Rapid kinetics procedures

3 ways to start a reaction :

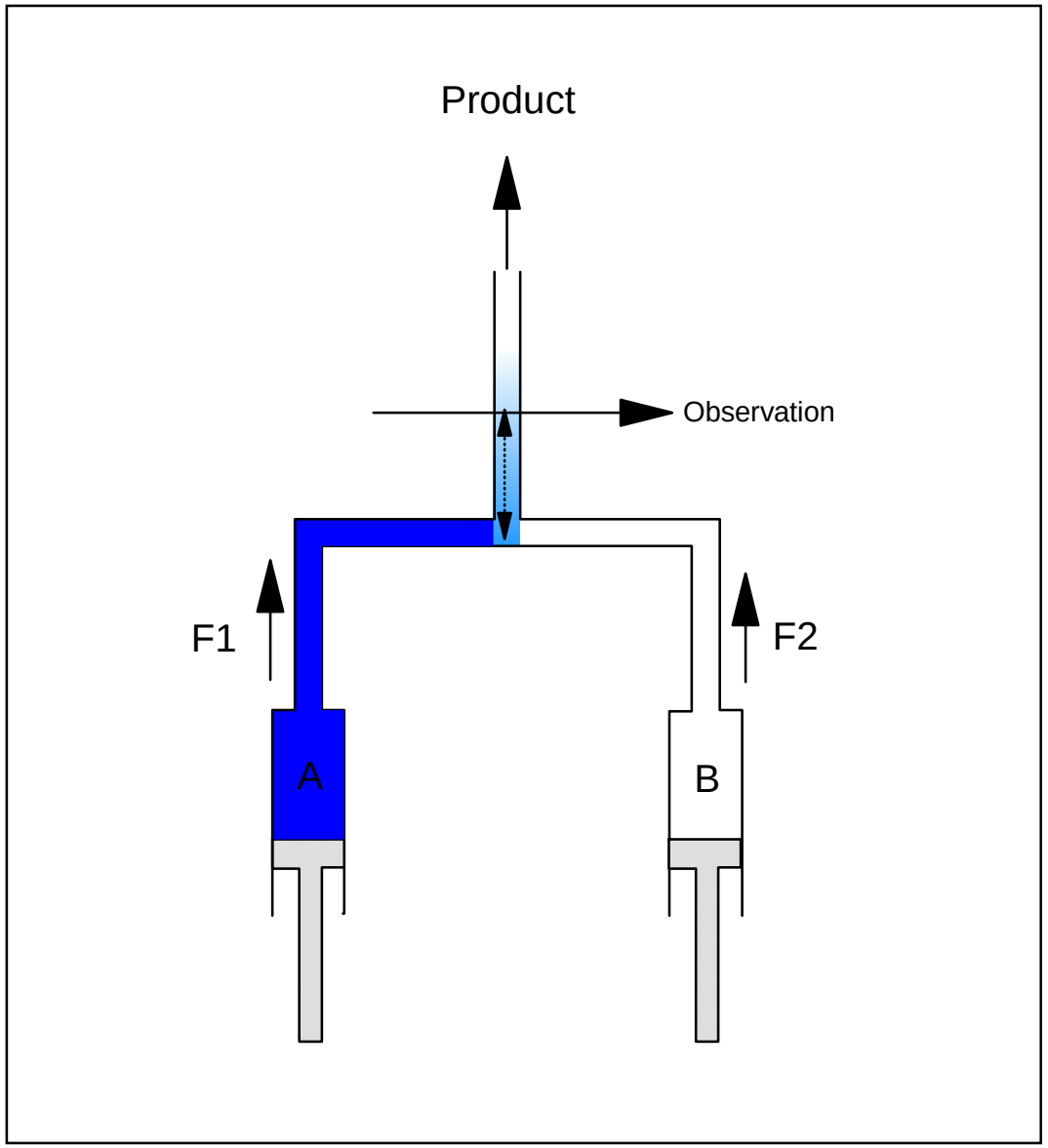
- Mixing
- Light
- Temperature

} Continuous recordings → Stopped-flow

3 ways to stop a reaction

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

Stopped-flow technique



Pushing

↓

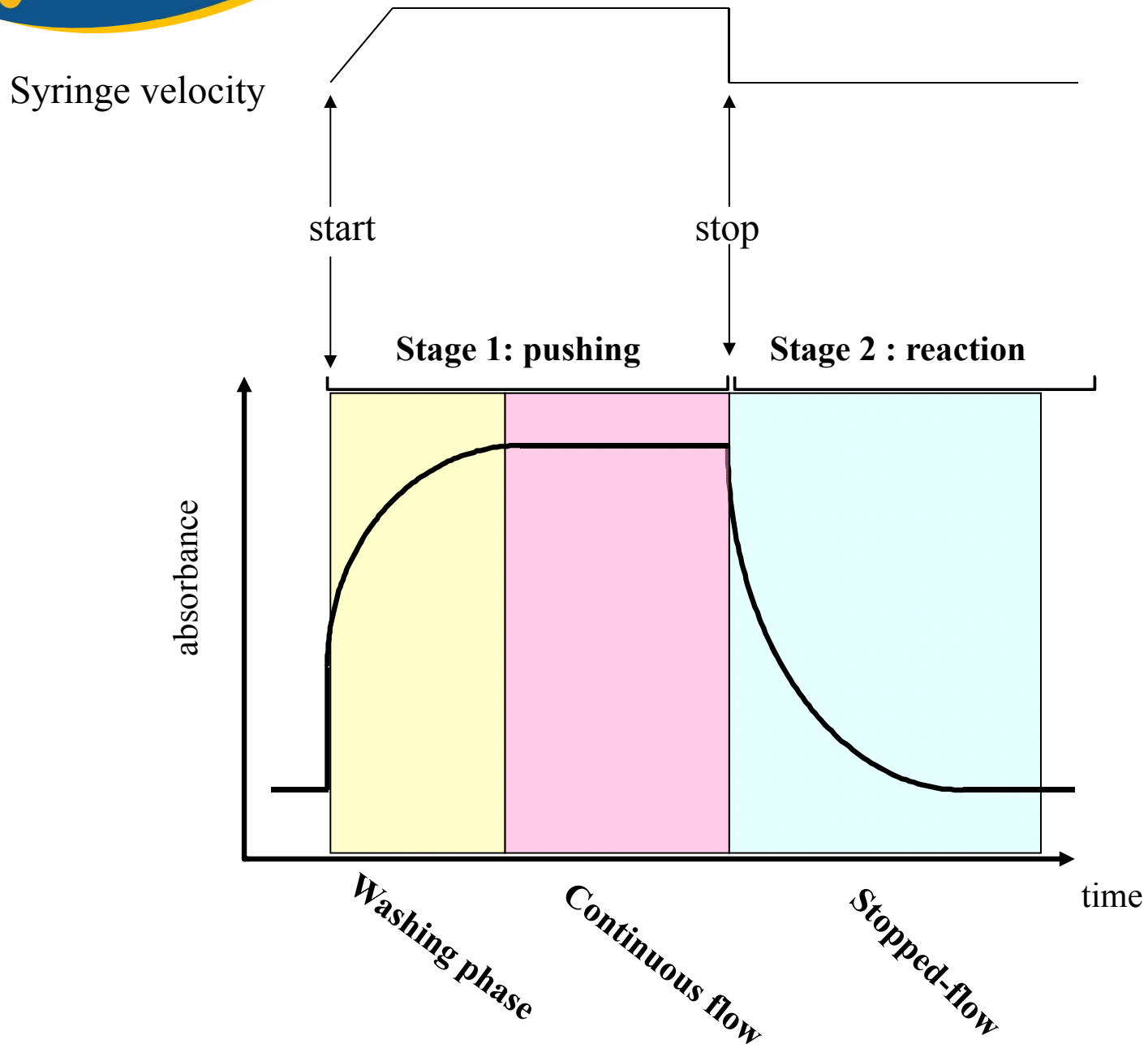
Mixing

↓

Stop of the flow

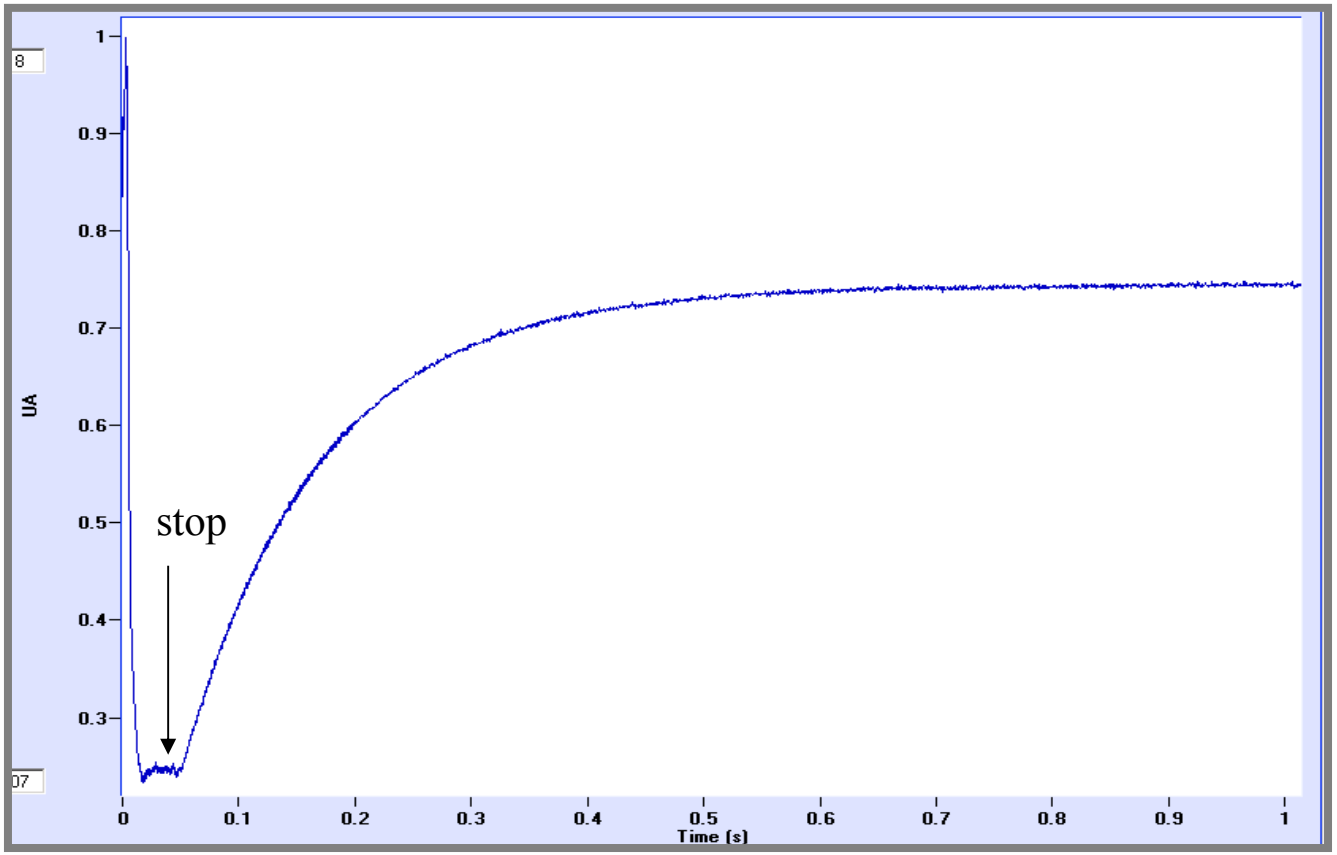
↓

observation

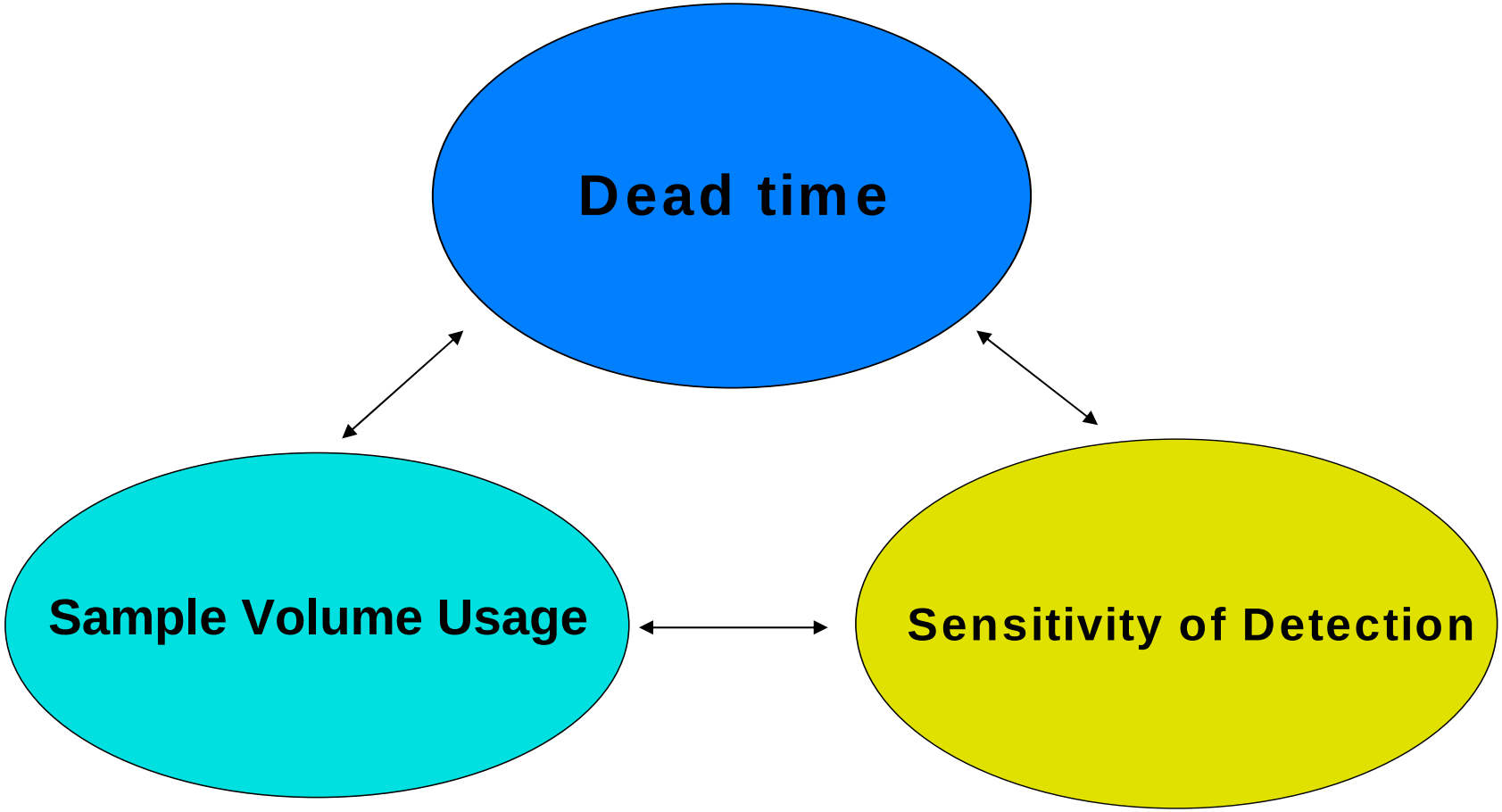

Syringe velocity
start
stop
Stage 1: pushing
Stage 2 : reaction
absorbance
time
Washing phase
Continuous flow
Stopped-flow

Typical stopped-flow trace : DNPA hydrolysis by NaOH

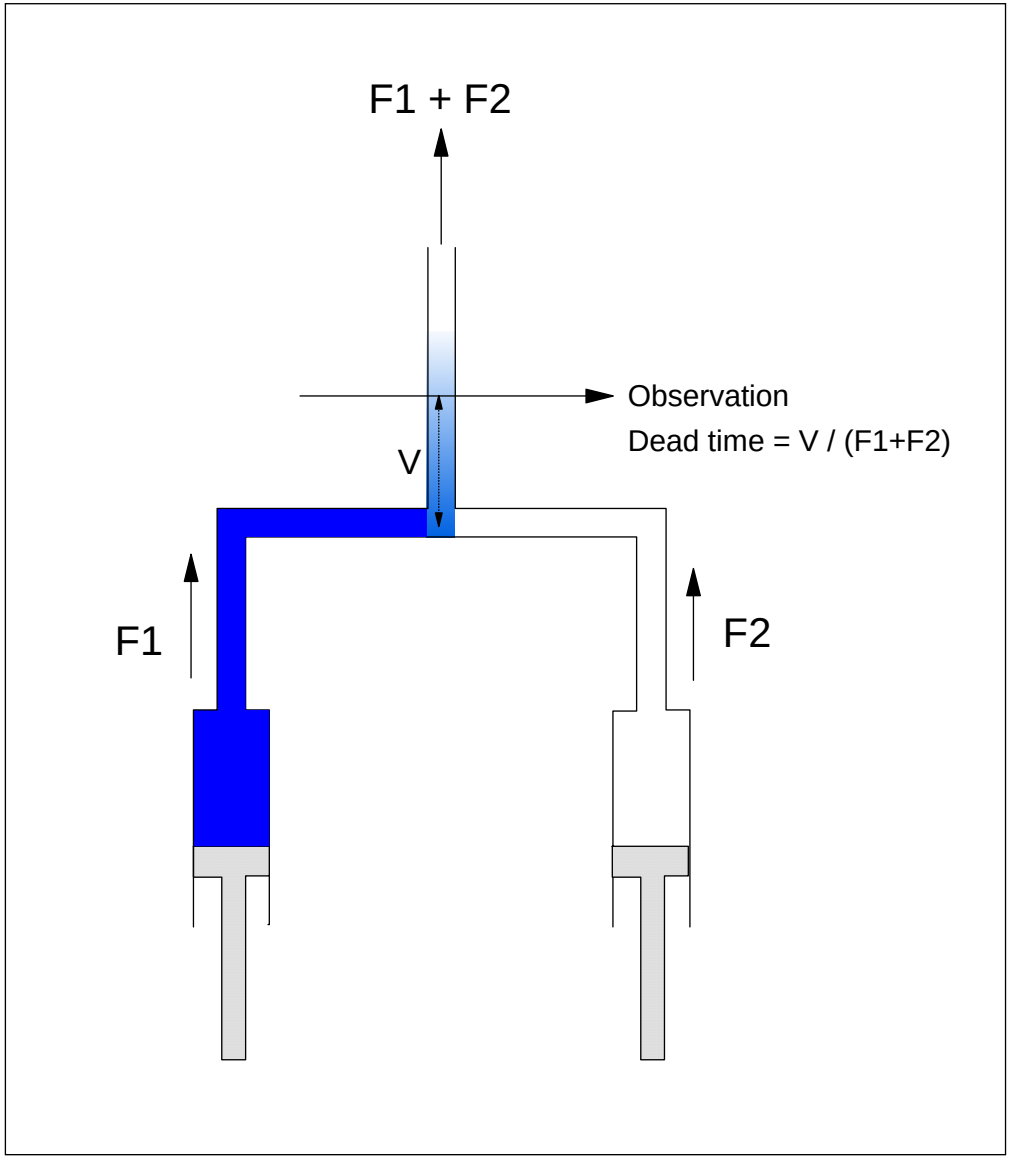
Followed by absorbance at 325 nm
1:1 mixing ratio, total volume 200 µl, 10 ml/s
