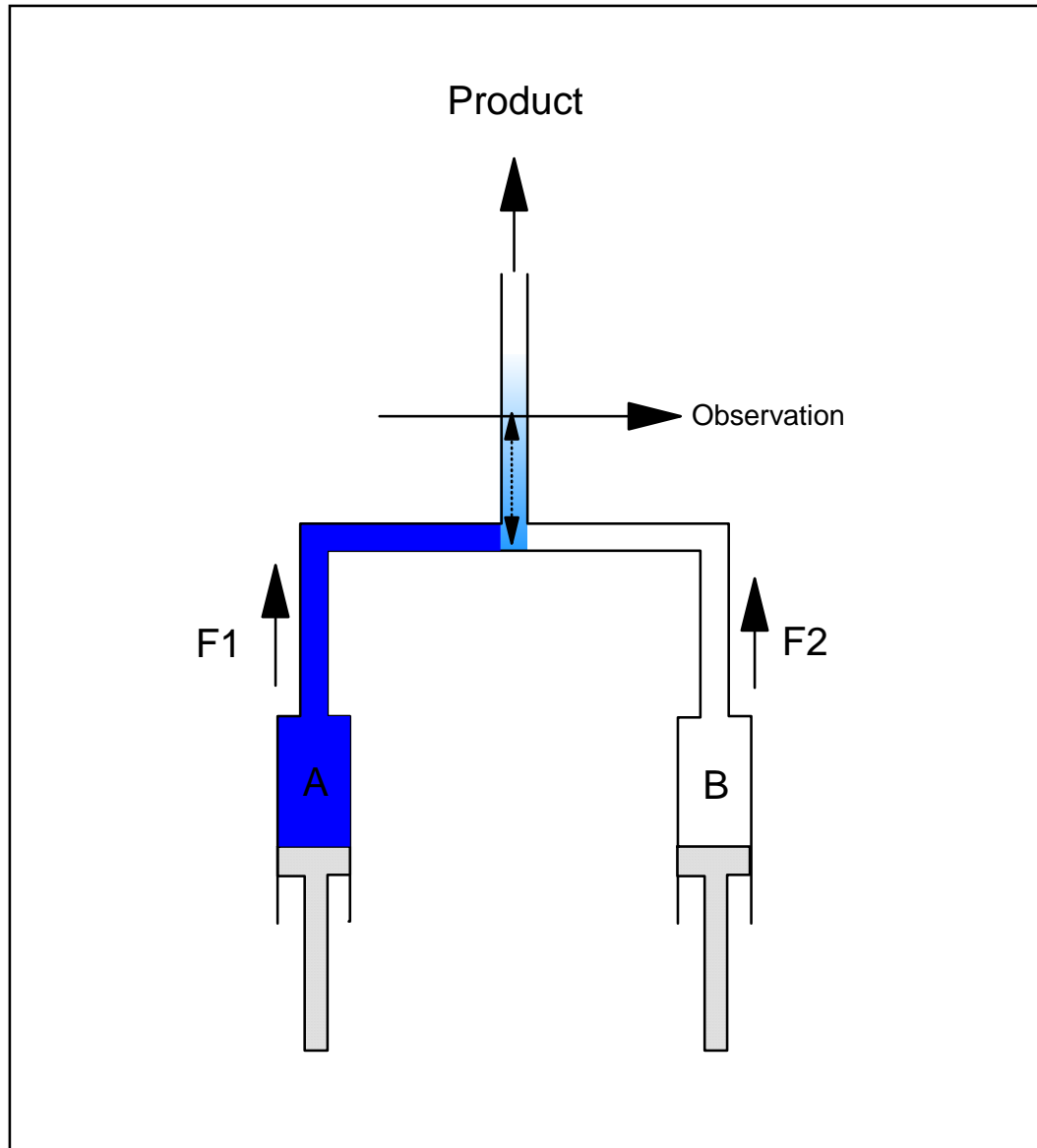
3 ways to start a reaction :

- Mixing
 - Light
 - Temperature
- } Continuous recordings → Stopped-flow

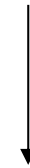
3 ways to stop a reaction

- Mixing → Quenched-flow
- Light → optical quench
- Temperature (freezing) → Freeze quench