Acquisition 50 msec before stop



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**

Example of dead time

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

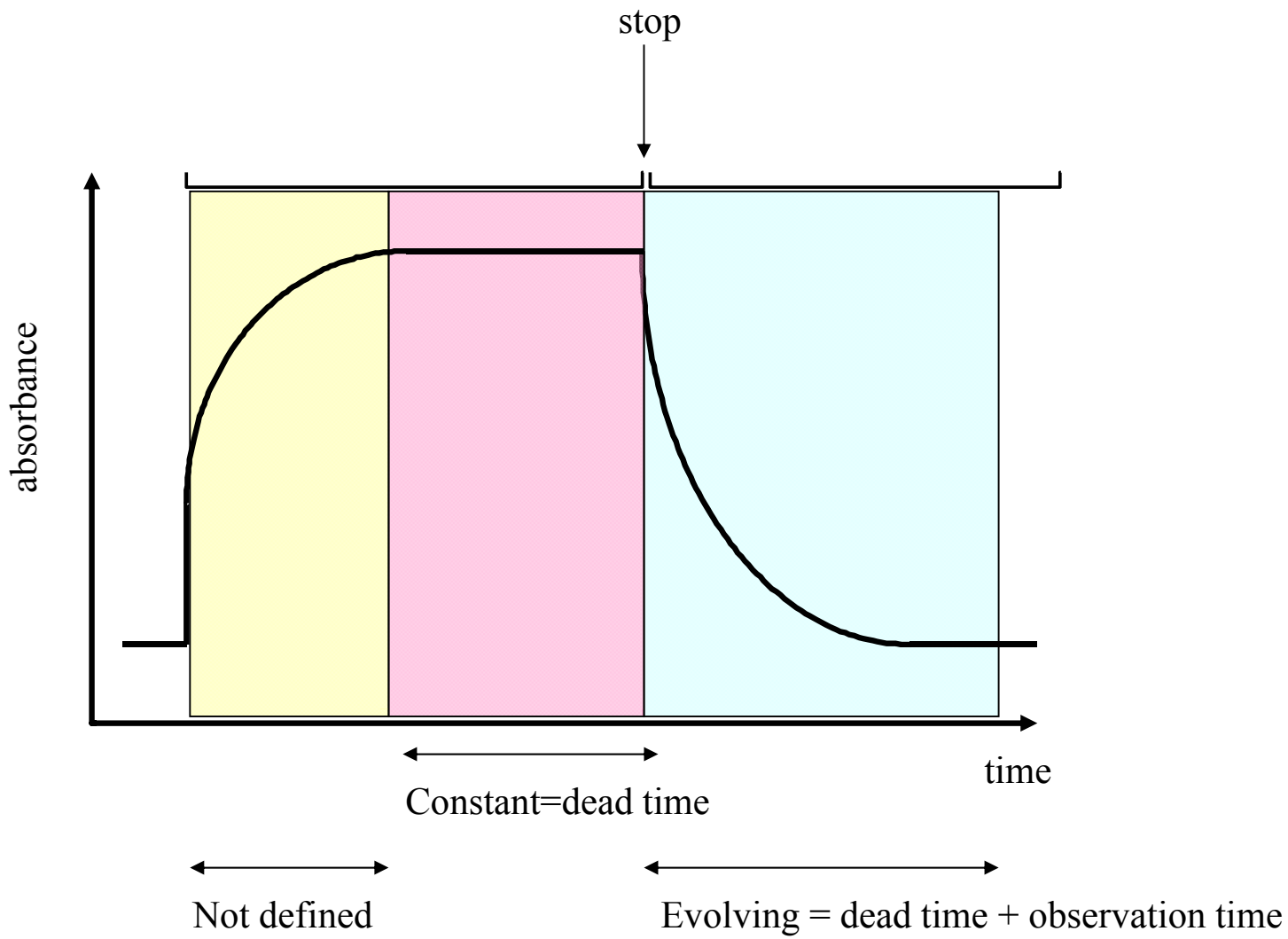
Irina Smirnova, Vladimir Kasho, Junichi Sugihara, and H. Ronald Kaback

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

Methods

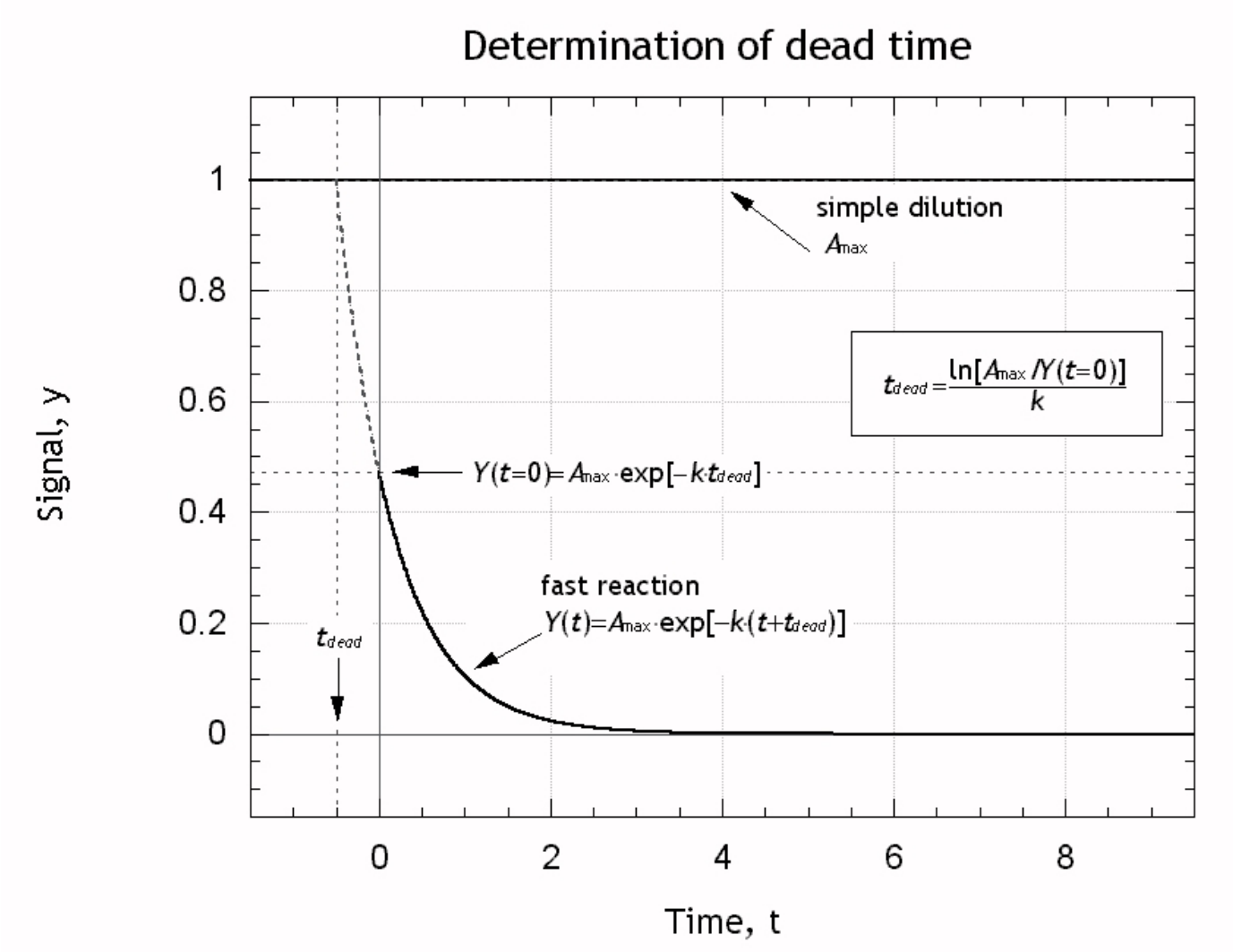
Fluorescence Measurements. Steady-state Trp fluorescence of 0.5 μM protein was monitored at excitation wavelength 295 nm on a SPEX Fluorolog 3 spectrofluorometer at room temperature as described (14). Time traces were recorded at emission wavelength 335 nm. Stopped-flow measurements were performed at 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