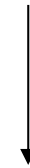
Stopped-flow technique



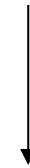
Pushing



Mixing

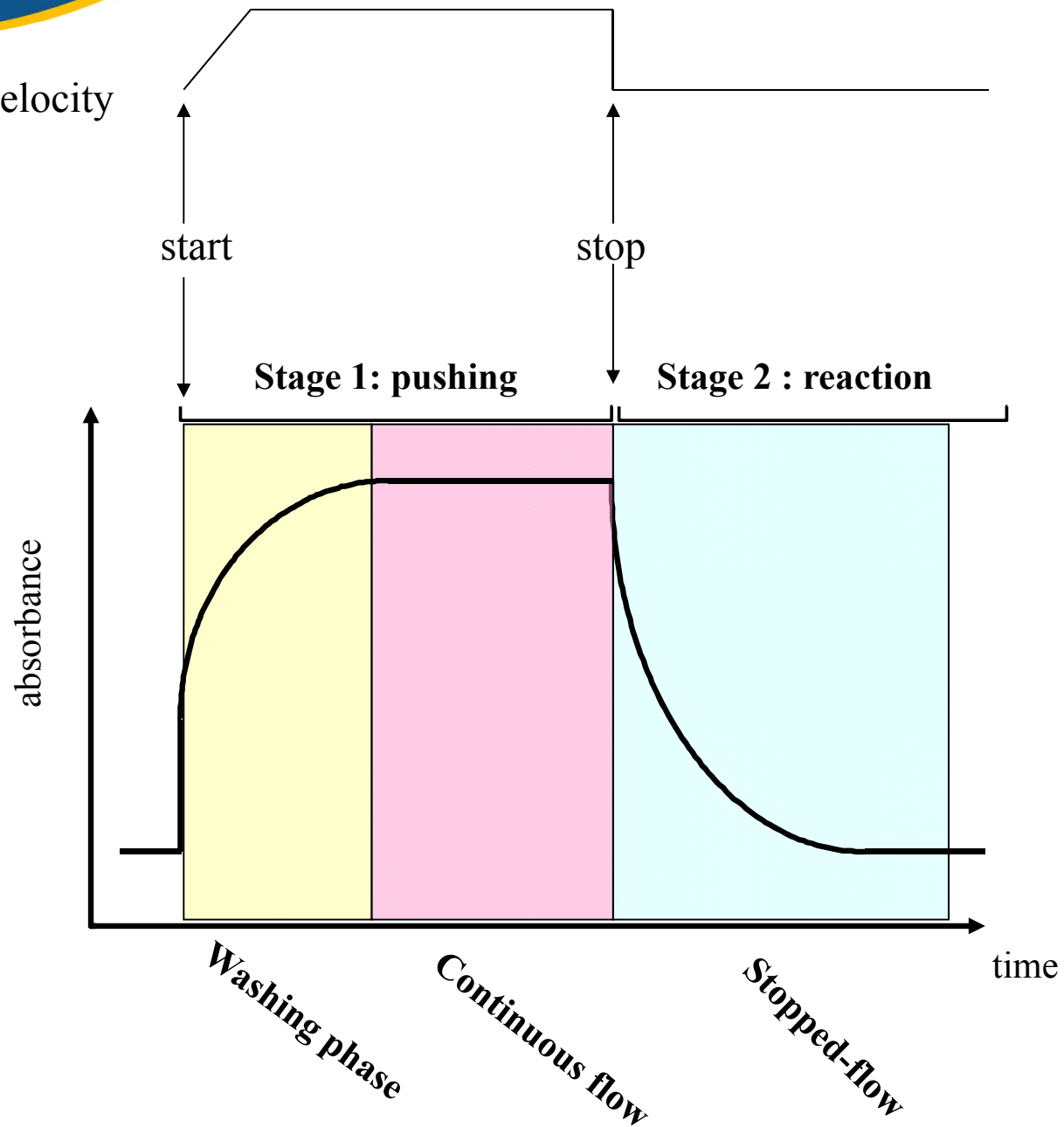


Stop of the flow



observation

Syringe velocity

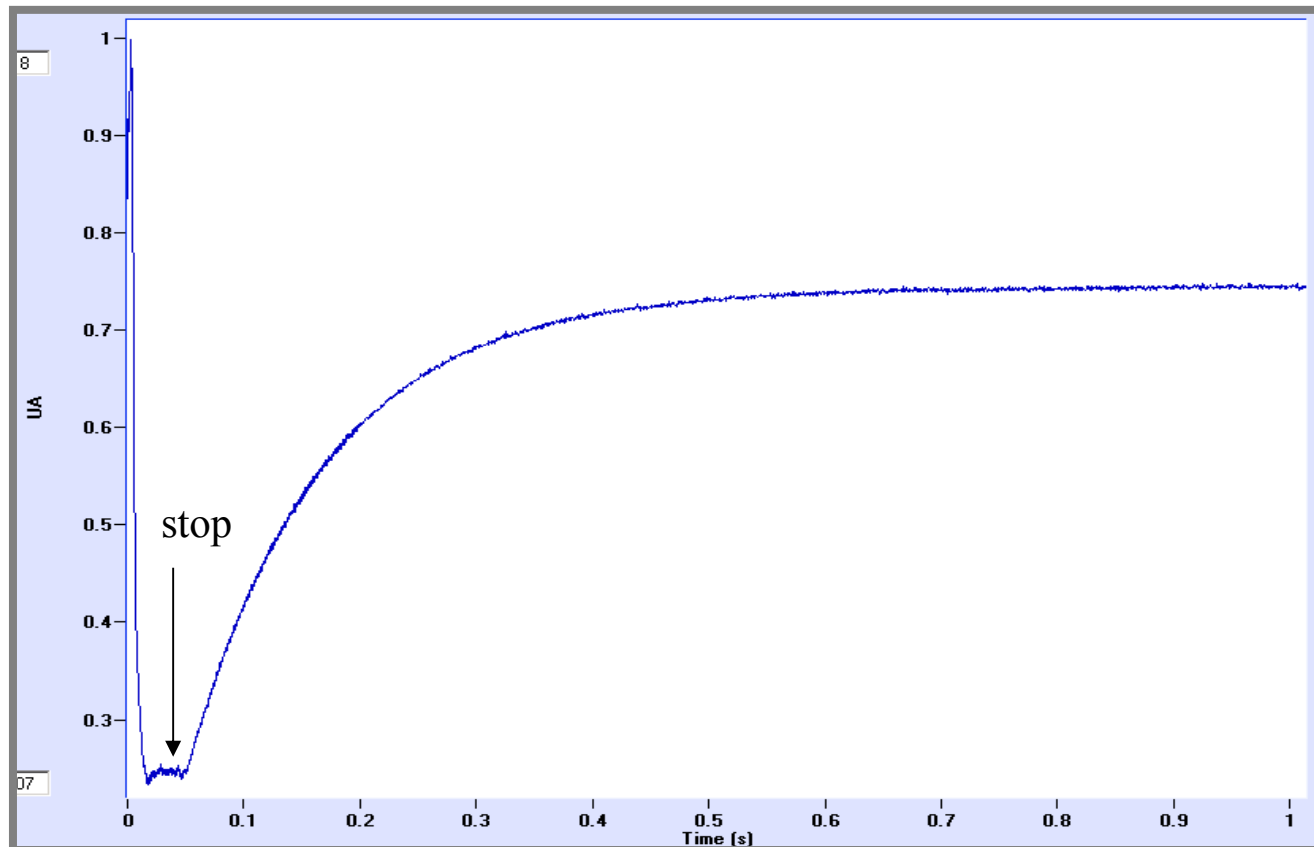


Typical stopped-flow trace : DNPA hydrolysis by NaOH

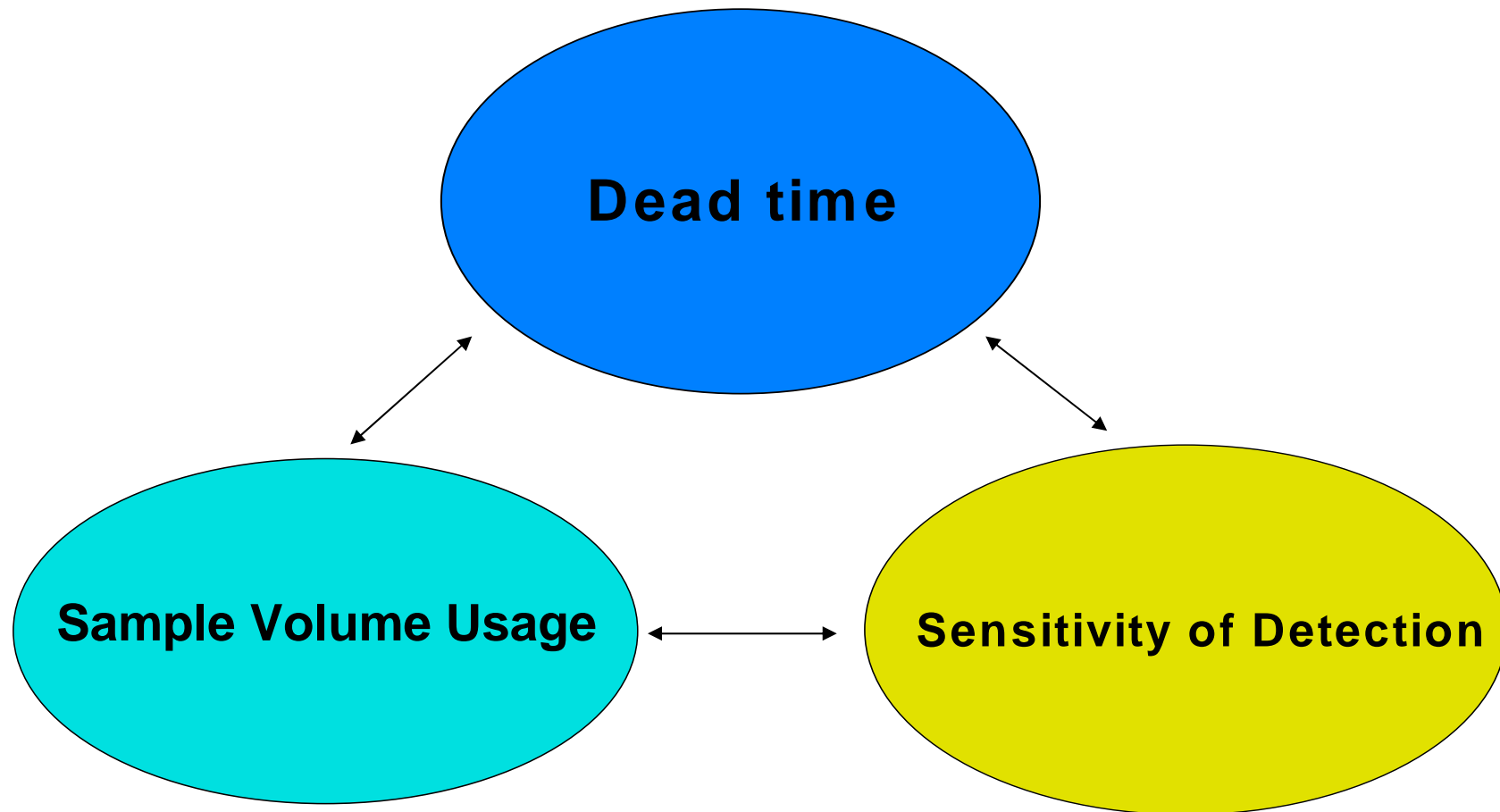
Followed by absorbance at 325 nm

1:1 mixing ratio, total volume 200 μ l, 10 ml/s

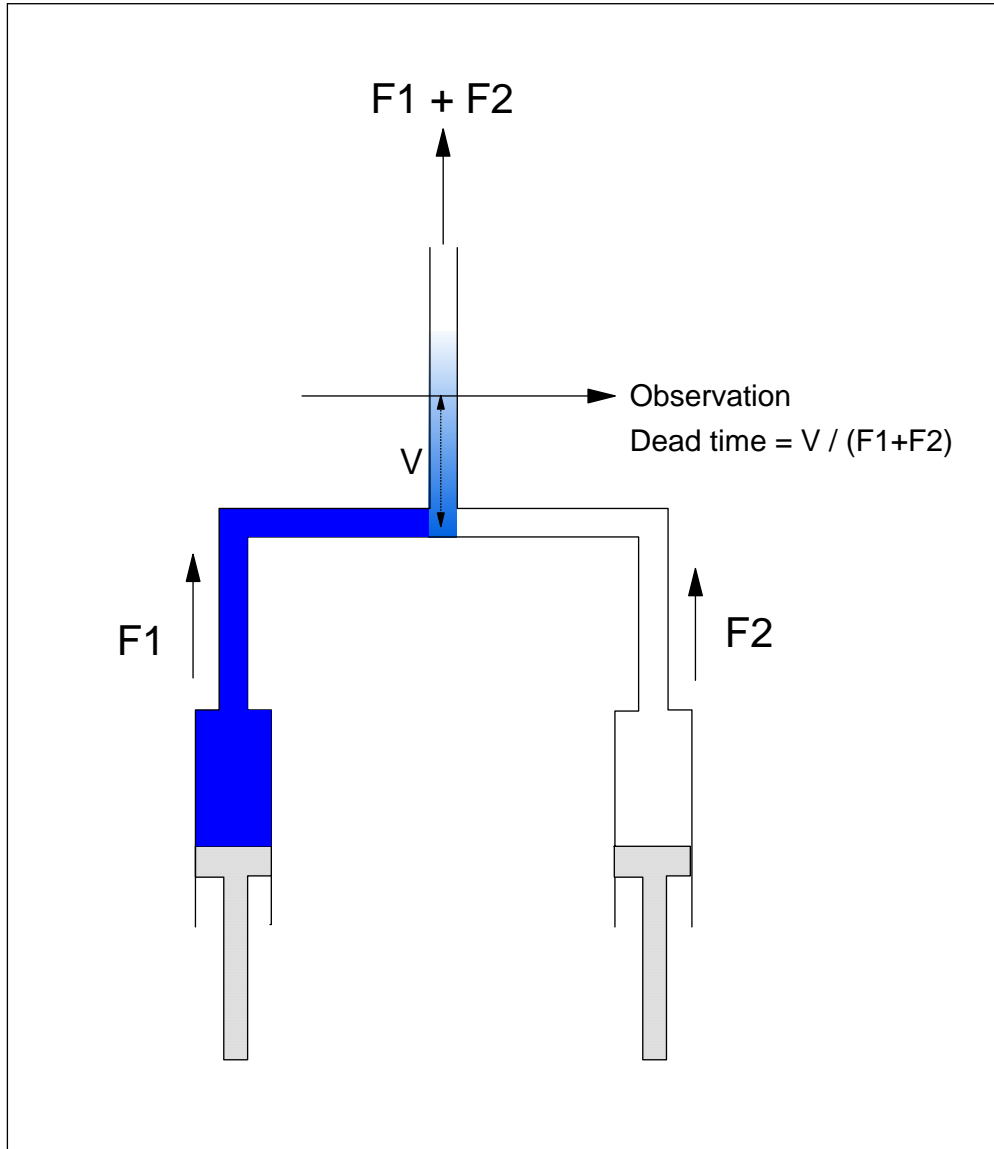
Acquisition 50 msec before stop



CRITERIA IN STOPPED-FLOW INSTRUMENTATION



A stopped-flow experiment is always associated to a dead time



DEAD TIME

=

- **Age of the solution at the observation point**
- **Time to go from mixing point to observation point during the pushing phase**
- **Part of the kinetics not observed**

Opening the periplasmic cavity in lactose permease is the limiting step for sugar binding

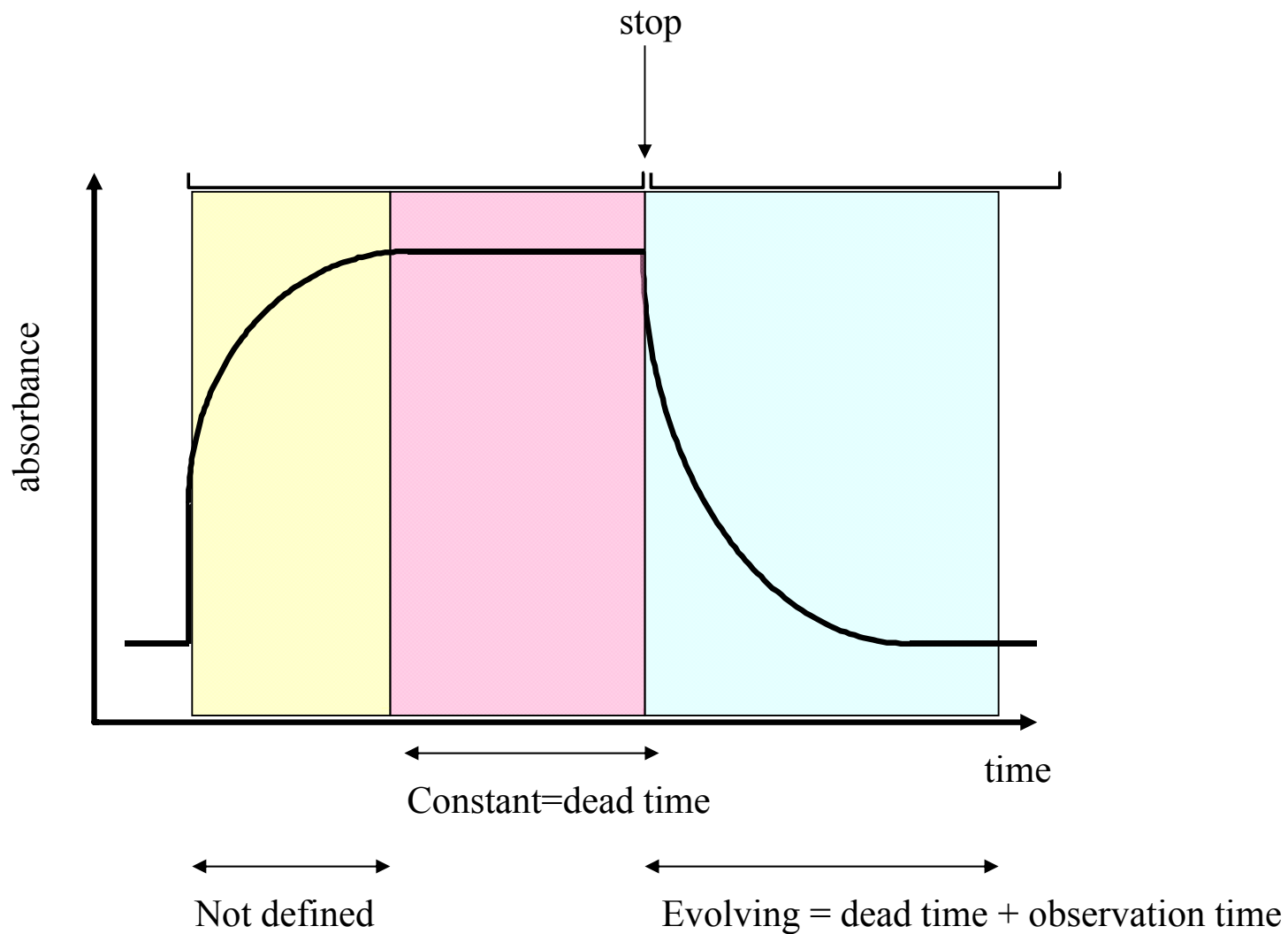
[Irina Smirnova](#), [Vladimir Kasho](#), [Junichi Sugihara](#), and [H. Ronald Kaback](#)

PNAS, 2011, vol 108, n°137, 15147-15151

Methods

Fluorescence Measurements. Steady-state Trp fluorescence of 0.5 μM protein was monitored at excitation wavelength 295 nm on a SPEX Fluorolog 3 spectrofluorometer at room temperature as described (14). Time traces were recorded at emission wavelength 335 nm. Stopped-flow measurements were performed at 25°C on an SFM-300 rapid kinetic system equipped with either a microcuvette (dead time 0.22 ms) or a TC-50/10 cuvette (dead time 1.1 ms), and an MOS-450 spectrofluorimeter (Bio-Logic). Excitation wavelength was 295 nm with a 320-nm cutoff filter for emission. The final concentration of protein after mixing was 0.5–2 μM . Measurements with purified protein in detergent were done in 0.02% DDM and 50 mM NaP_i (pH 7.5), citrate/ NaP_i (pH 6.0), or 3-(cyclohexylamino)-1-propanesulfonic acid (pH 9.3), as indicated. Experiments with proteoliposomes were carried out in 50 mM NaP_i (pH 7.5). For dissolving proteoliposomes, DDM was added to a final concentration of 0.3%, and after 10 min, the samples were used in stopped-flow experiments. Typically, 10–20 traces were recorded for each data point, averaged and fitted with an exponential equation using the built-in Bio-Kine32 software package, or by using Sigmaplot 10 (Systat Software). All concentrations given are final after mixing.