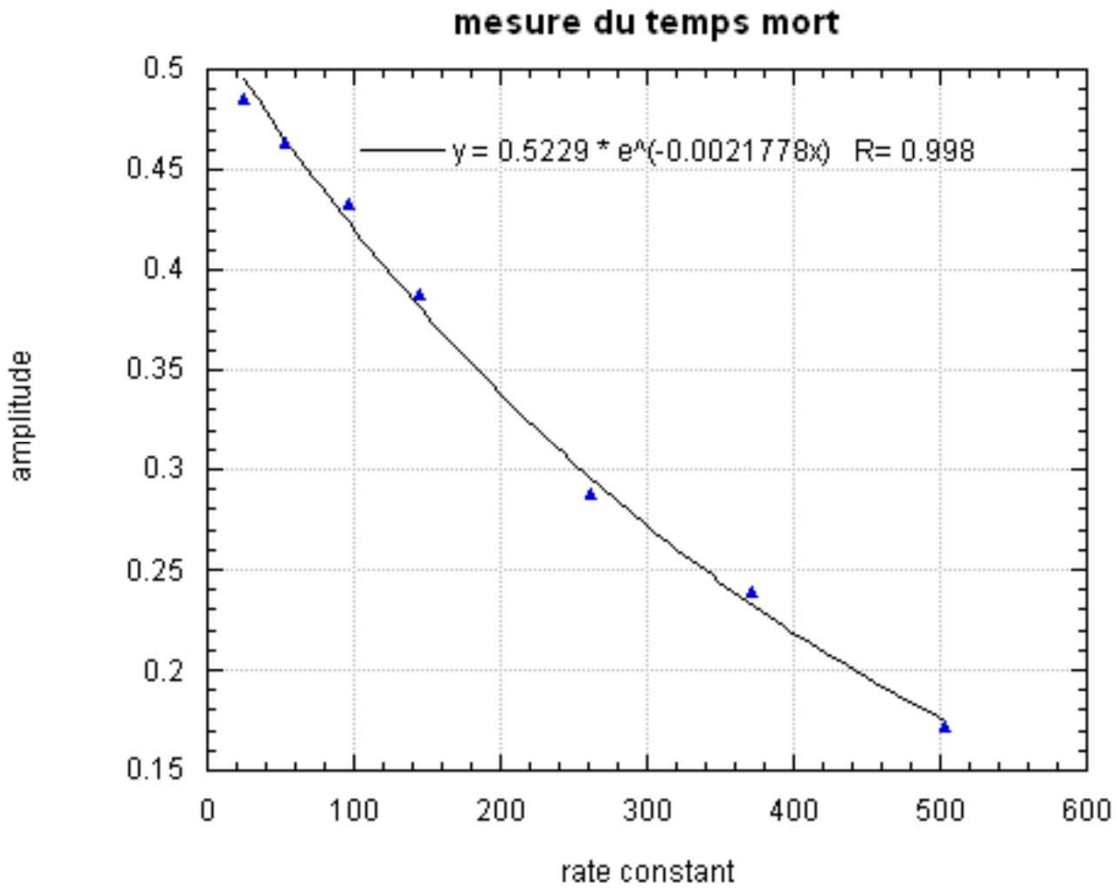
REACTION TIME

A quick and convenient way to measure dead time…



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.

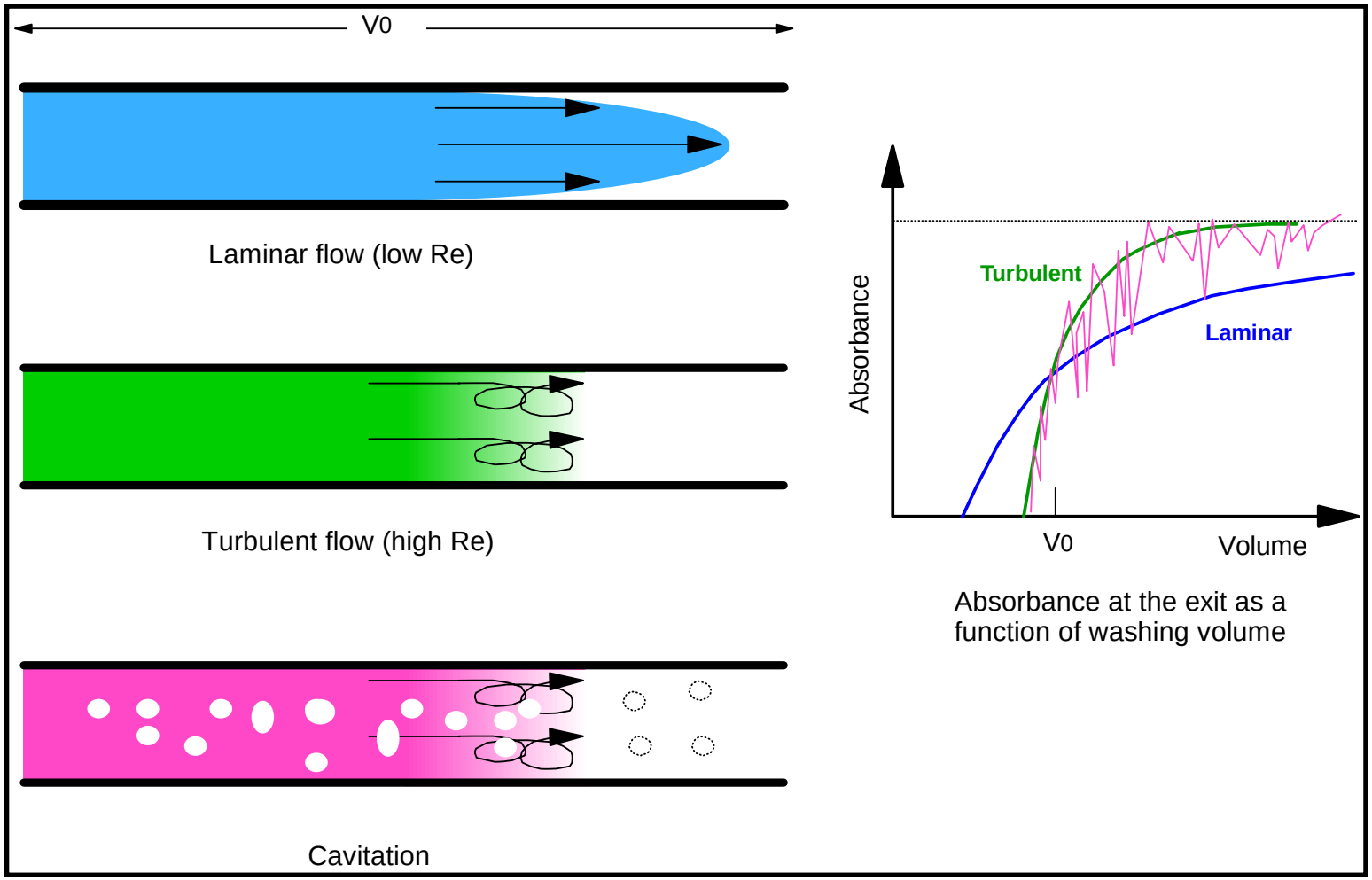


Sample volume usage

Depends on :