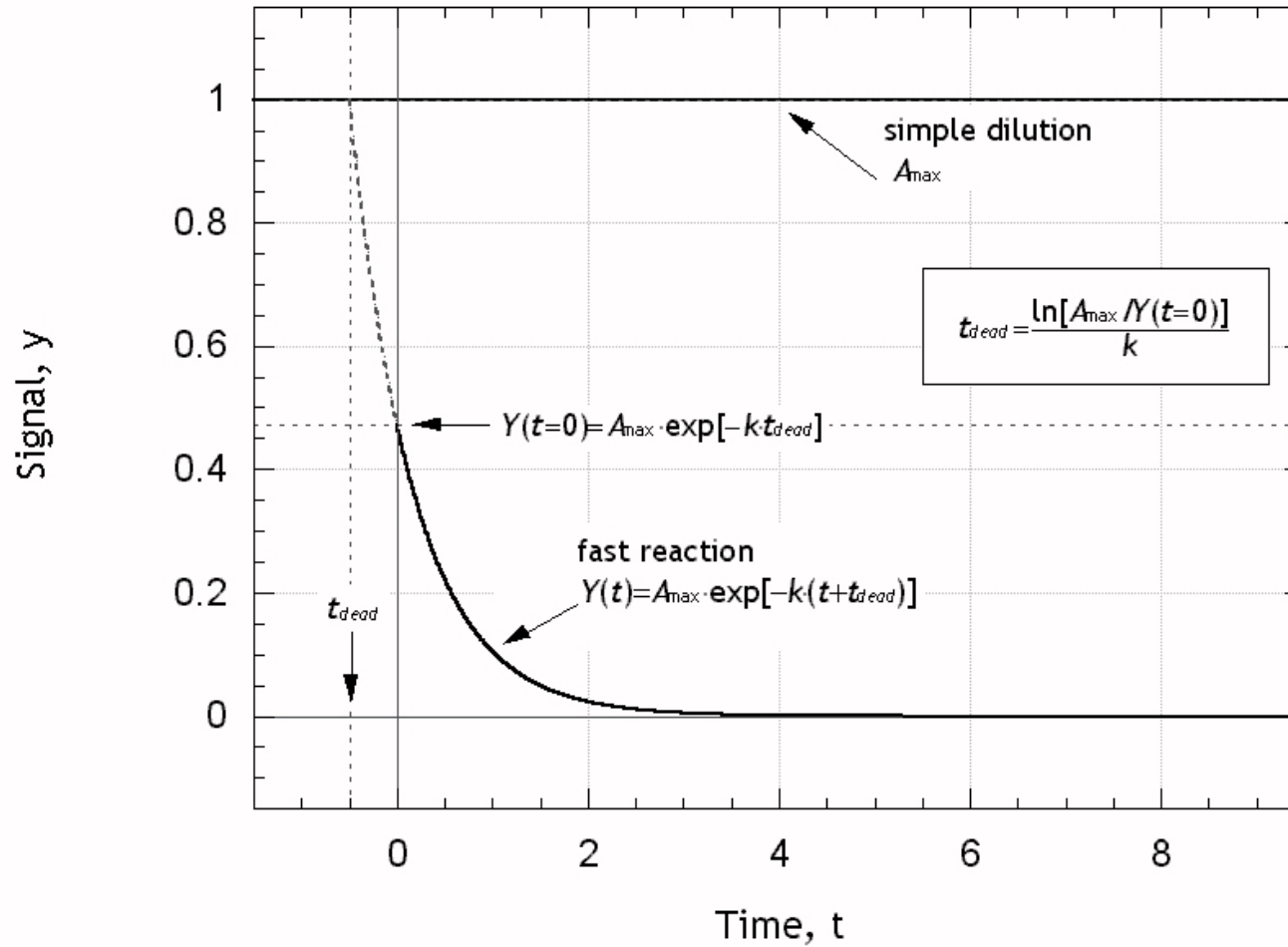
Determination of experimental dead time



REACTION TIME

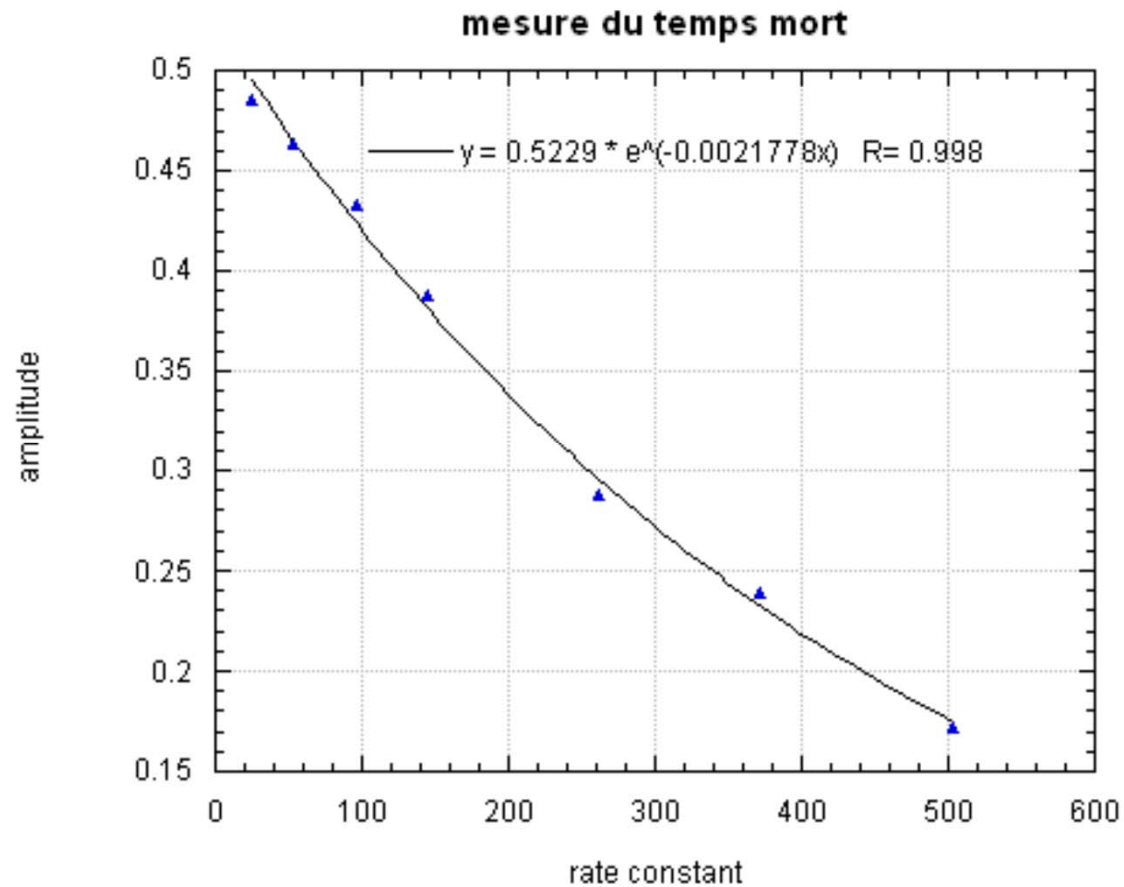
A quick and convenient way to measure dead time...

Determination of dead time



A more complete technique (ideal for new user or teaching)

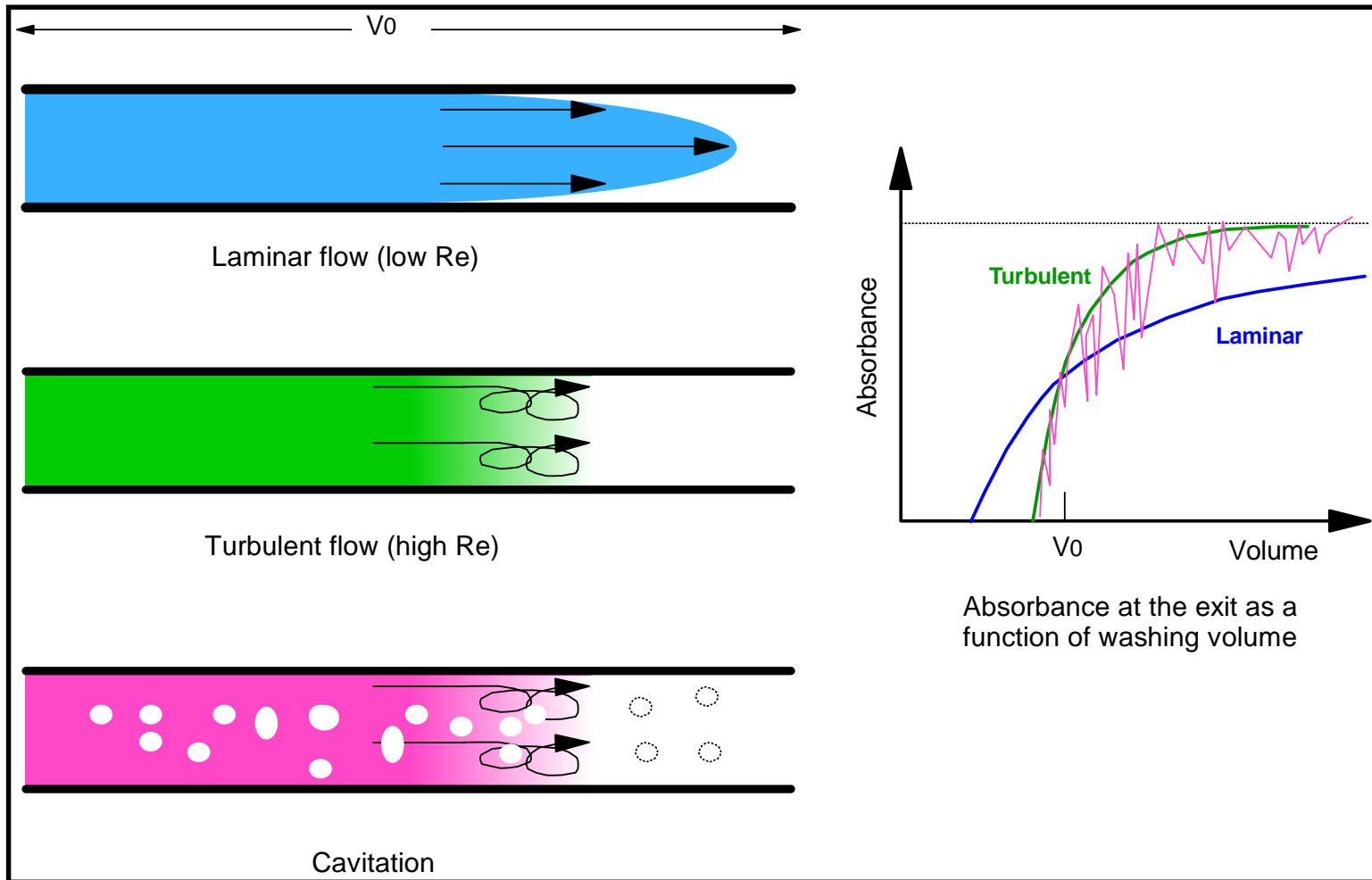
DCIP reduction by ascorbic acid at different concentrations using same mixing ratio and same flow rate.



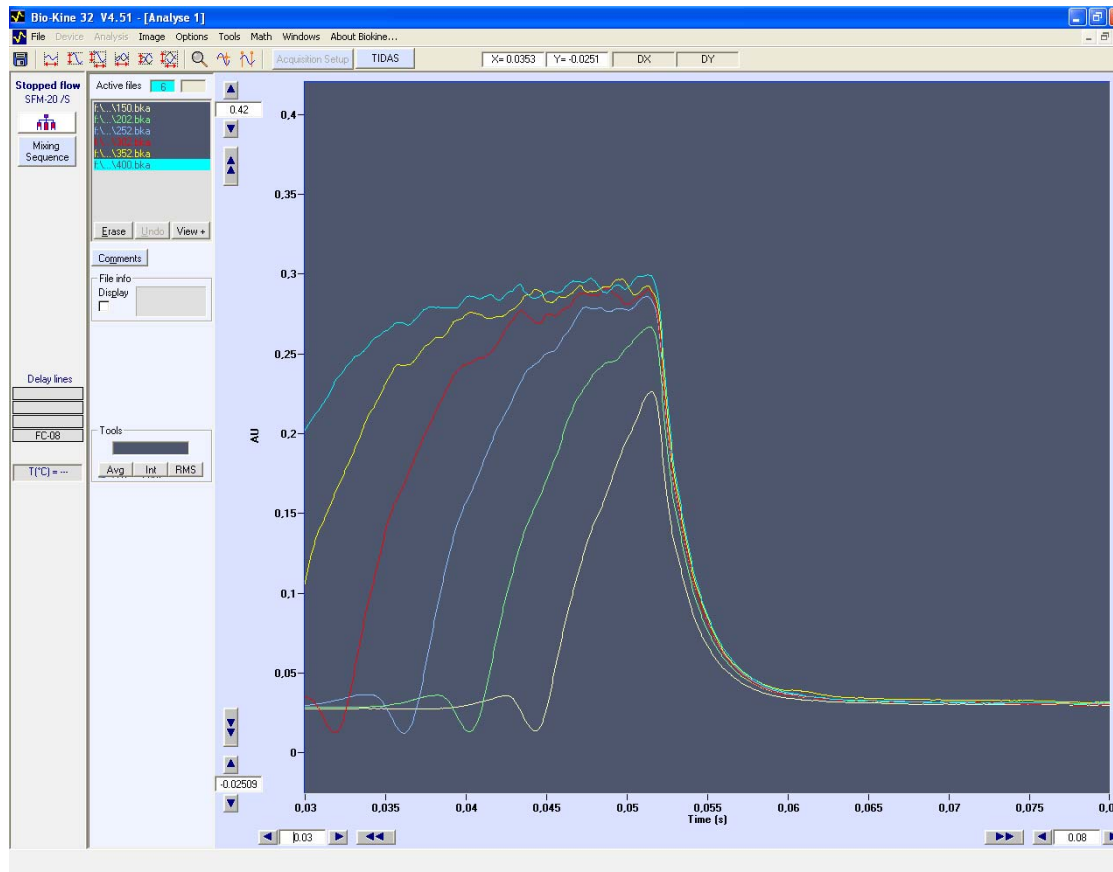
Sample volume usage

Depends on :

- design on mixer
- dead volume of observation cell (from 4 to 50 μ l)
- precision of pushing mechanism
- mixing sequence (single or double mixing)



minimum volume to inject : CUVETTE LIMITATION
fast reaction, same shot but reducing total volume

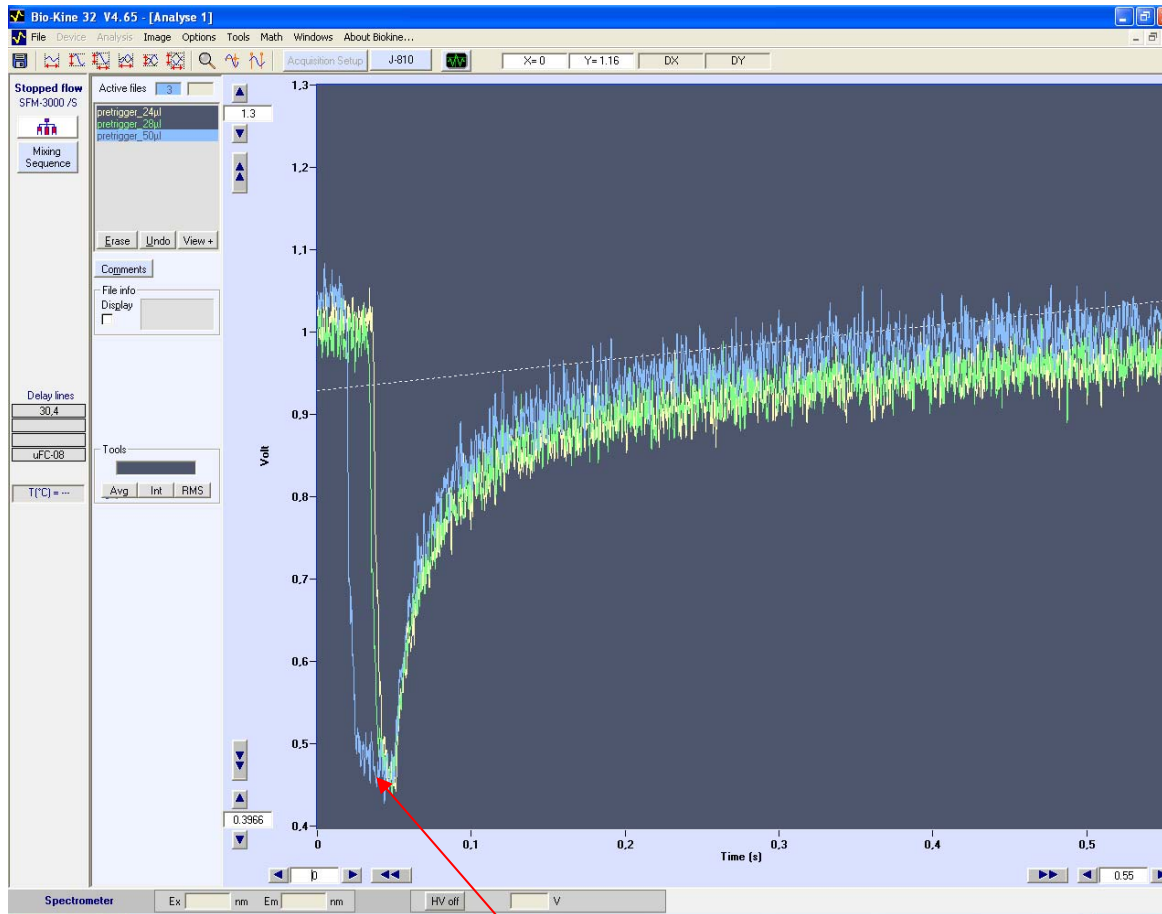


Loss of amplitude
=
Bad washing

MIMIMUM VOLUME = 3-4 times the volume of cuvette

minimum volume to inject : SYRINGE LIMITATION

Small cuvette (4 μ l) , same reaction but we change volume per syringe



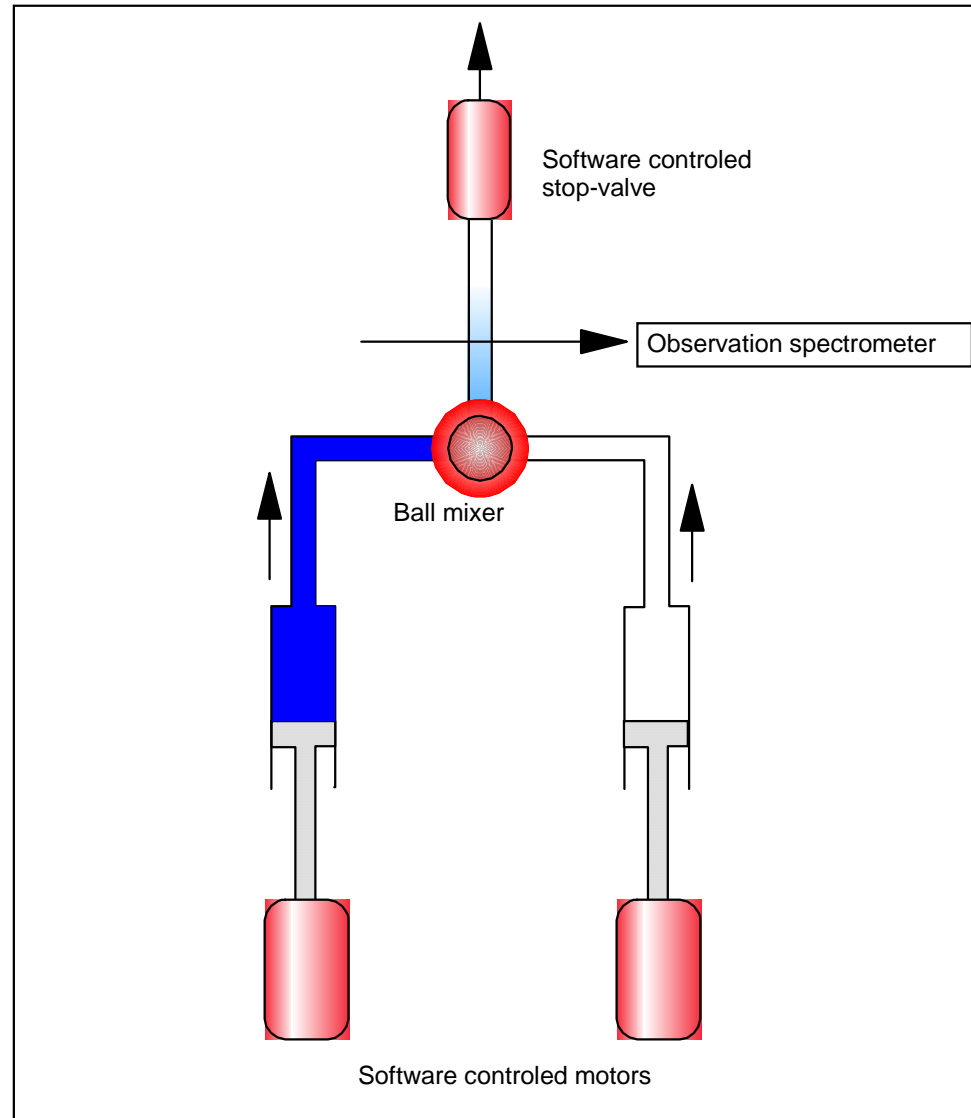
Vol of sample	rate (s-1)	amplitude
50 μ l	32,3	0,332
28 μ l	32,4	0,331
24 μ l	32,3	0,332

Decrease of volume until limit of stationary state

Key points of Stopped-flow technology

- **Mixer**
- **Pushing method**
- **Stopping method**

Bio-Logic stopped-flow design



- **Berger ball mixer**

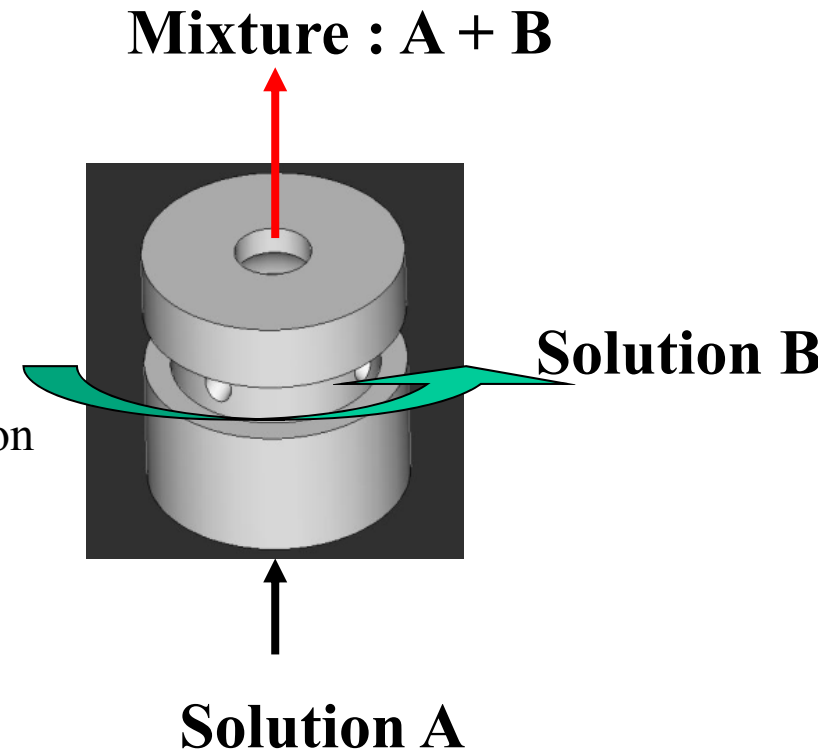
- Mixing of viscous solution (up to 2000 cp)
 - High mixing ratio (1:100)
 - Flow rate range (1 to 20 ml/s)
 - Designed to create turbulence
 - HDS mixer available to avoid back convection

- **Independant stepping motors**

- Independant control of syringes
 - Total control of flow rate and volume
 - Easy change of mixing ratio
 - Upgrades possibilities (QF, titration...)

- **Stop electrovalve**

- Synchronization: valve closure with stop of motors
 - Full control of pressure
 - Software control



Design of a stopped-flow sequence Single mixing experiment

Mixing sequence

Mixing ratio	Volume	Total flow rate
S1: 2	Total volume / shot: 141 μL	4.95 mL/s
S2: 1	S1: 94 μL @ 3.3 mL/s	Default
S3:	S2: 47 μL @ 1.7 mL/s	
S4:	S3: μL @ mL/s	
	S4: μL @ mL/s	

Start of data acquisition

At stop
 At 50 ms before the stop

Sequence

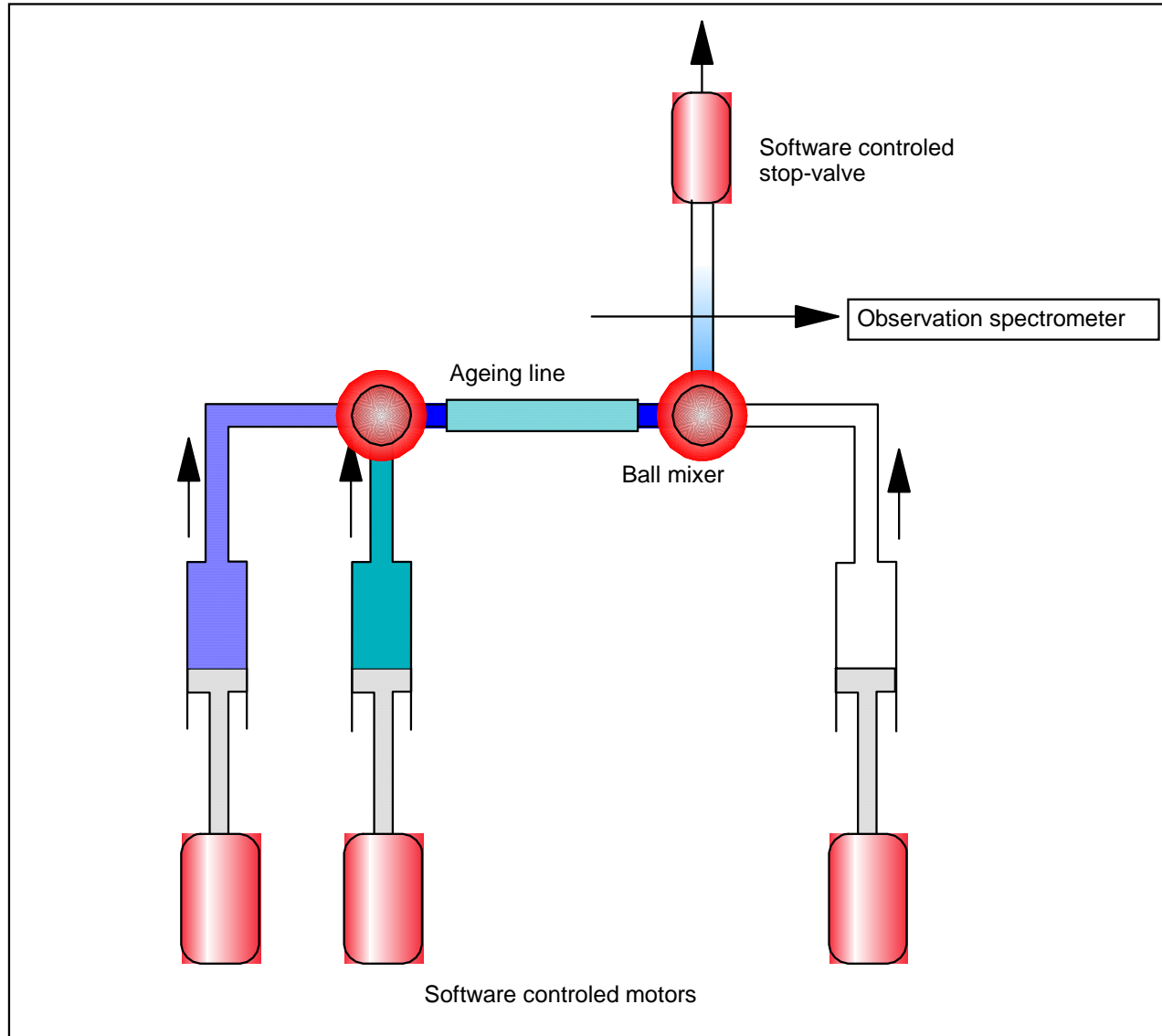
Ready

Estimated dead time : 0.8 ms

Configuration

	Content of syringes	Initial concentration	Final concentration
Syringe 1	10 ml DCIP	100 μM	66.667 μM
Syringe 2	10 ml Ascorbic acid	20 mM	6.667 mM
Syringe 3	----		
Syringe 4	----		

Load Save As Comments Print SFM Options Close



Distinguishing between two-state and three-state models for ubiquitin folding

B. Krantz, T. Sosnick
 Biochemistry 2000, 39, 11696-11701

Double mixing to reduce Proline-related phase

Ub has 3 trans-proline residues whose isomerization in the unfolded state is known to produce a slow folding population.

This slow phase makes accurate measurement of fast phase difficult in single mixing refolding.

Krantz and Sosnick designed a double mixing (N→U→N) so proline related phase is reduced.

For the double-jump measurements, the native protein in 2 M GdmCl was mixed with 7.4 M GdmCl to a final concentration of 4.7 M GdmCl. After a 10 s delay (about 10 unfolding half-lives), the ~40 μL denatured protein solution contained in the aging loop was diluted with buffer to yield the final denaturant concentration. Continuous-flow