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

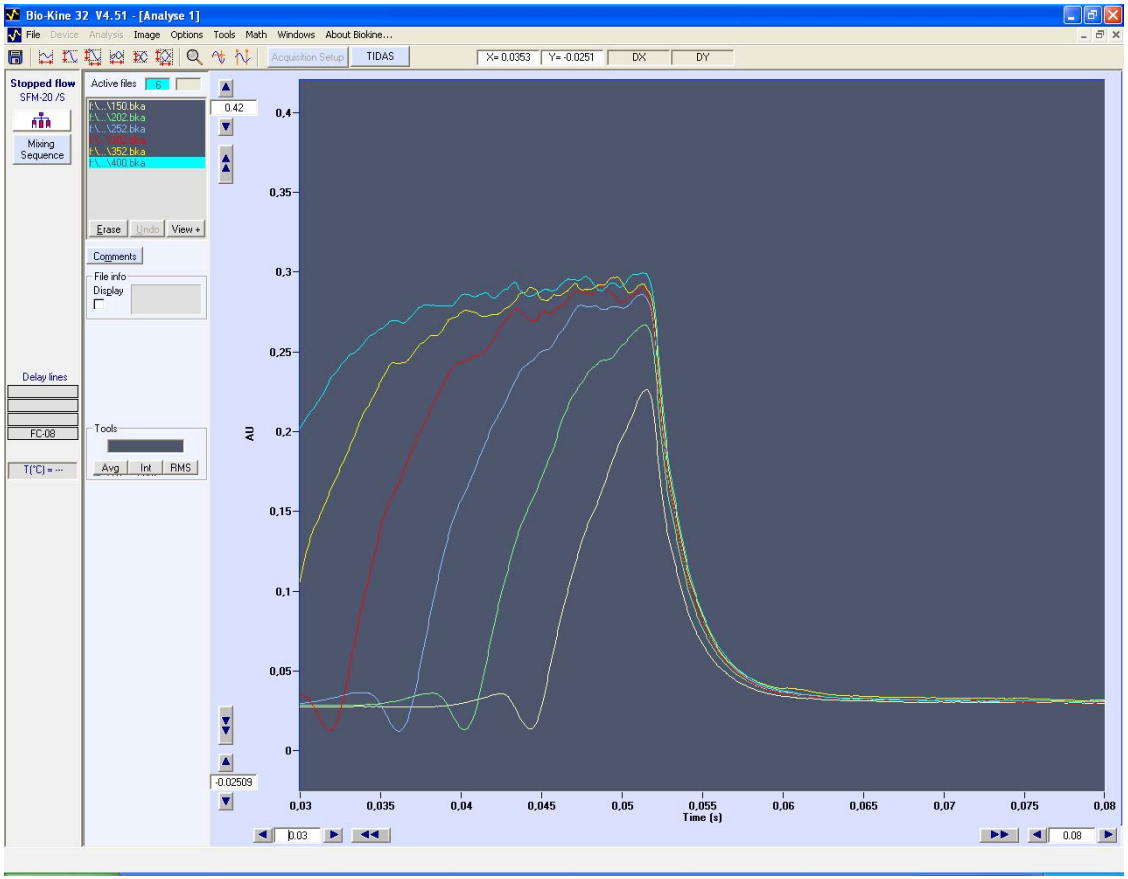
Mixing efficiency



Absorbance at the exit as a function of washing volume

minimum volume to inject : CUVETTE LIMITATION

fast reaction, same shot but reducing total volume



Loss of amplitude

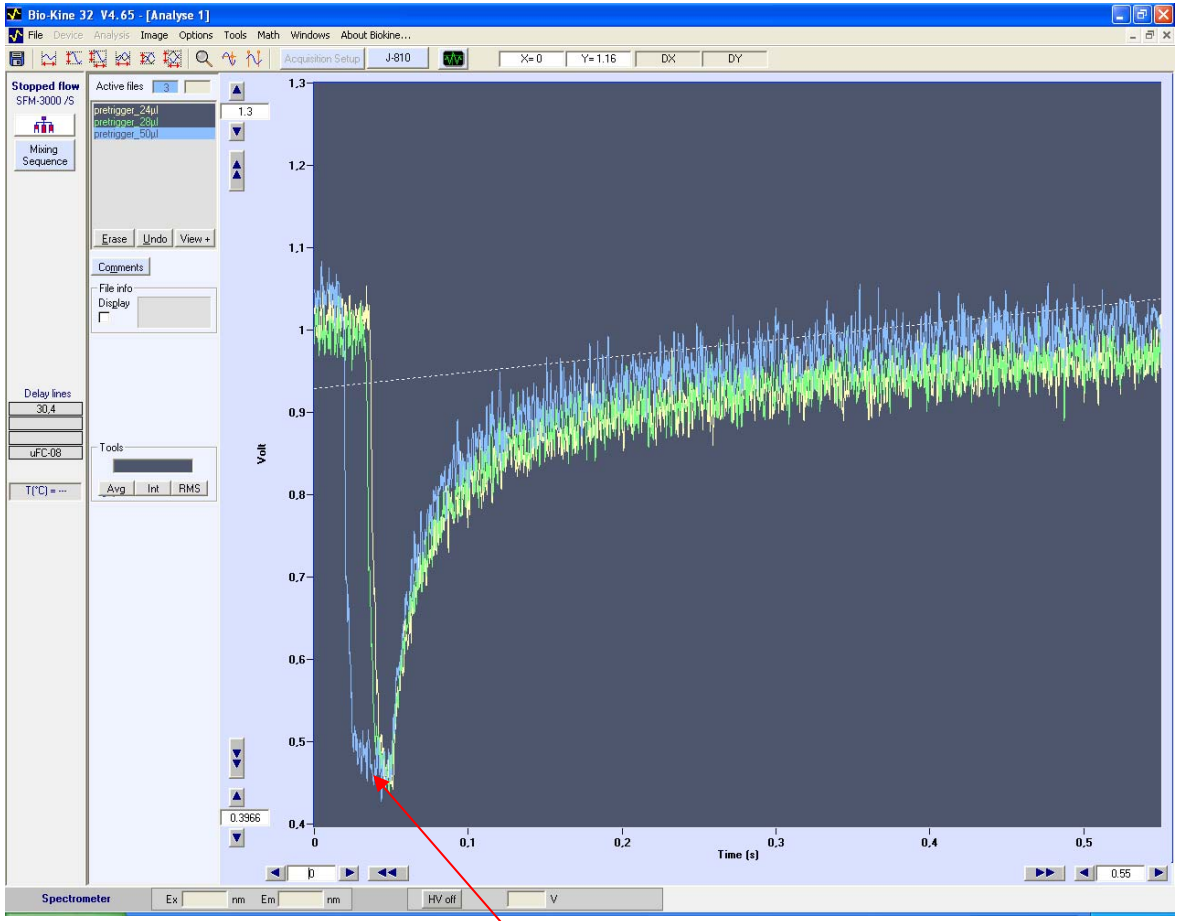
=

Bad washing

MIMIMUM VOLUME = 3-4 times the volume of cuvette

minimum volume to inject : SYRINGE LIMITATION

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



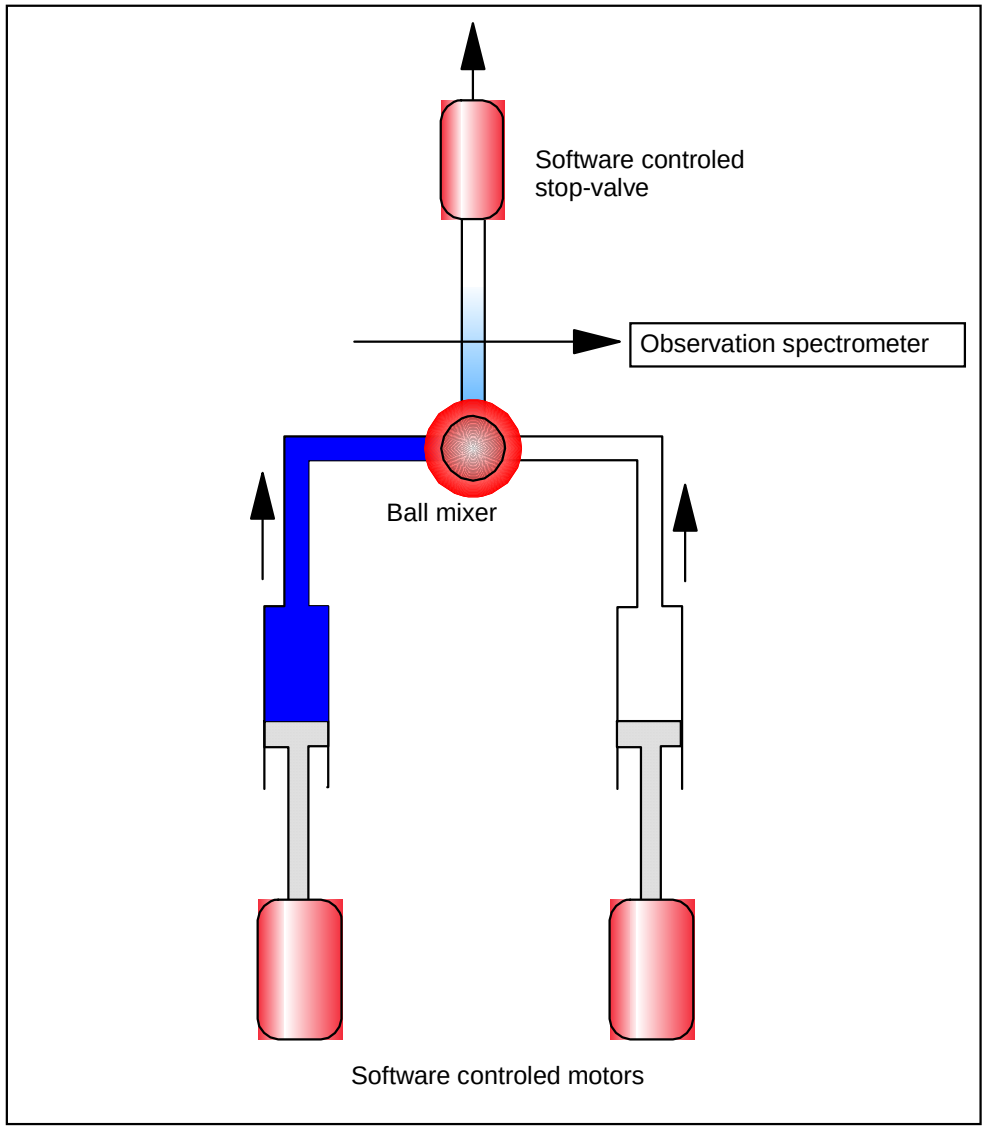
Vol of sample	rate (s-1)	amplitude
50 µl	32,3	0,332
28 µl	32,4	0,331
24 µl	32,3	0,332

Decrease of volume until limit of stationnary state

Key points of Stopped-flow technology

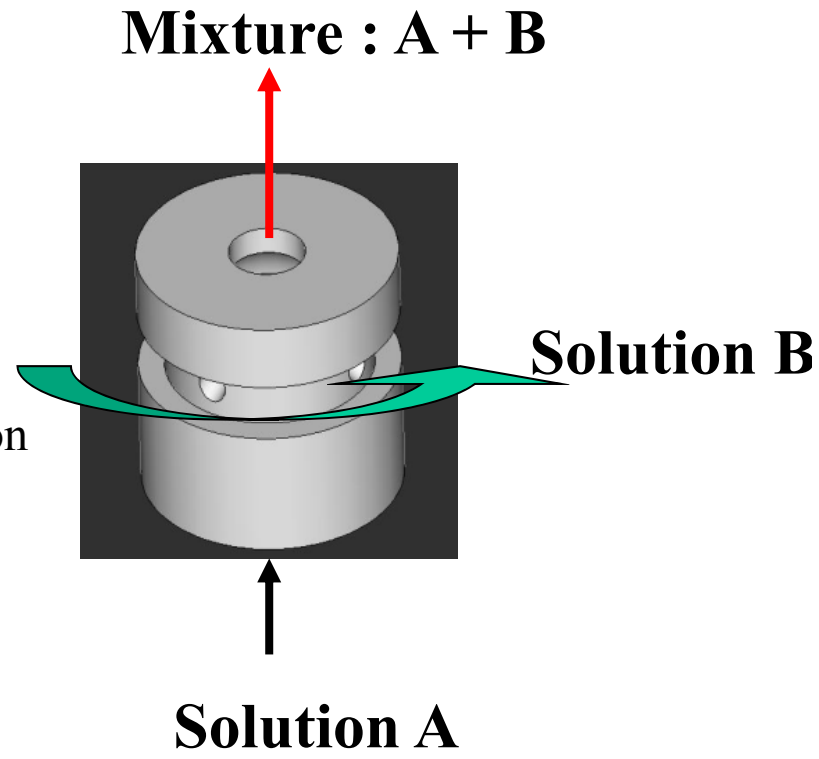
- **Mixer**
- **Pushing method**
- **Stopping method**