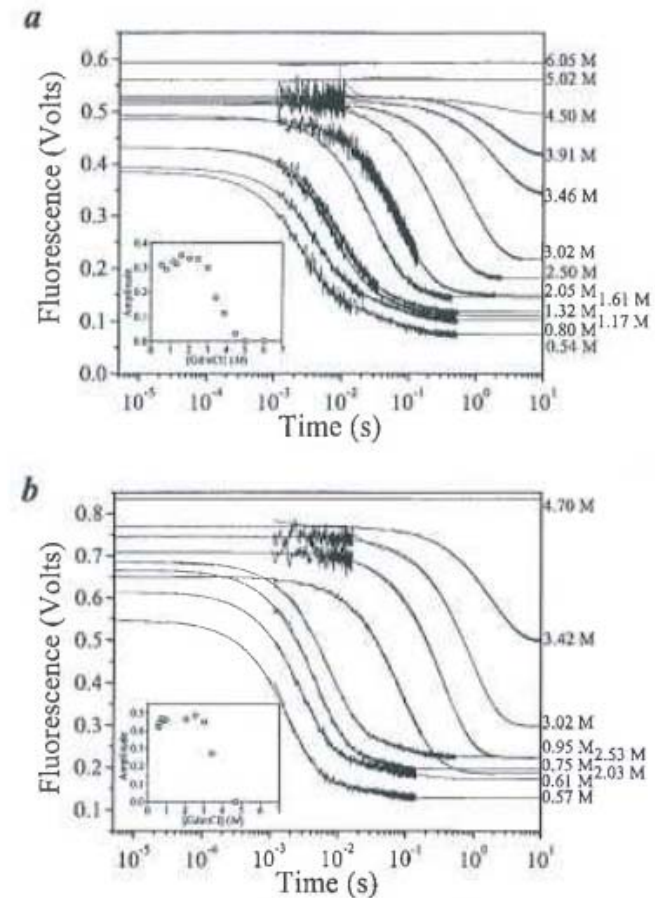
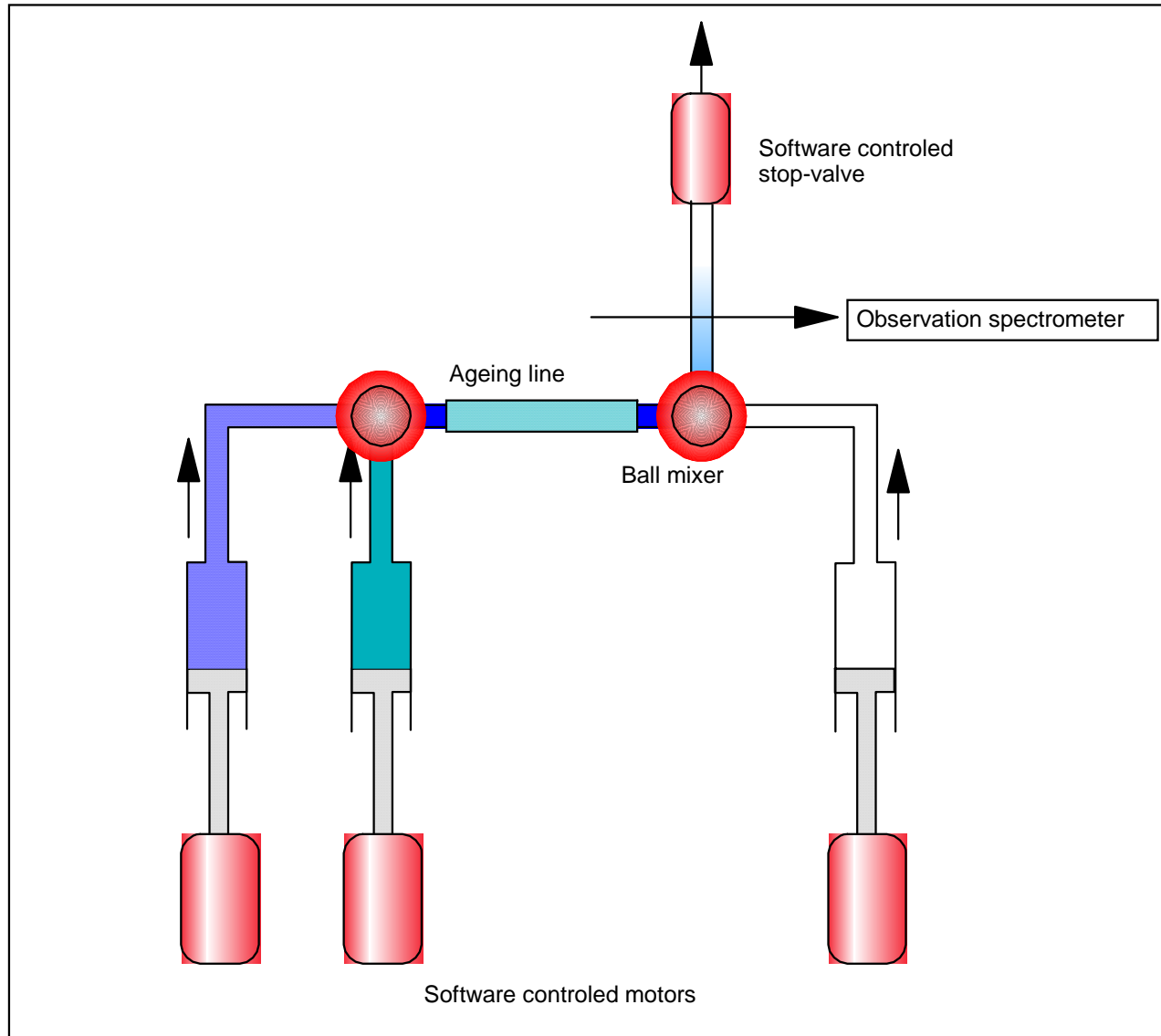
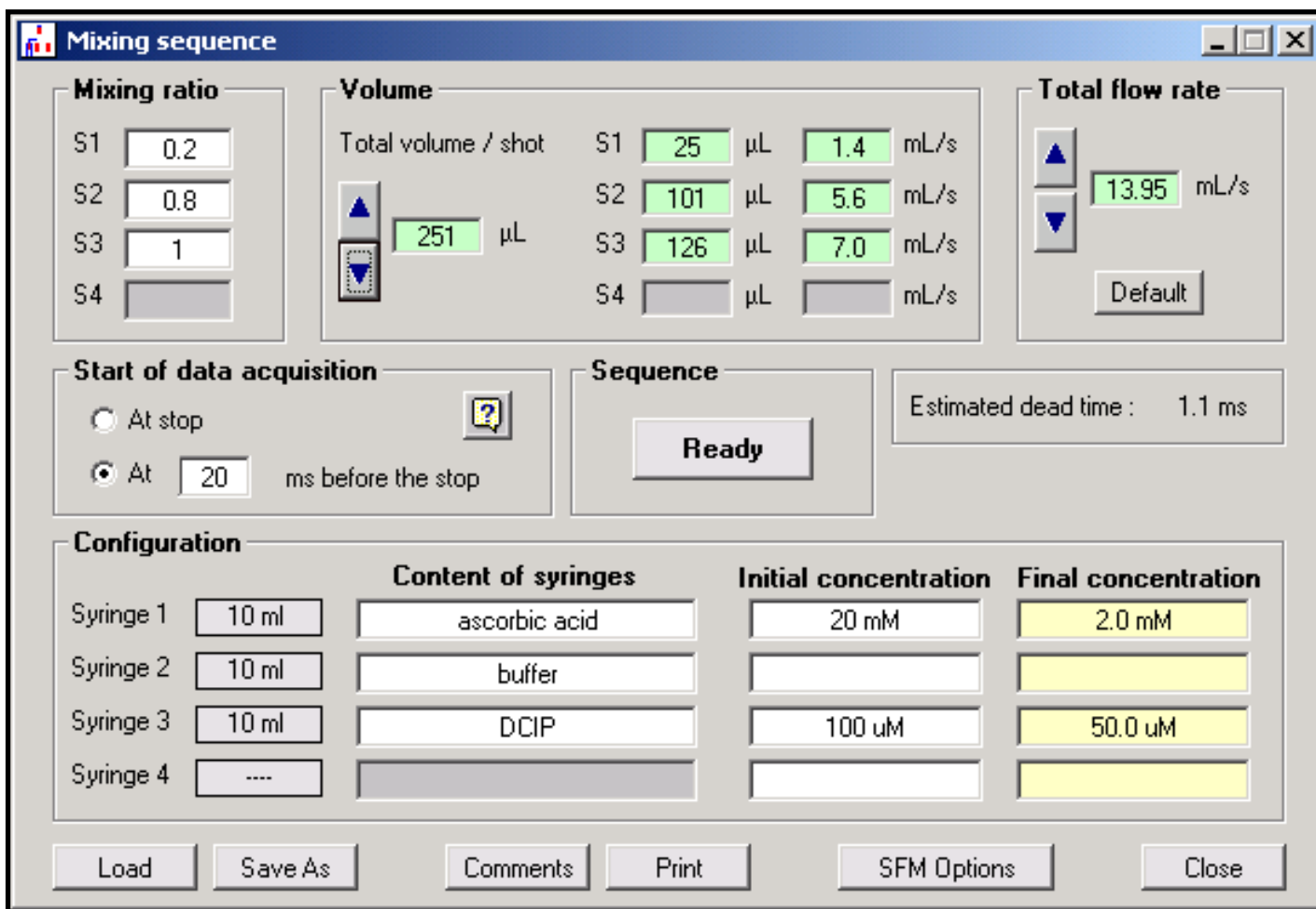


FIGURE 2: Denaturant dependence of folding at pH 5.0, 25 °C. Raw data traces in (a) single-jump refolding experiments and (b) double-jump refolding experiments which eliminate extraneous proline phases. The noncoincidence of the fully folded values largely represents the denaturant dependence of the native state (see Figure 3b). Inset: total amplitude change. In the two-state model, the change should be constant given that the slopes of the denaturant-dependent baselines of the denatured and native states are equivalent, as is the case (Figure 3b). The data, sampled every 50 μs, are binned into increasing longer time intervals which results in the decrease in the noise level.



Design of a stopped-flow sequence Variable ratio mixing



Mixing ratio

S1	0.2
S2	0.8
S3	1
S4	

Volume

Total volume / shot: 251 μL

S1	25 μL	1.4 mL/s
S2	101 μL	5.6 mL/s
S3	126 μL	7.0 mL/s
S4		

Total flow rate

13.95 mL/s

Default

Start of data acquisition

At stop

At 20 ms before the stop

Sequence

Ready

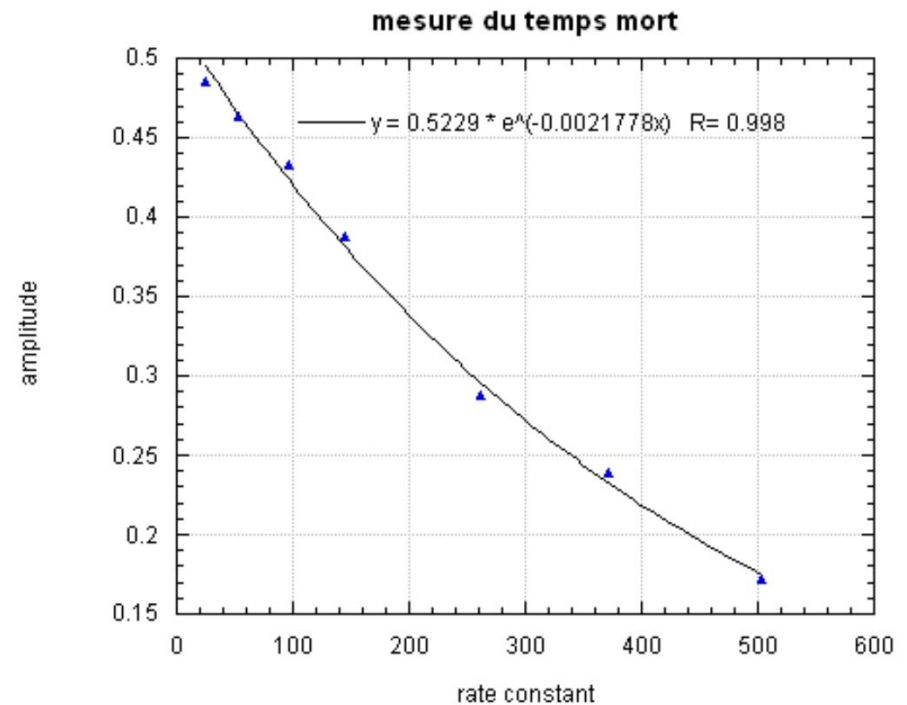
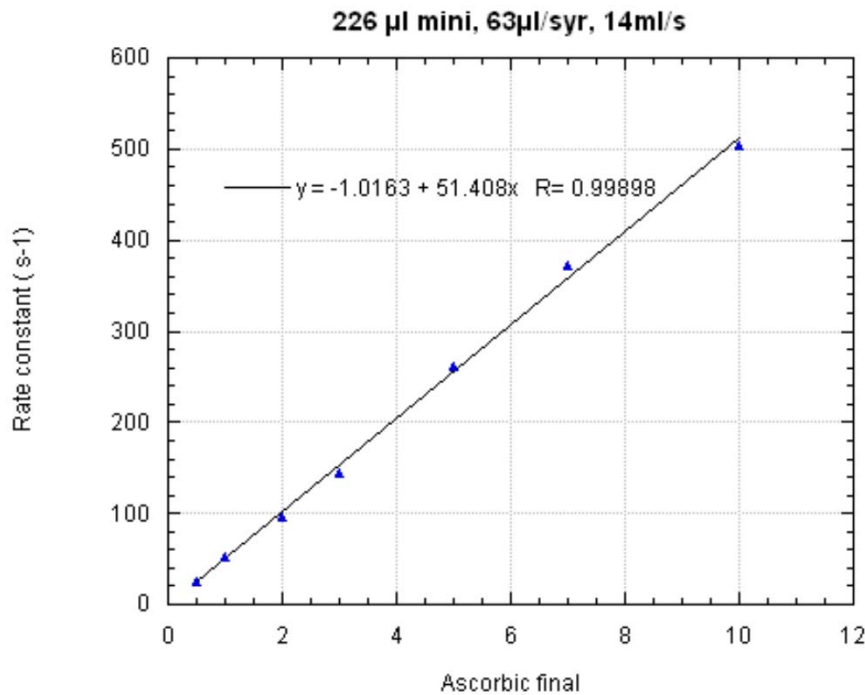
Estimated dead time : 1.1 ms

Configuration

	Content of syringes	Initial concentration	Final concentration
Syringe 1	10 ml, ascorbic acid	20 mM	2.0 mM
Syringe 2	10 ml, buffer		
Syringe 3	10 ml, DCIP	100 μM	50.0 μM
Syringe 4	----		

Buttons: Load, Save As, Comments, Print, SFM Options, Close

Cuvette TC-100/10, flow rate 14ml/s
 Mixing ratio from 0:10:10 to 19:1:20 without reloading
 (1:1 to 1:39 dilution)



Chevron plots, concentration dependence studies

Concentration dependence studies

Concentration dependence studies

	Content of syringes	Initial concentration	Mixer 3 conditions	
Syringe (2)	buffer	Dil. -	Ratio (A +Diluant) 1	Concentration Amax : 10.0 mM
Syringe (3)	ascorbic	A0 20 mM	Ratio B 1	Concentration B : 0.5 μM
Syringe (4)	DCIP	B0 1.0 μM	Total flow rate 12 ml/s	Estimated dead time : 1.3 ms

Mode

Ratio steps Auto
 Concentration steps Auto

Variation Concentration in Mixer 2

Concentration A (mM) 1
 Step number 5
 Step value (mM) 1
 Final value (mM) 5.0

Start next acquisition step at the end of measurement
 after 5 sec
 manual continuation

Start of data acquisition At stop
 Pre-trigger

Load Print Save As SFM Options Infos... Edit Sequence

Global sequence

Autovariation concentration steps mode

Step	Ratio Dil.	Ratio A	Ratio B	Concent.[A]	Volume Dil.	Volume A	Volume B
1	0.9	0.1	1	1	450	50	500
2	0.8	0.2	1	2	200	50	250
3	0.7	0.3	1	3	116.7	50	166.7
4	0.6	0.4	1	4	90	60	150
5	0.5	0.5	1	5	75	75	150

Repeat number 2 Total volume / Syringe 1863.3 μl 570 μl 2433.3 μl

Ready Close

Automatic double mixing

Double mixing experiments

Double mixing experiments

	Content of syringes	Initial concentration	Final concentration
Syringe (1)	A	A0 10 mM	1.667 mM
Syringe (2)	B	B0 2 nM	0.333 nM
Syringe (3)	C	C0 100 µM	66.667 µM

Phase 1 conditions	Phase 3 conditions	Start next acquisition step
Ratio A: 1	Empty Delay line by using: Syringe (B)	<input checked="" type="radio"/> at the end of measurement
Ratio B: 1	Ratio Delay line: 1 Ratio C: 2	<input type="radio"/> after 5 sec
Total flow rate: 14 ml/s		Start of data acquisition
Estimated dead time: 0.3 ms Minimum ageing time: 15.0 ms		<input checked="" type="radio"/> At stop <input type="radio"/> Pre-trigger

Load Print Save As SFM Options Infos... Edit Sequence

Global sequence

Structure of the sequence				Ageing times	
	Phase 1	Phase 2	Phase 3	Step	Age (ms)
Time (ms)	45.1	15	15	1	30
Syr.1 (µl)	105.2		0	2	100
Syr.2 (µl)	105.2		70.1	3	200
Syr.3 (µl)	0		140.3	4	500
				5	2000

Repeat number: 1 Total volume / Syringe: A 526 µl B 876.6 µl C 701.3 µl

Ready ▶ Close ✖

<p>Protein folding Conformational changes Substrate binding Enzyme kinetics Substrate and H₂O transport in vesicles</p>	}	<p>UV/Vis absorbance Light scattering Fluorescence Circular dichroism Fluorescence anisotropy FTIR X-ray and neutron scattering EPR Conductivity ...</p>
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Method	Information	Time resolution
Absorbance	Structure of chromophore	0.2 to 1 ms
Fluorescence	Environment of chromophore	0.2 to 1 ms
Fl. Anisotropy	Mobility of chromophore	0.2 to 1 ms
CD (Far-UV)	Secondary structure (low resolution)	2 to 5 ms
CD (Near UV)	Tertiary structure	2 to 5 ms
FTIR	Secondary structure (high resolution)	100 ms
H-D exchanges	Accessibility of residues to solvent	5 ms

Example extracted from :

The water permeability of *Arabidopsis* plasma membrane is regulated by divalent cations and pH
The Plant Journal (2002), 3(1), 71-81

Christophe Maurel and al.

Aim : to study regulation of water channels using Stopped-flow light scattering,
to measure vesicles shrinking created by an osmotic shock

Solutions :

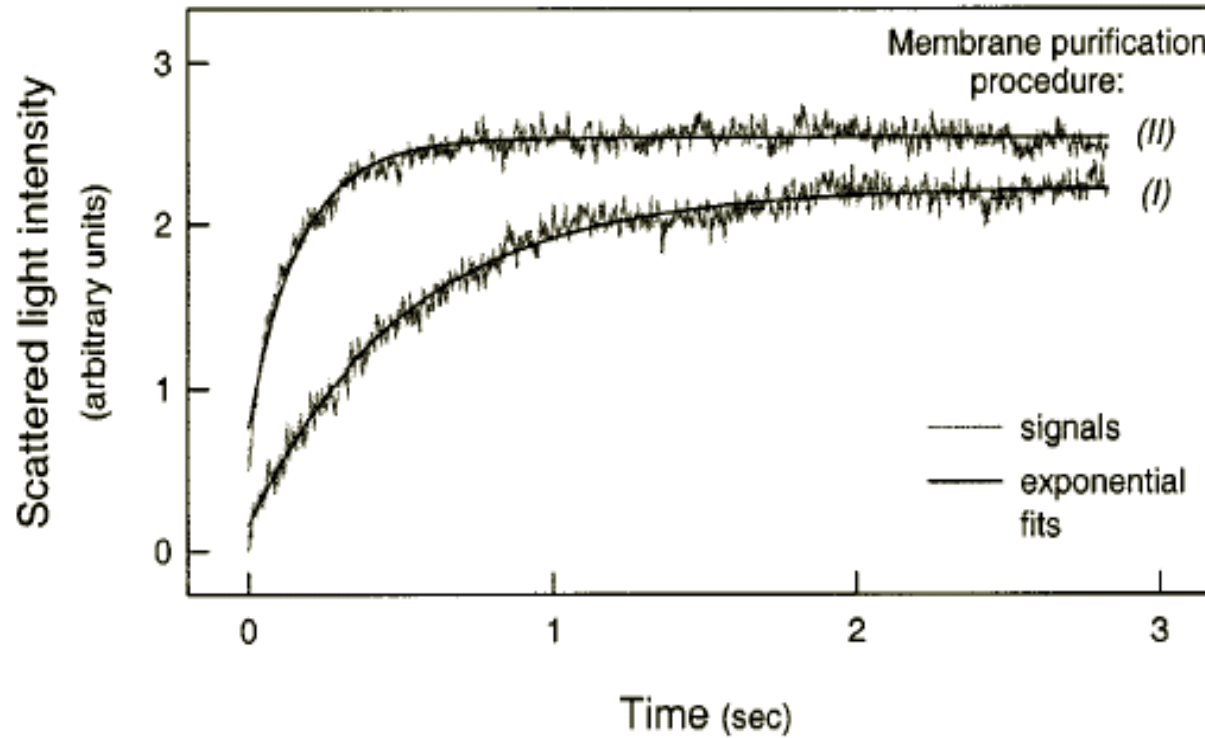
Solution 1 : vesicle into equilibrium media (50mM NaCl, 50mM mannitol, 10mM Tris pH=8.3)
(187 mosmol.kg⁻¹ H₂O)

Solution 2 : same media but with 500mM mannitol (693 mosmol.kg⁻¹ H₂O)

Mixing conditions :

1:1 mixing ratio leading to a 253 mosmol.kg⁻¹ H₂O inward osmotic gradient

Example of traces obtained



$$P_f = K \cdot V_0 / A_v \cdot V_w \cdot C_{out}$$

Where

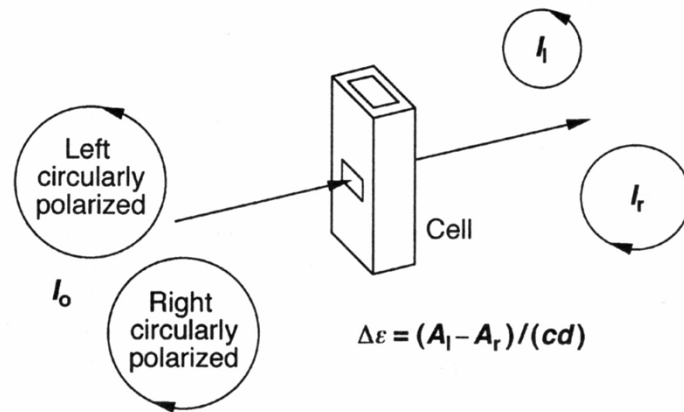
P_f = osmotic water permeability

V_0 = initial mean vesicle volume

A_v = mean vesicle surface

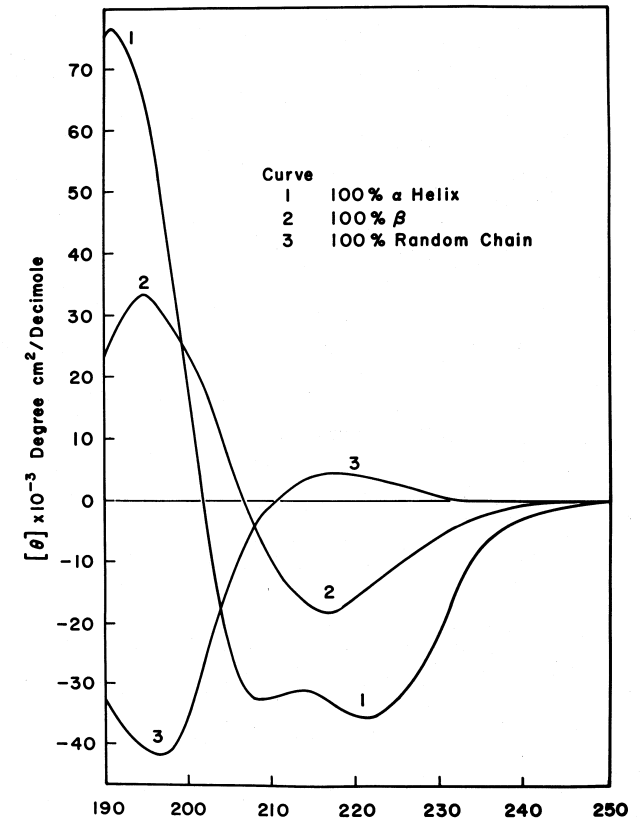
V_w = molecular volume of water

C_{out} = external osmolality

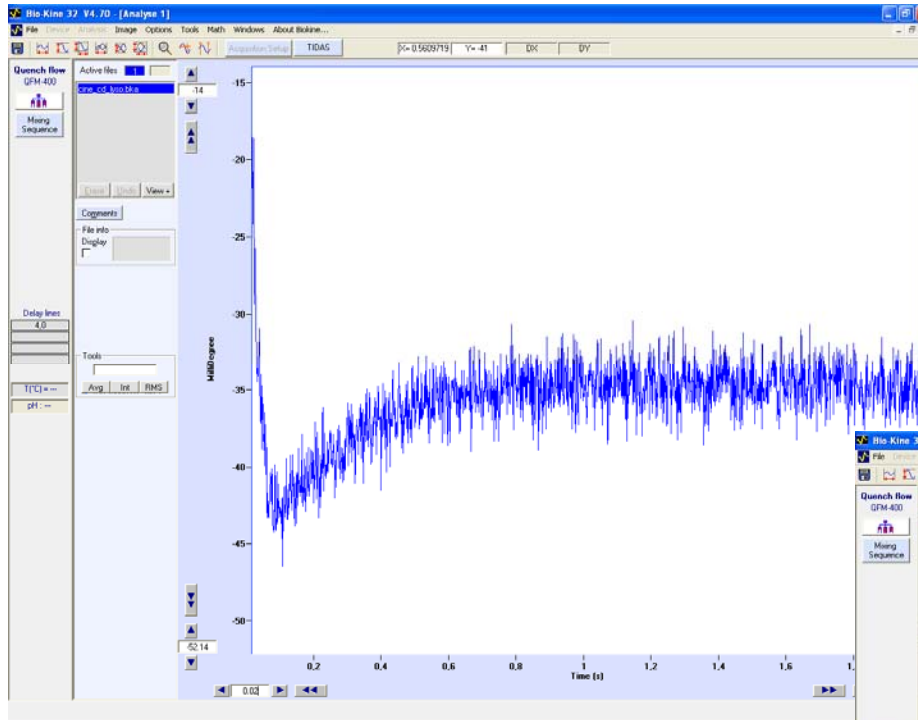


Circular dichroism is the difference between the absorptions for left, A_l , and right, A_r , circularly polarized light. Alternately left and right circularly polarized light with the initial intensity I_0 is passed through the sample cell. In most CD spectrometers, the difference between A_l and A_r is calculated using solely the final intensities, I_l and I_r

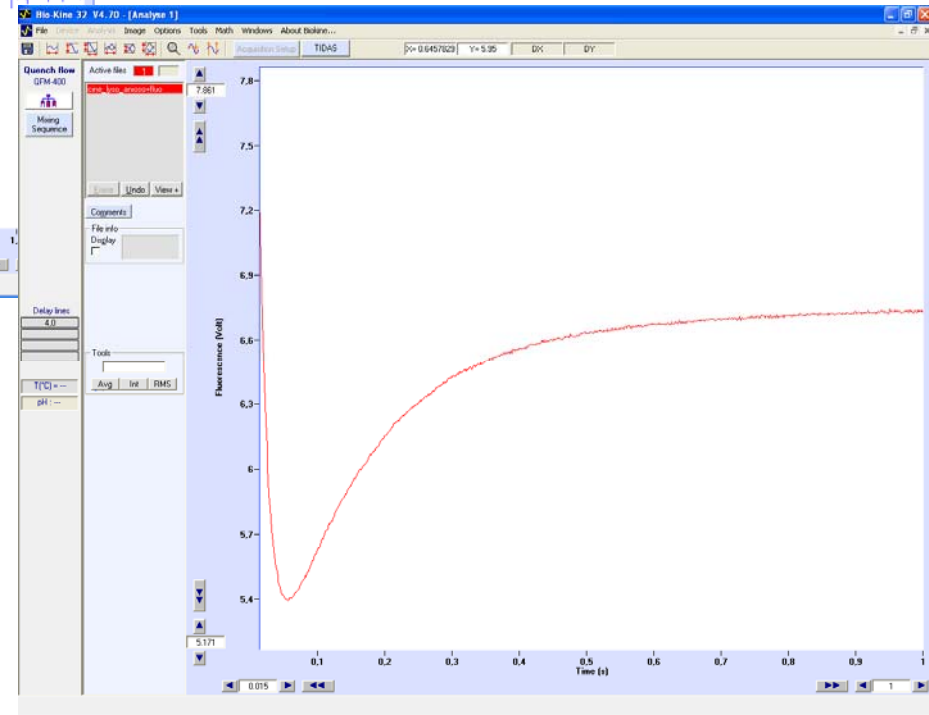
Circular Dichroism of Proteins—(Continued)



Lysozyme refolding

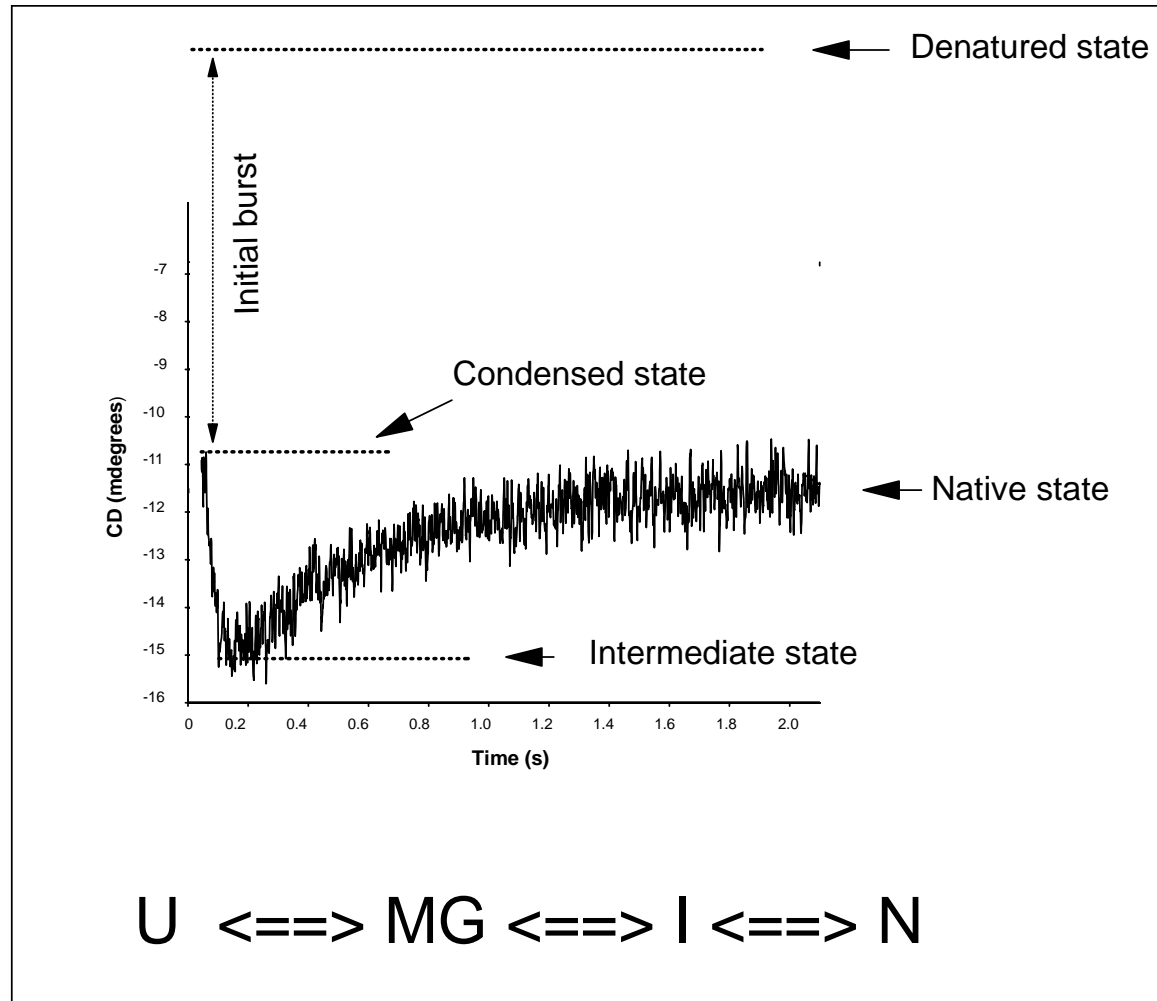


Alpha helix CD



Trp fluorescence

Lysozyme refolding



An unique technique for cold jumps experiments
Application: refolding experiments