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



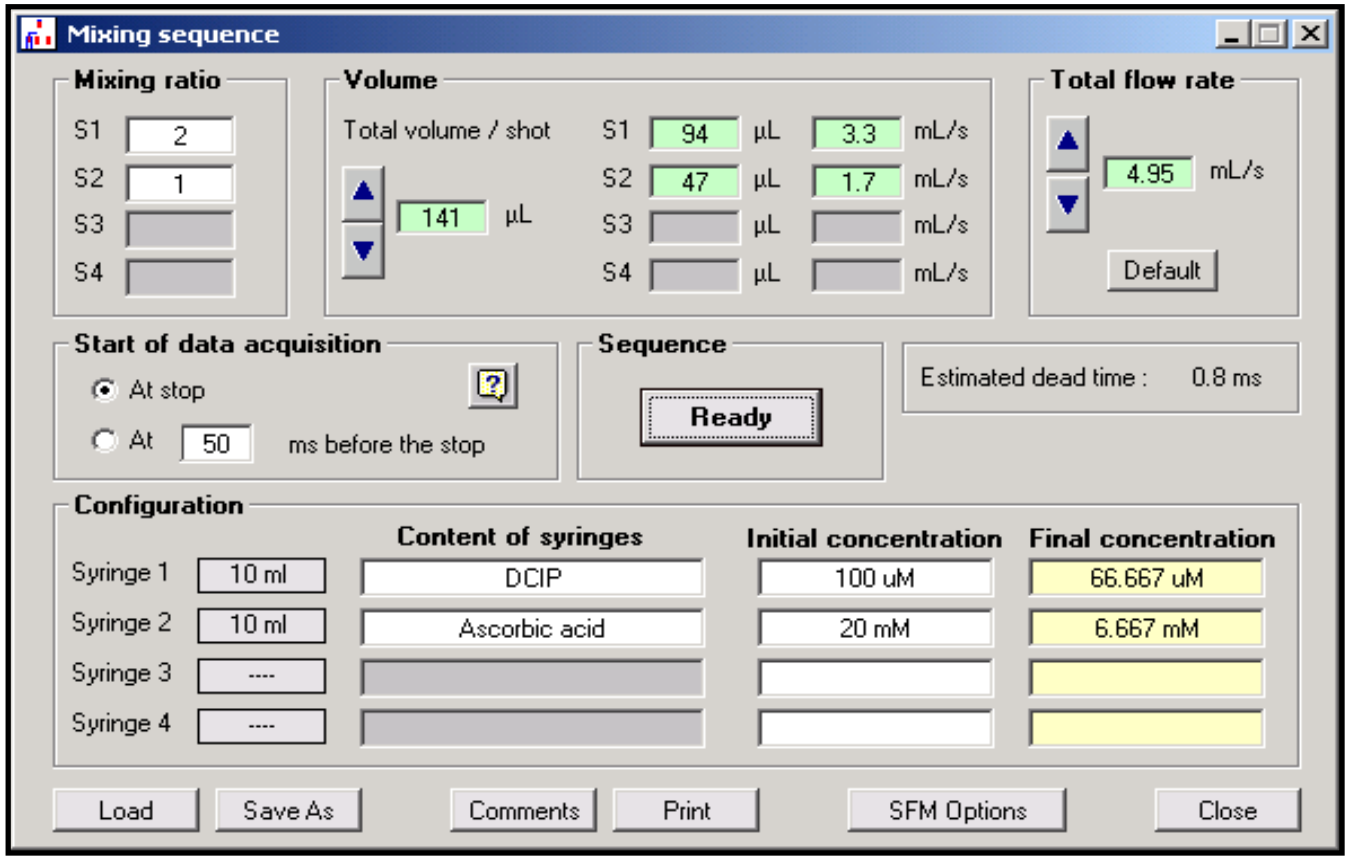
- **Independant stepping motors**

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

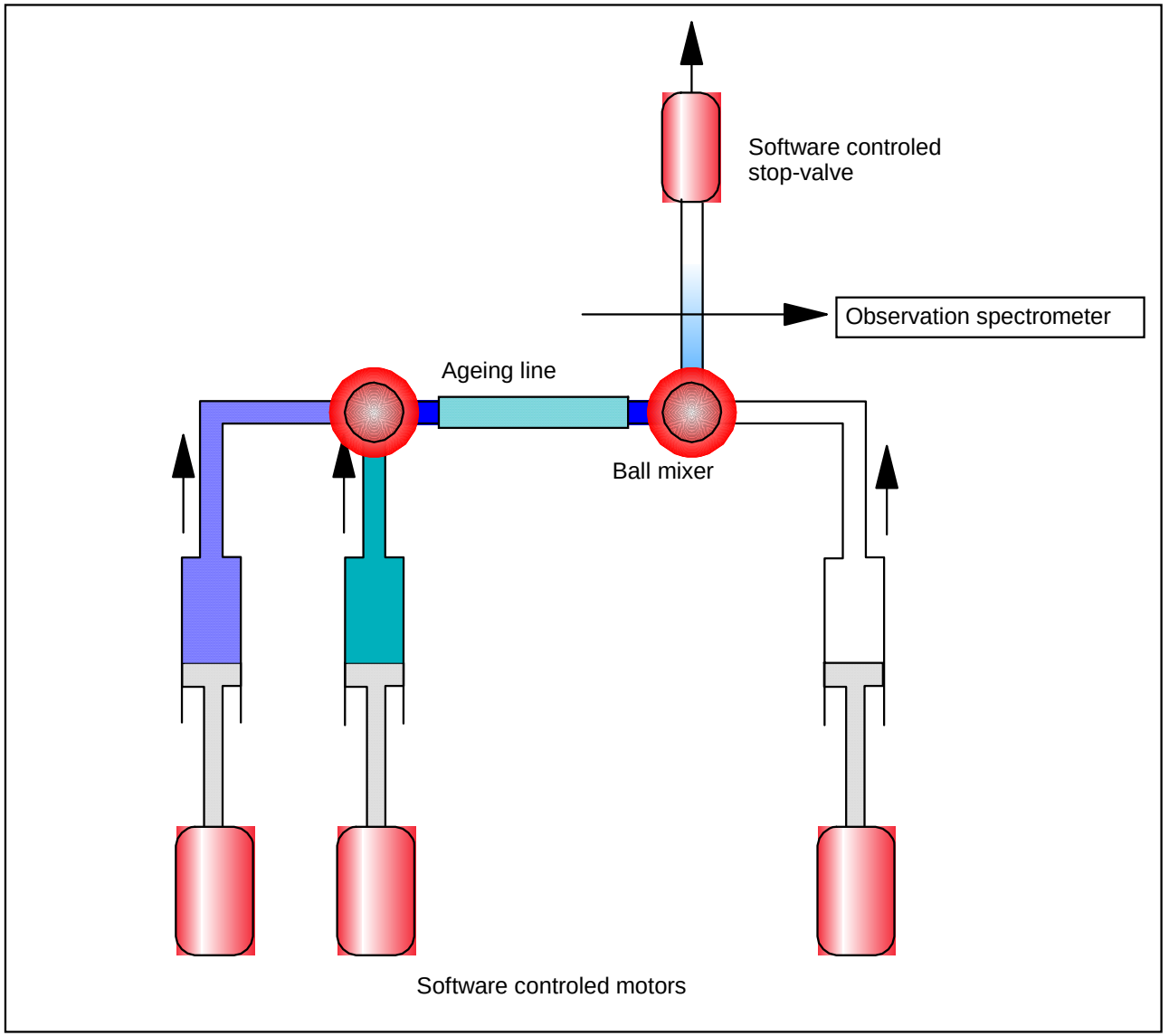
- **Stop electrovalve**

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

Design of a stopped-flow sequence
Single mixing experiment



Double mixing



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

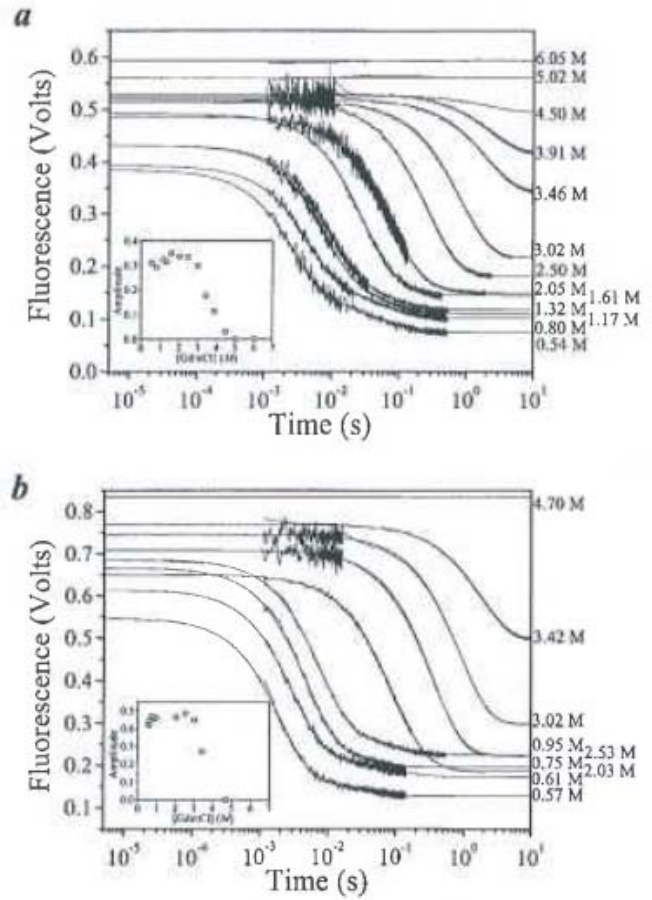
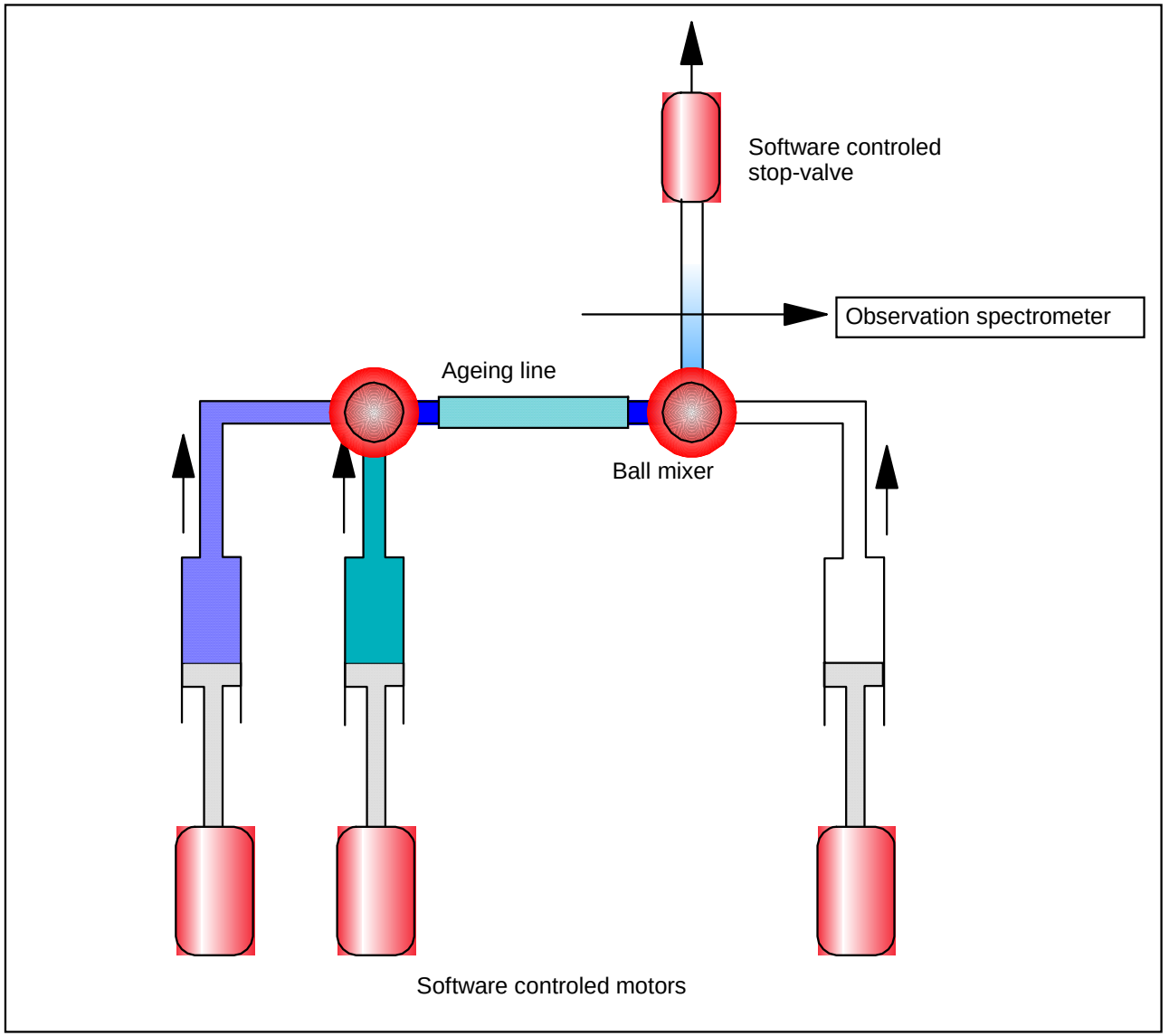
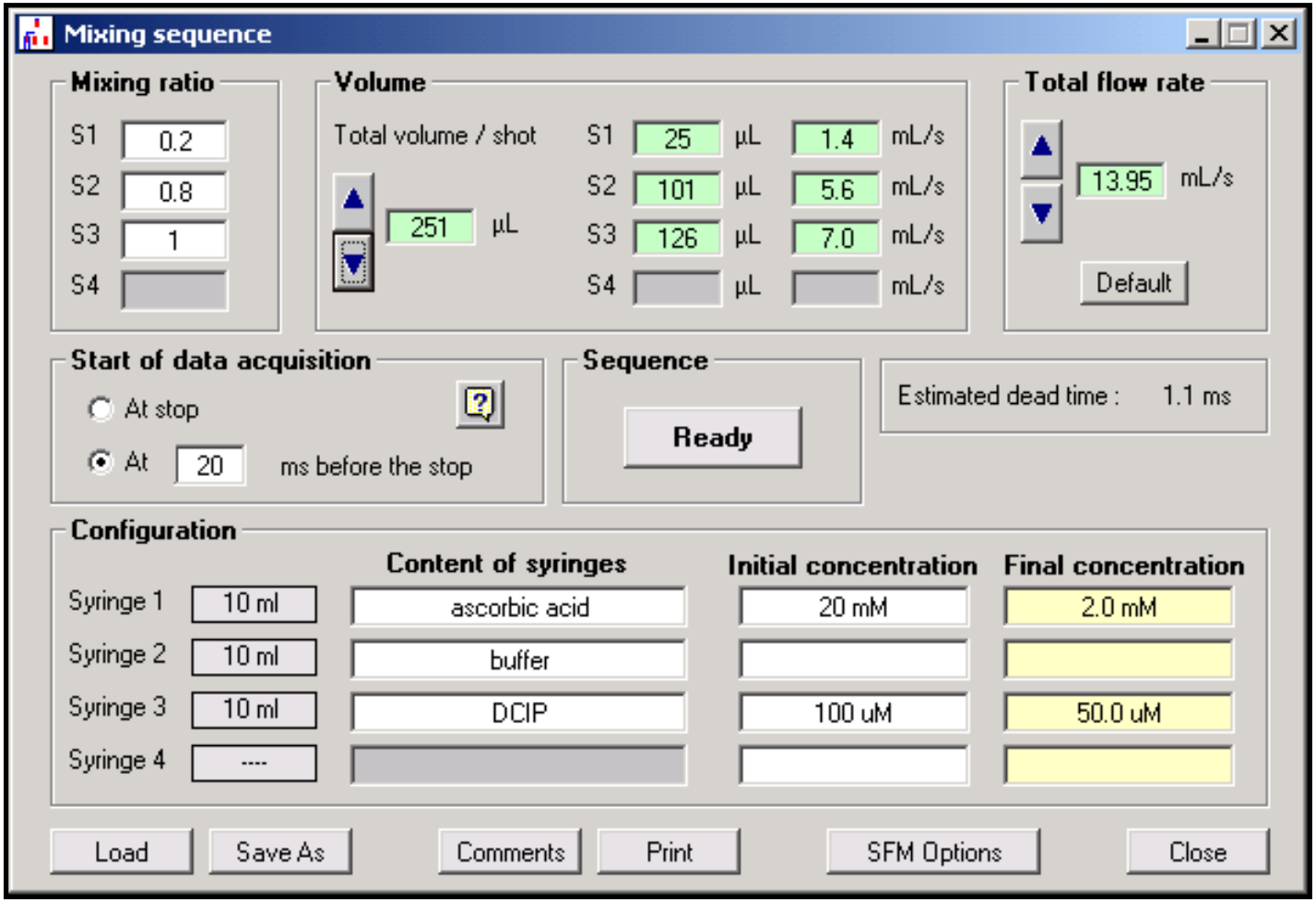


FIGURE 2: Denaturant dependence of folding at pH 5.0, 25 °C. Raw data traces in (a) single-jump refolding experiments and (b) double-jump refolding experiments which eliminate extraneous proline phases. The noncoincidence of the fully folded values largely represents the denaturant dependence of the 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.