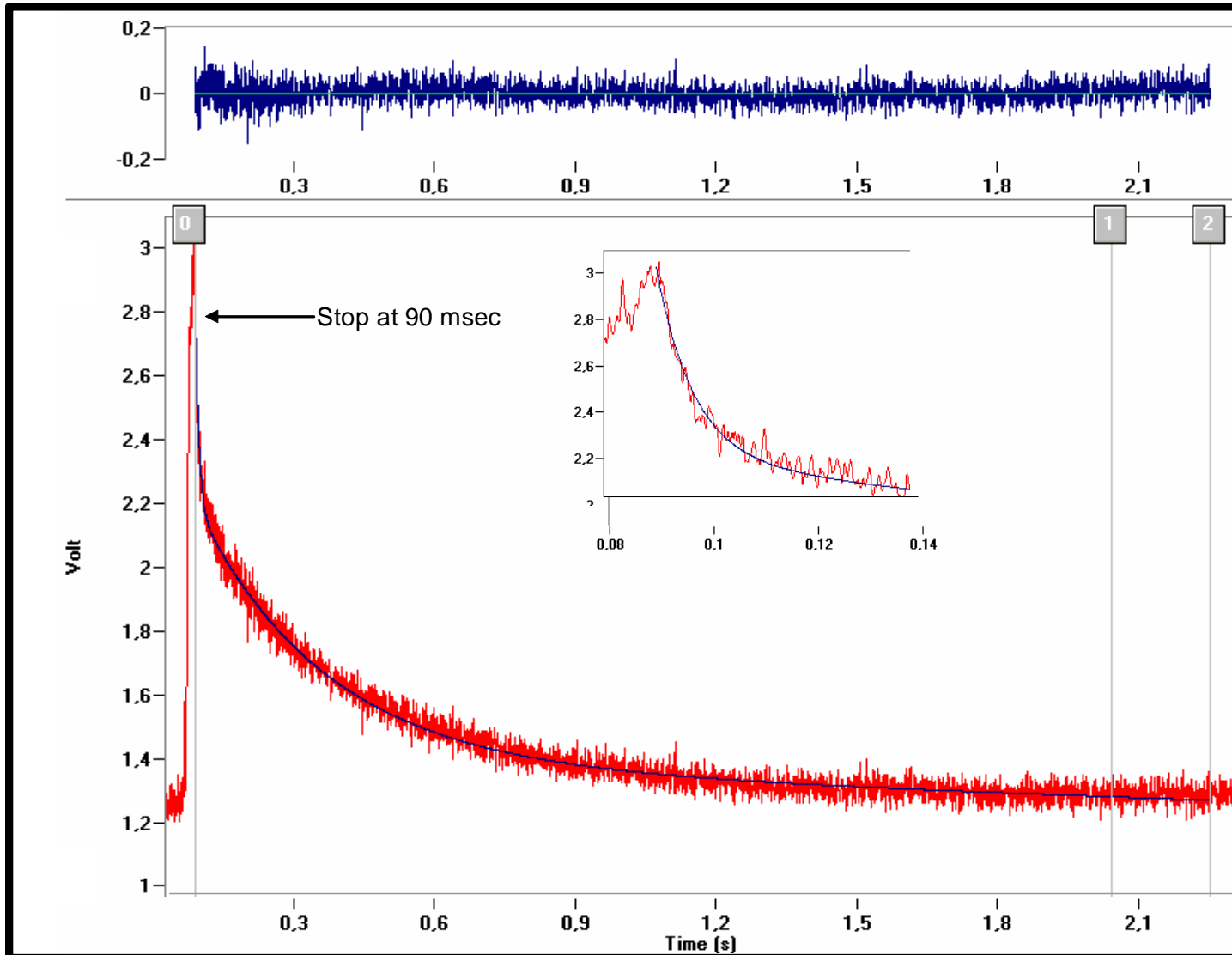


Classical T-jump

Maximum amplitude : 4-5°C
Only hot jumps induced by fast laser heating
T stability: only few ms

mT-jump

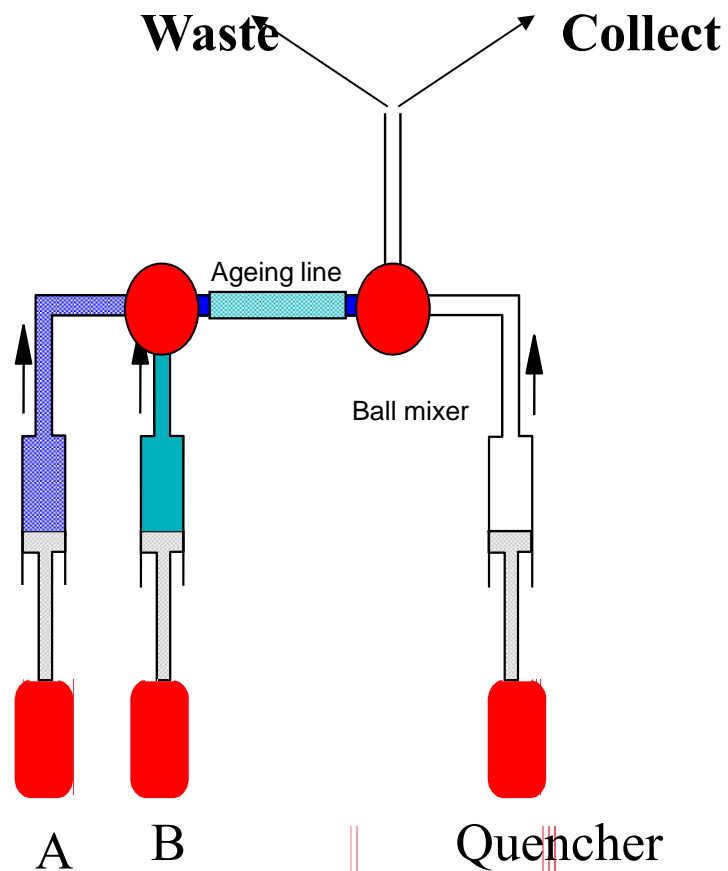
- ms time resolution
- Cold and hot jumps : up to +40°C
- Compatible with all stopped-flow models
- Compatible with all spectrometers
- T stability: variation < 1% per 30 seconds



Cytochrome c refolding : T-jumps from 85°C to 60°C

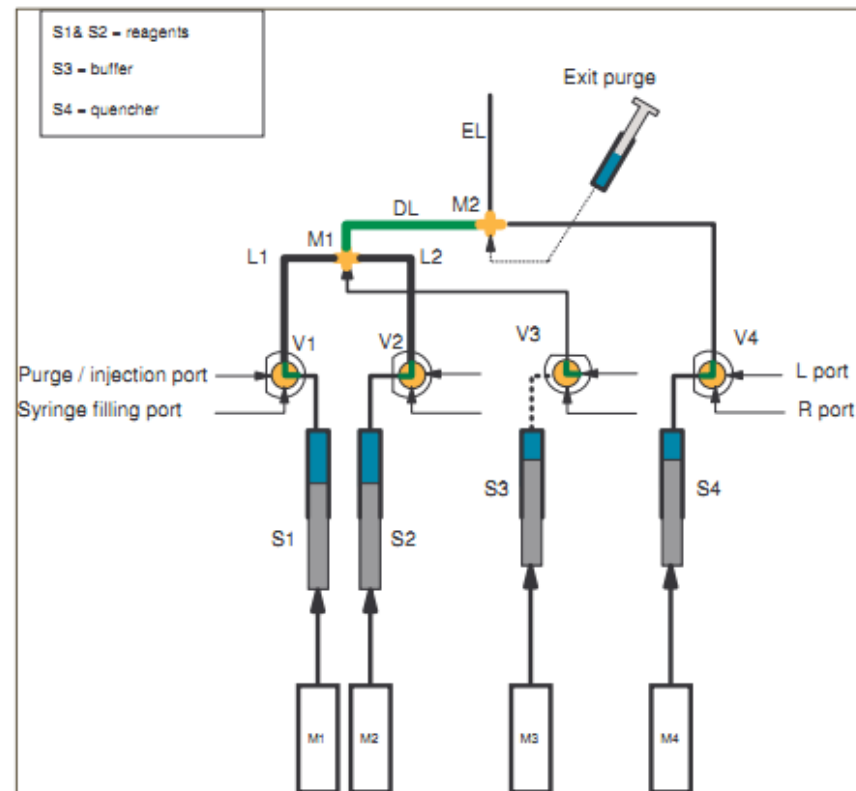
QUENCH-FLOW

Classical QF instrument



$$A = V_{DL} / (F_1 + F_2) \text{ or Interrupted mode}$$

Microvolume QF instrument



One delay line for all ageing times

10-15 μ l of sample only

Exemple of single mixing quench-flow sequence Using micro-volume quench flow

Quenched Flow Program - *.*

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
time (ms)	375	500			
Syr. 1 (µl)		20			
Syr. 2 (µl)		20			
Syr. 3 (µl)	30				
Syr. 4 (µl)		20			
Ageing	Manual	Auto	Auto	Auto	Auto
Synchro 1	Off	Off	Off	Off	Off
Synchro 2	Off	Off	Off	Off	Off

Syringes Volumes (ul)

- Syr. 1: 20
- Syr. 2: 20
- Syr. 3: 30
- Syr. 4: 20

Auto Ageing time
50 ms

Phase : 1/5 Total Volume : 30 µl Total Flow Rate : 0,08 Delay Line : 4 ul

Insert Phase Remove Phase Clear sequence

Syringes contents

Syringe 1	A
Syringe 2	B
Syringe 3	buffer
Syringe 4	quencher

Sequence

Ready

Single shot
 Repeat sequence

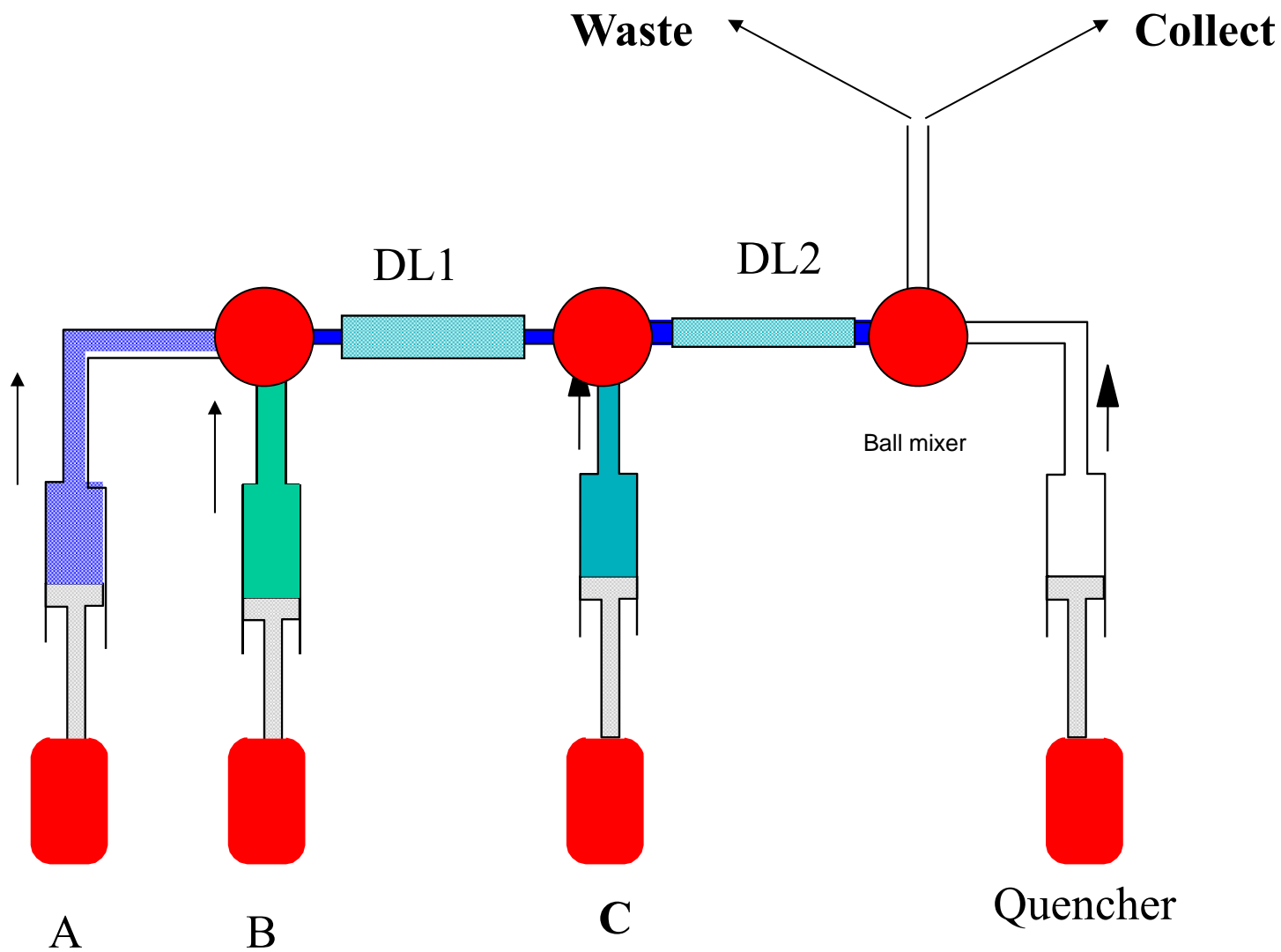
Repeat sequence

Repeat phase: 1
Repeat number: 1
Time between shots (s): 1

Times (ms):