Double mixing for concentration studies

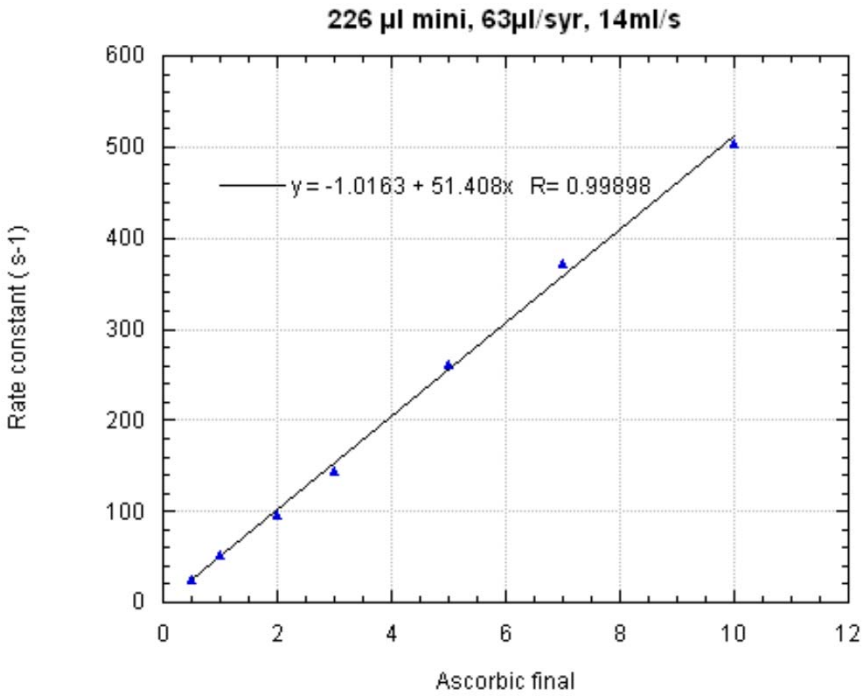


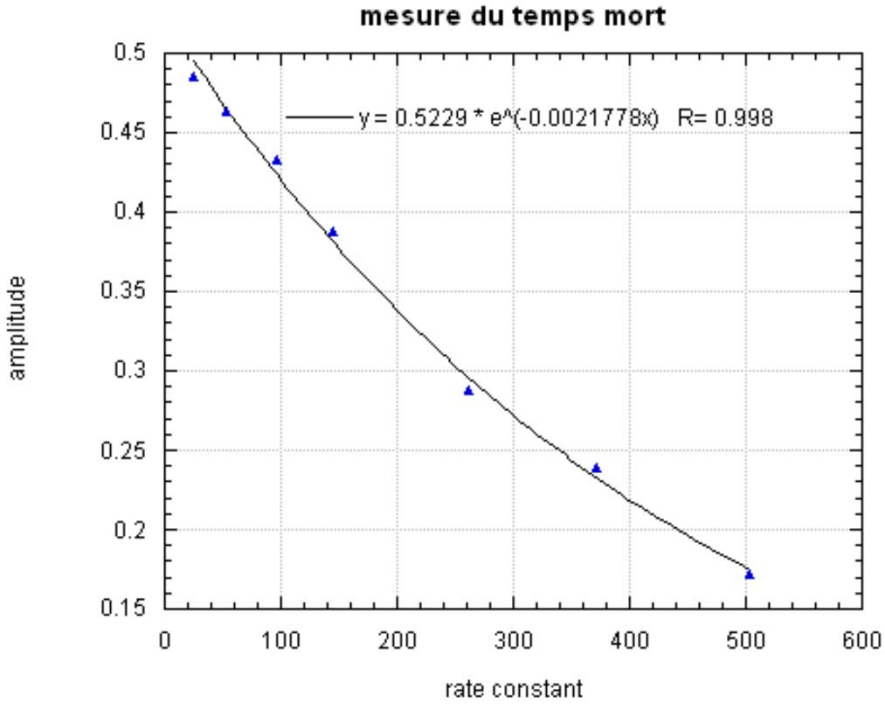
Design of a stopped-flow sequence
Variable ratio mixing



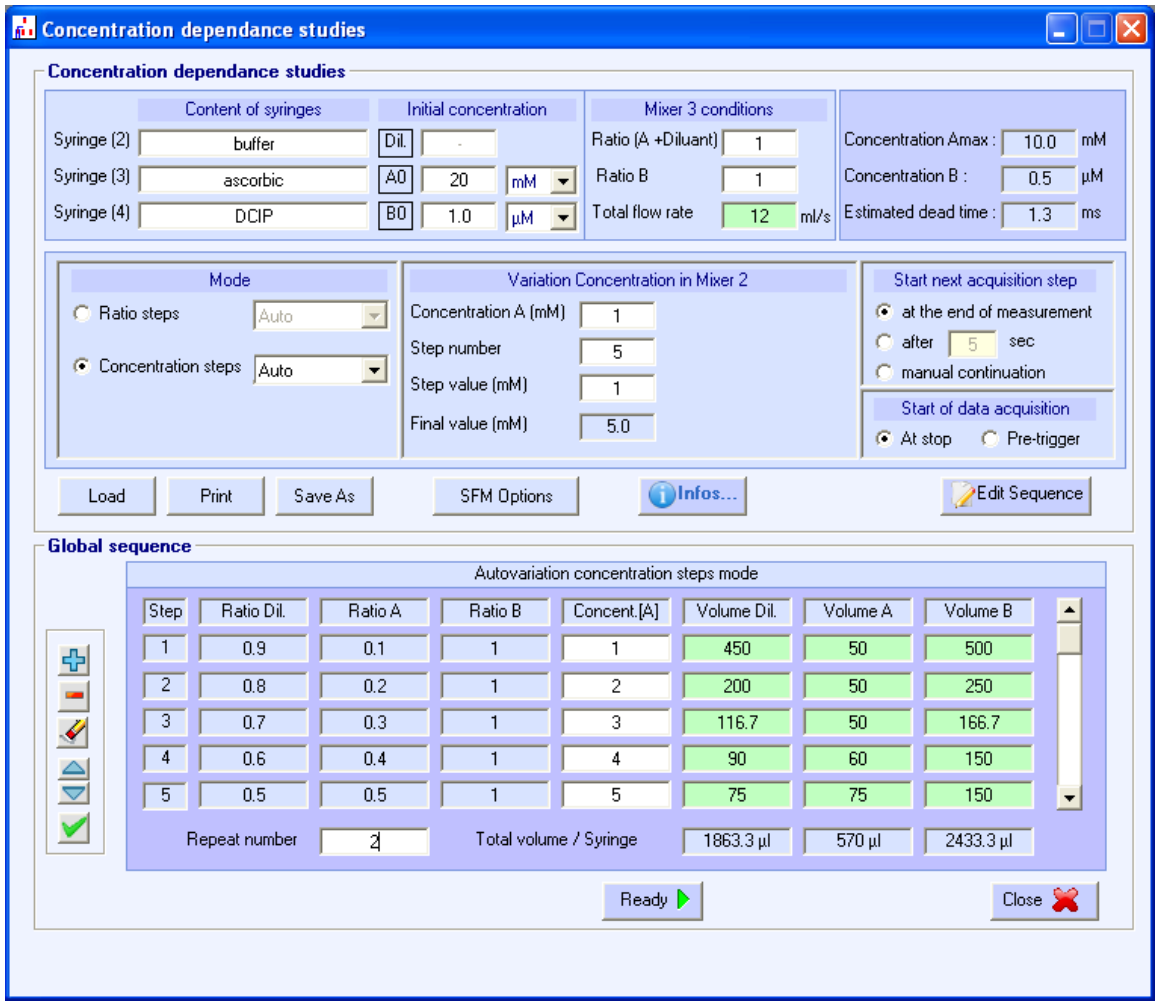
Concentration dependence studies

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)

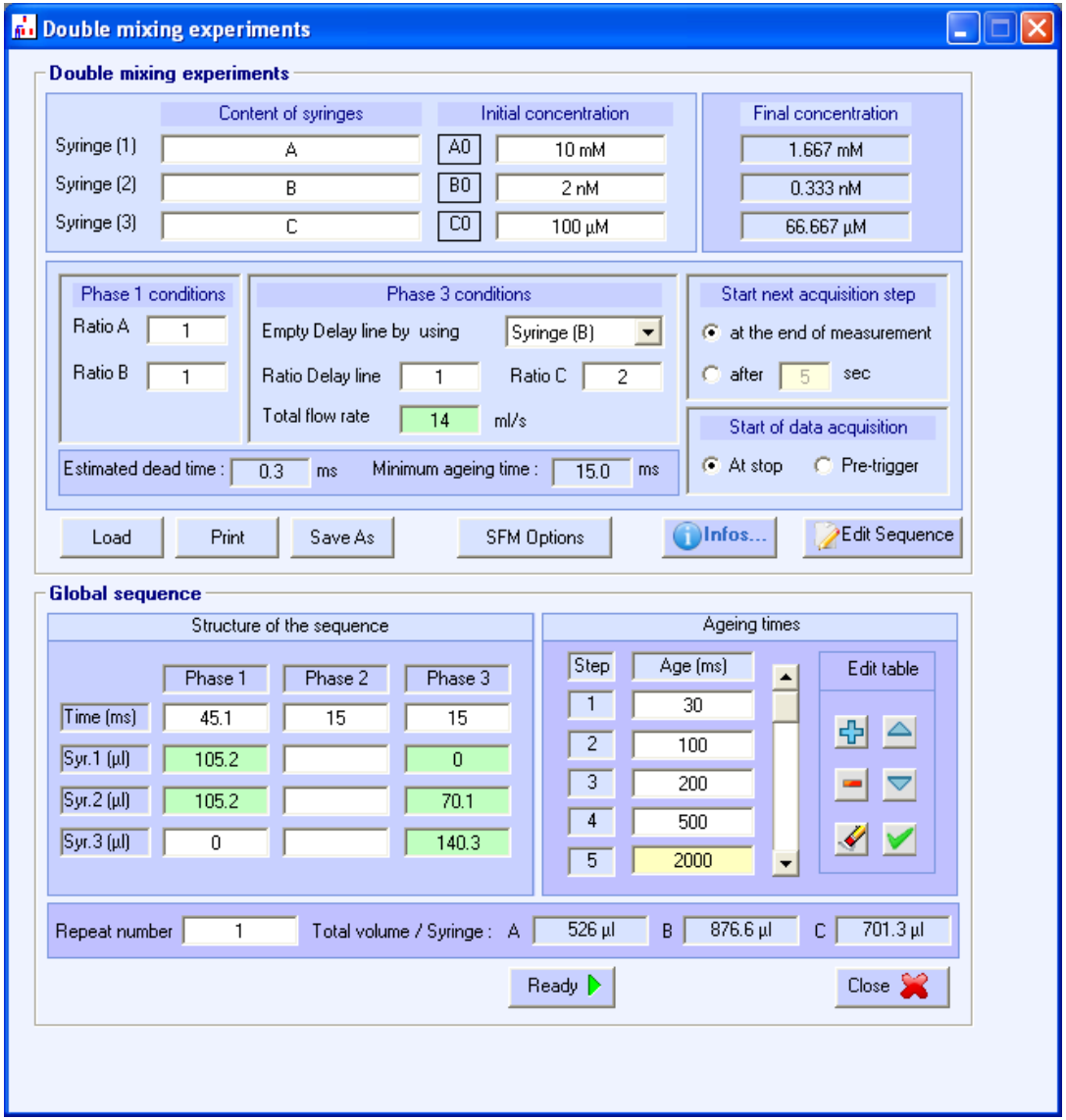




Chevron plots, concentration dependence studies



Automatic double mixing



Applications of stopped-flow

Protein folding
Conformational changes
Substrate binding
Enzyme kinetics
Substrate and H_2O transport in vesicles

→

UV/Vis absorbance
Light scattering
Fluorescence
Circular dichroism
Fluorescence anisotropy
FTIR
X-ray and neutron scattering
EPR
Conductivity
…

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 1 : water transport

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 :

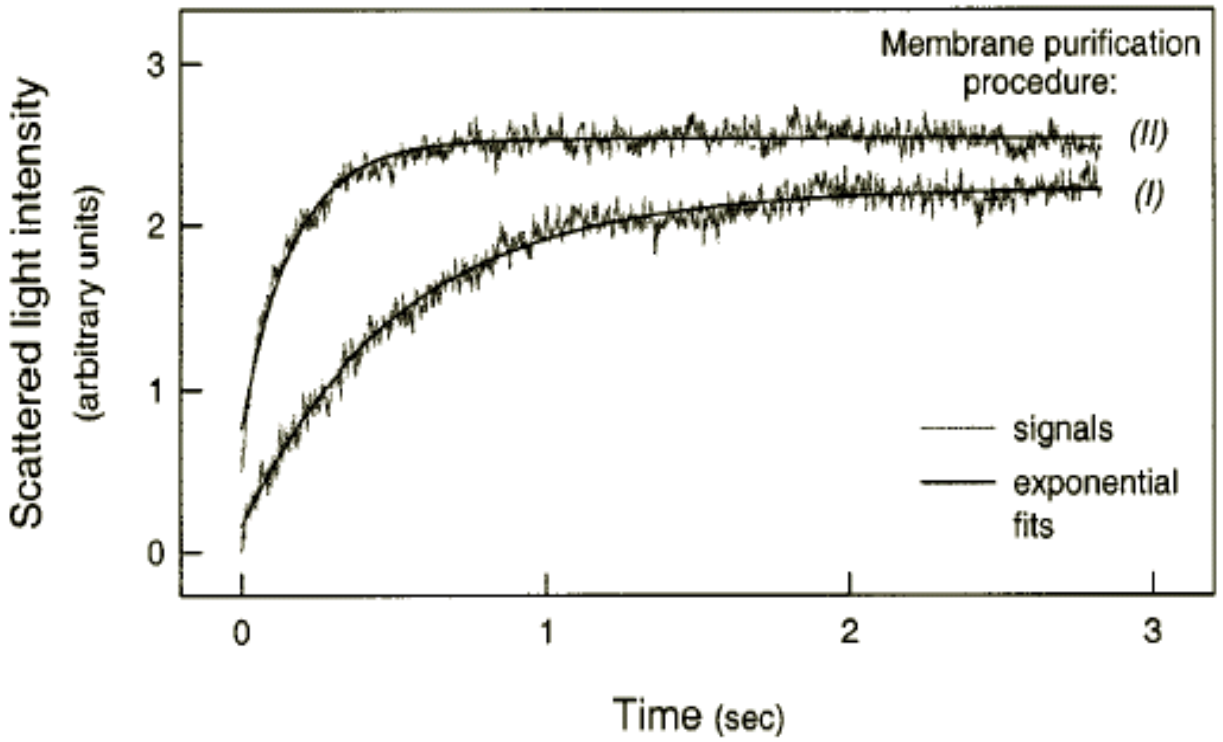
Solution 1 : vesicle into equilibrium media (50mM NaCl, 50mM mannitol, 10mM Tris pH=8.3)
(187 mosmol.kg^{-1} H_2O)

Solution 2 : same media but with 500mM mannitol (693 mosmol.kg^{-1} H_2O)

Mixing conditions :

1:1 mixing ratio leading to a 253 mosmol.kg^{-1} H_2O inward osmotic gradient

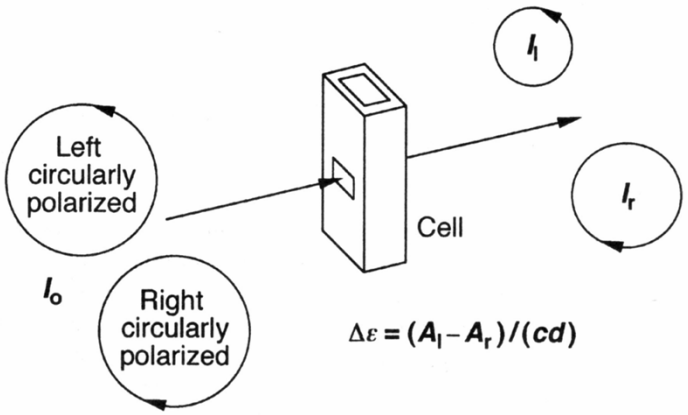
Example of traces obtained



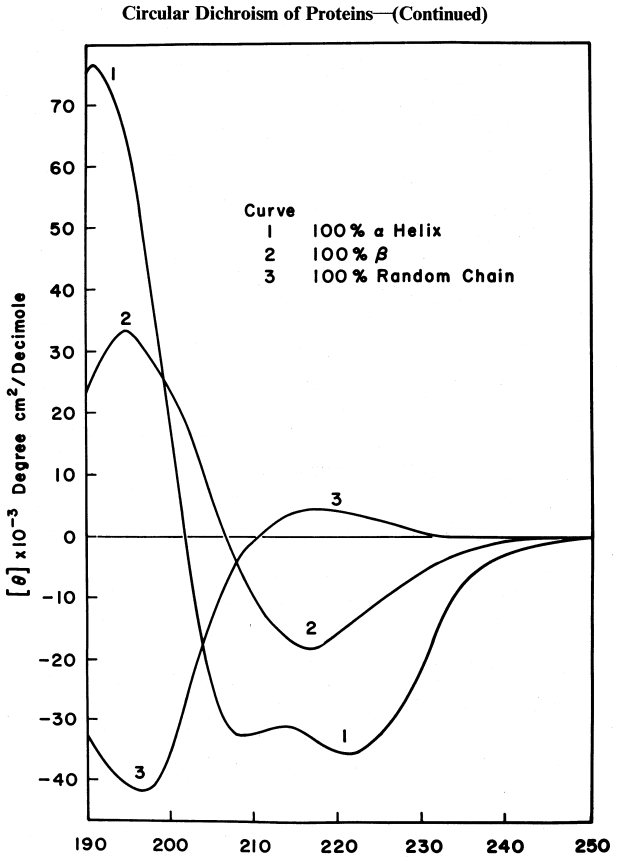
$$P_f = K.V_0/A_v.V_w.C_{out}$$

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

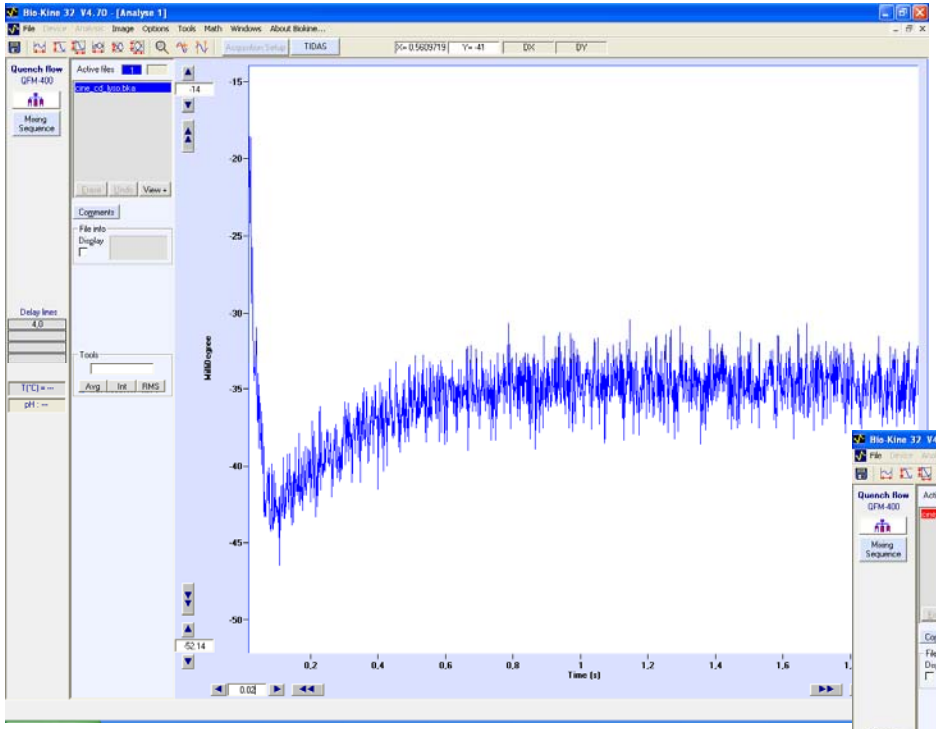
Example 2 : refolding, CD+fluo



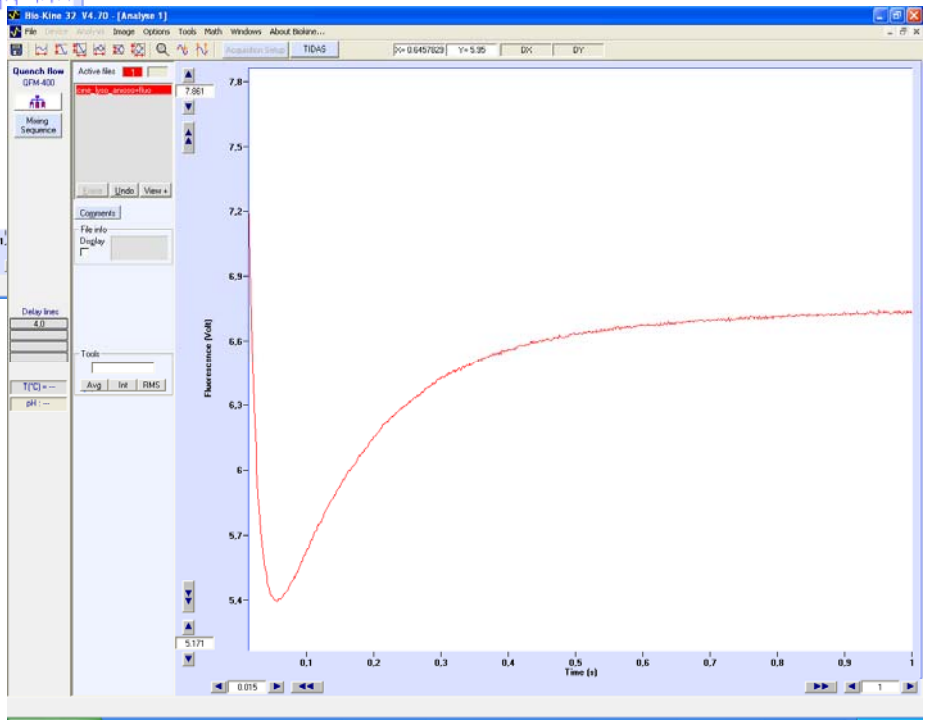
Circular dichroism is the difference between the absorptions for left, A_l, and right, A_r, circularly polarized light. Alternately left and right circularly polarized light with the initial intensity I_o is passed through the sample cell. In most CD spectrometers, the difference between A_l and A_r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