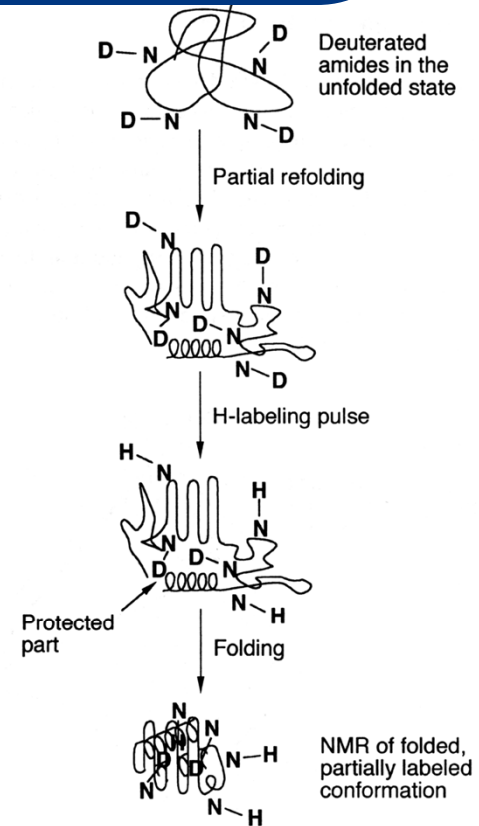
Load Save As Comments Print SFM Options Close

Double mixing quenched-flow instrument

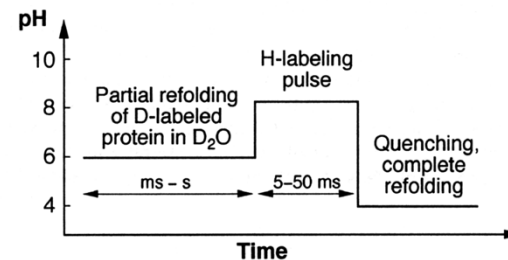


EXAMPLE

H/D exchange experiment



Kinetic H/D exchange experiment. Prior to the refolding reaction, all exchangeable protons were replaced by deuterons in the unfolded state. Refolding is initiated with deuterated buffer. After partial refolding, the protein solution is mixed with H₂O. Parts of the protein which are protected at this time stay deuterated. Then H/D exchange is quenched with buffer of low pH and refolding is completed prior to NMR measurement.



pH profile of a typical kinetic H/D exchange experiment (quenched-flow).

H/D exchange exemple

Quenched Flow Program - *.*

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Total Volumes (µl)
time (ms)	100	300	15	10		100
Syr. 1 (µl)	100					650
Syr. 2 (µl)	500		90	60		150
Syr. 3 (µl)			90	60		300
Syr. 4 (µl)			180	120		
Valve	Waste	Waste	Waste	Collect	Waste	
Synchro 1	Off	Off	Off	Off	Off	

Phase : 4/5 Volume : 60 µl Flow Rate : 6,0 ml/s

Syringes contents

Syringe 1	protein in D Gnd
Syringe 2	D buffer
Syringe 3	water
Syringe 4	buffer pH=4

Shots

Single

Multiple

Drive Sequence

1(1:20)

Ageing Times

DL1 27,4 ms

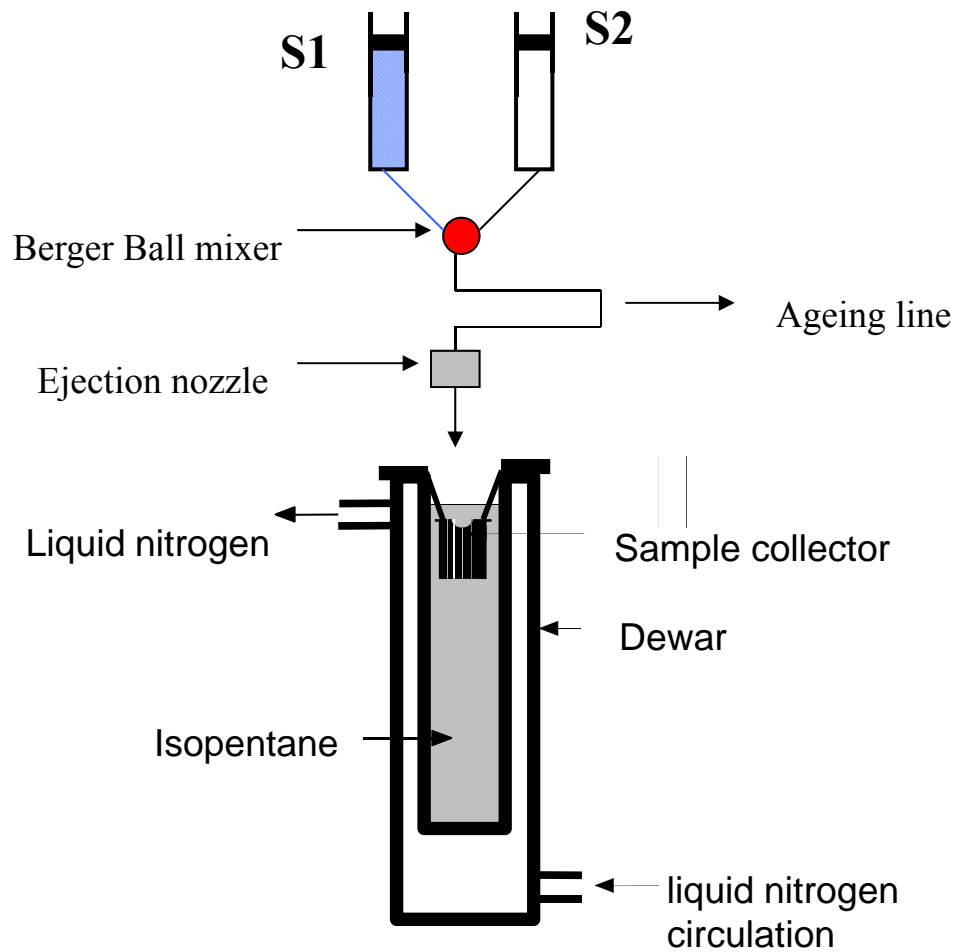
DL2 5,0 ms

EDL

Load Save As Comments Print SFM Options Close

Delay line 1 = 164.3 µl
 Delay line 2 = 59.8 µl

Partial refolding = 327.4 msec
 H-labelling = 5 msec



Reaction time

=

Ageing time + flying time + freezing time



Applications of chemical quenched-flow

Protein folding
Conformational changes
Substrate binding
Enzyme kinetics
Second messenger studies

Radioactive labelling
H/D exchange followed by NMR
H/D exchange followed by Mass spectrometry
Chromatography
Gel electrophoresis
...

Applications of freeze quench

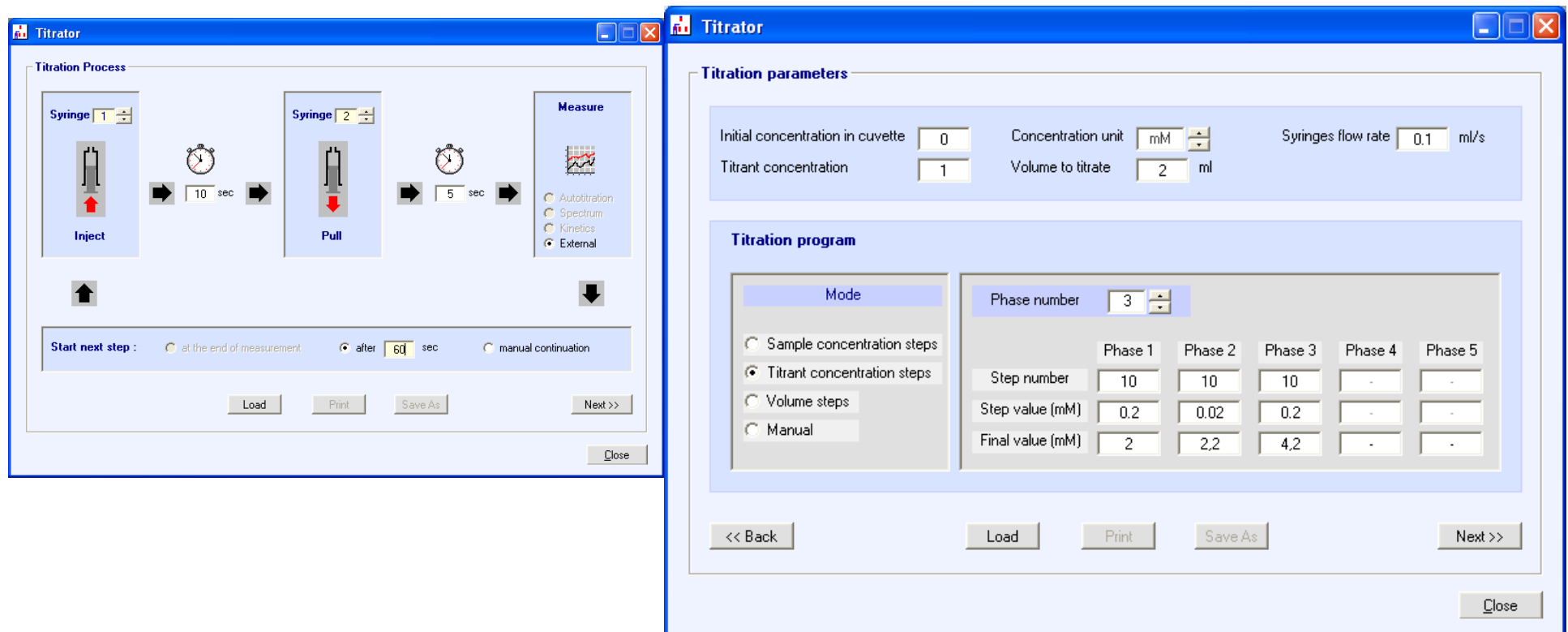
**Trapping of kinetic intermediates
by ultra-fast freezing**

Solid state NMR
EPR
EXAFS
Mössbauer
...

Your stopped-flow is also a titration unit

Full automation of titration

Possibility to use one syringe to pull solution and to work at constant volume



Titration Process

Syringe 1 → 10 sec → Syringe 2 → 5 sec → Measure

Inject → Pull → Measure

Start next step : at the end of measurement after 60 sec manual continuation

Buttons: Load, Print, Save As, Next >>, Close

Titration parameters

Initial concentration in cuvette: 0 Concentration unit: mM Syringes flow rate: 0.1 ml/s

Titrant concentration: 1 Volume to titrate: 2 ml

Titration program

Mode: Sample concentration steps Titrant concentration steps Volume steps Manual

Phase number: 3

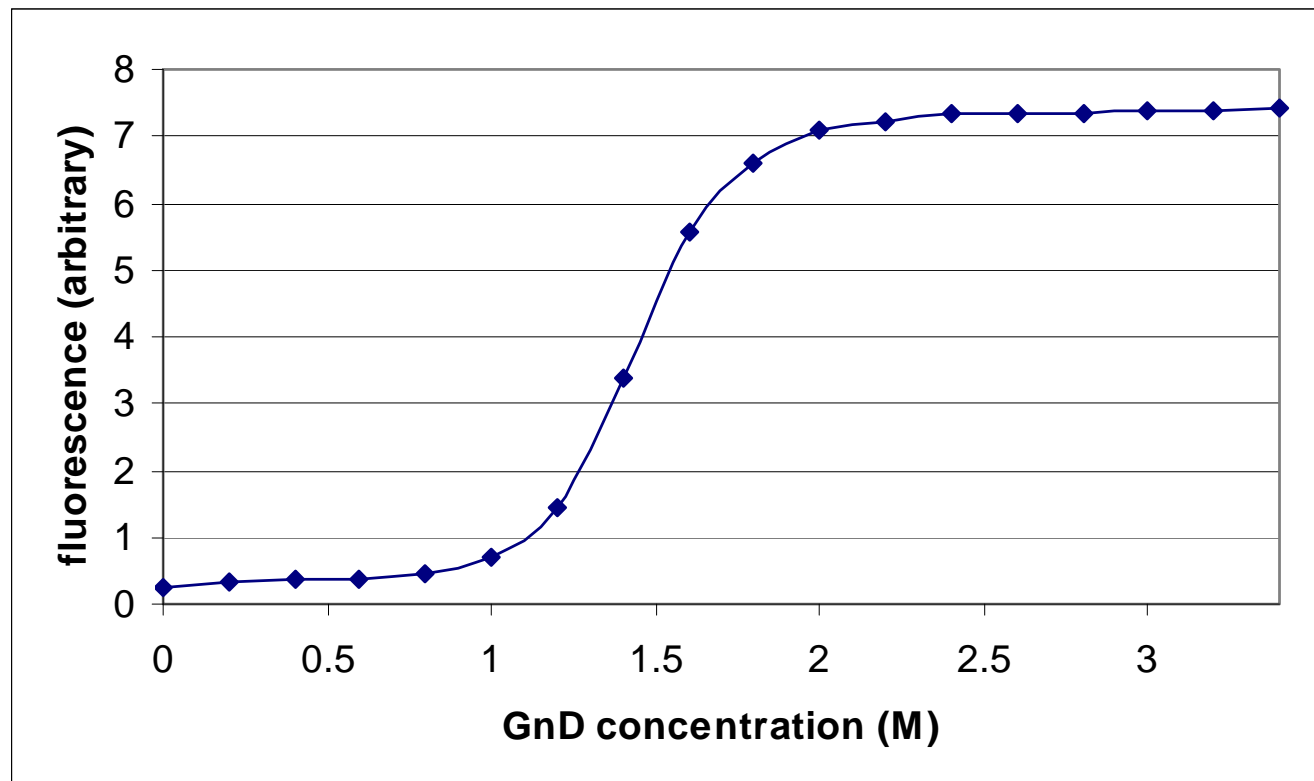
	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5
Step number	10	10	10	-	-
Step value (mM)	0.2	0.02	0.2	-	-
Final value (mM)	2	2,2	4,2	-	-

Buttons: << Back, Load, Print, Save As, Next >>, Close

Exemple : Titration of cytochrome c from horse heart Determination of refolding conditions for SF

Detection, excitation 290 nm, detection >320 nm

Cyt C is unfolded in GndHCl 8M and titrate by native cyt C



Stepping motors : the basis of a versatile kinetics station