Lysozyme refolding

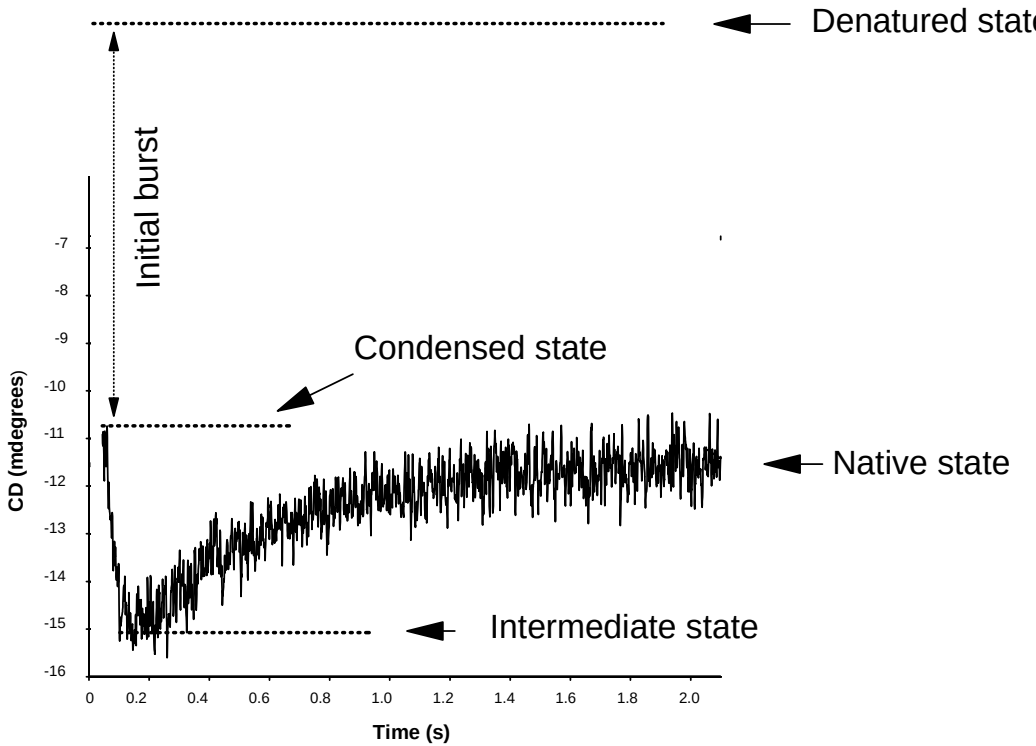


Alpha helix CD



Trp fluorescence

Lysozyme refolding



U <==> MG <==> I <==> N

mT-jump

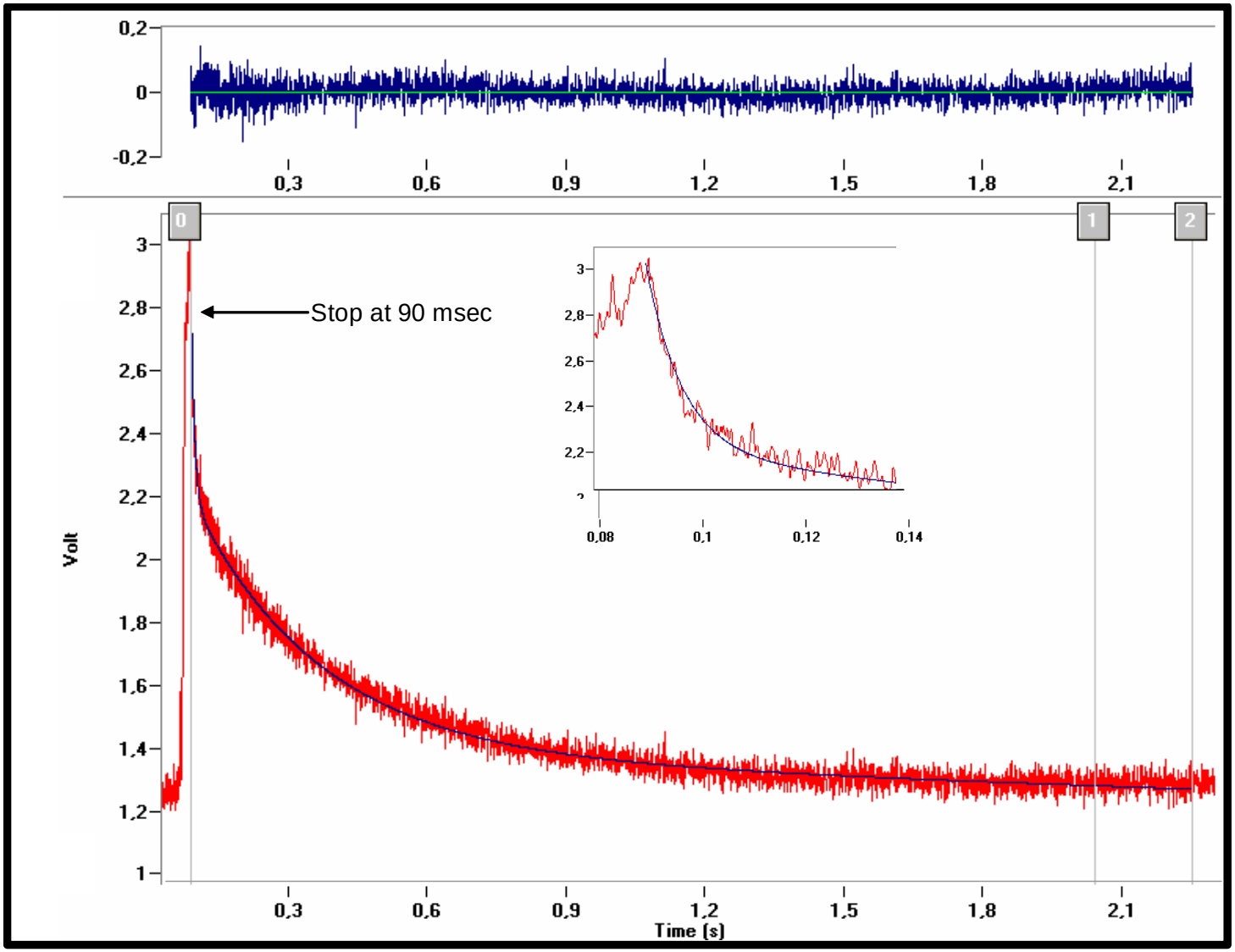
An unique technique for cold jumps experiments
Application: refolding experiments

Classical T-jump

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

mT-jump

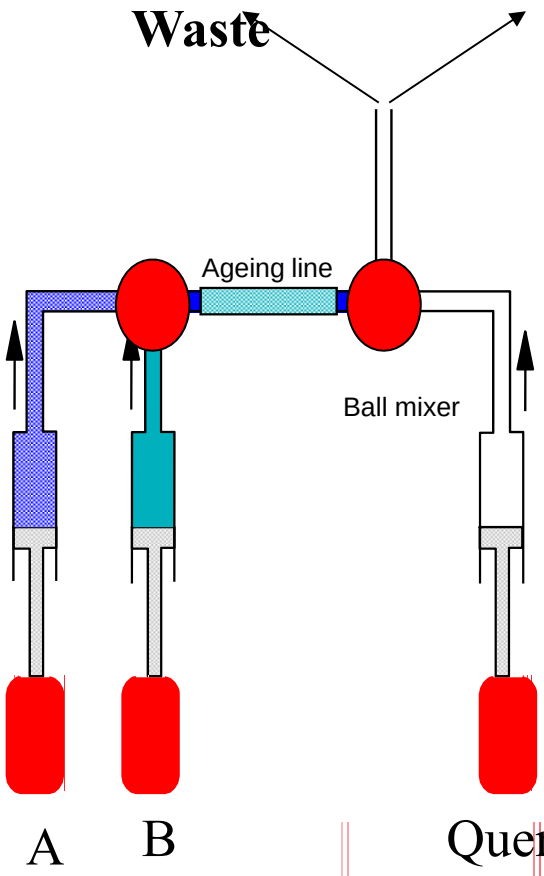
- ms time resolution
- Cold and hot jumps : up to +40°C
- Compatible with all stopped-flow models
- Compatible with all spectrometers
- T stability: variation < 1% per 30 seconds



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

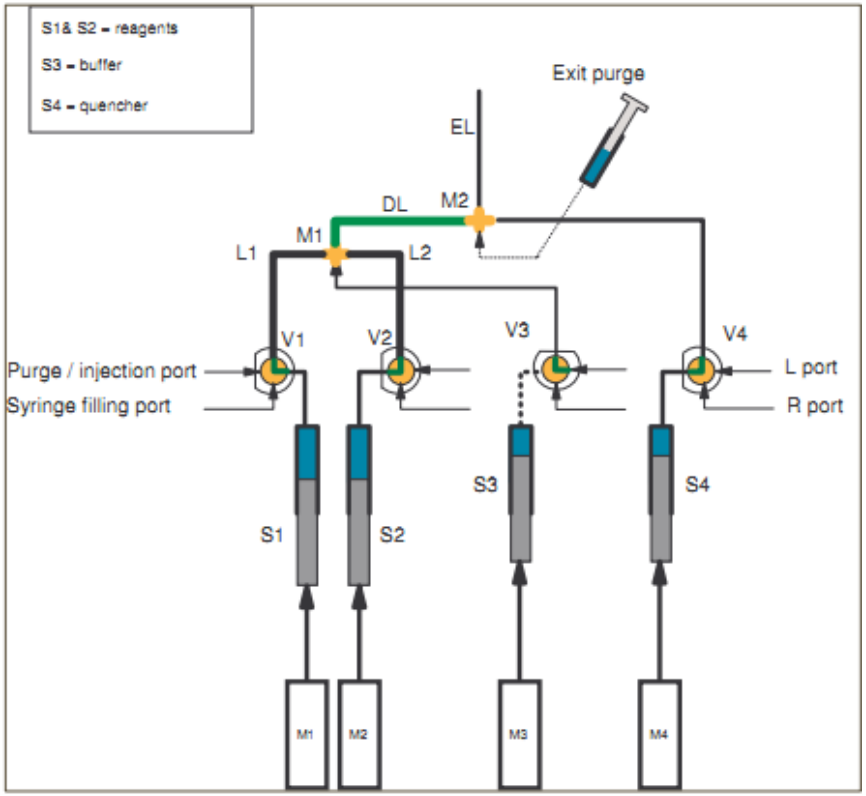
QUENCH-FLOW

Classical QF instrument



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

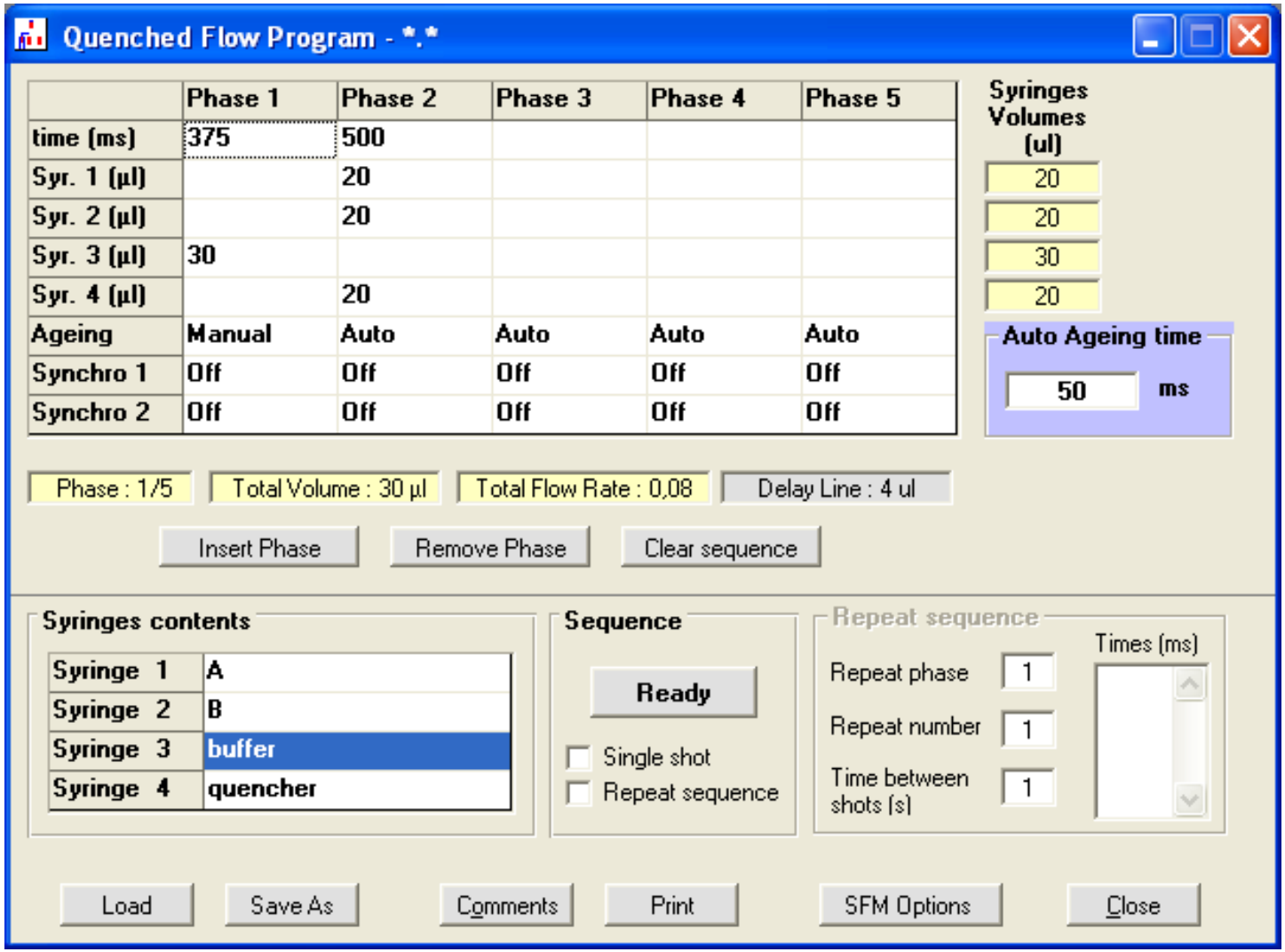
Microvolume QF instrument



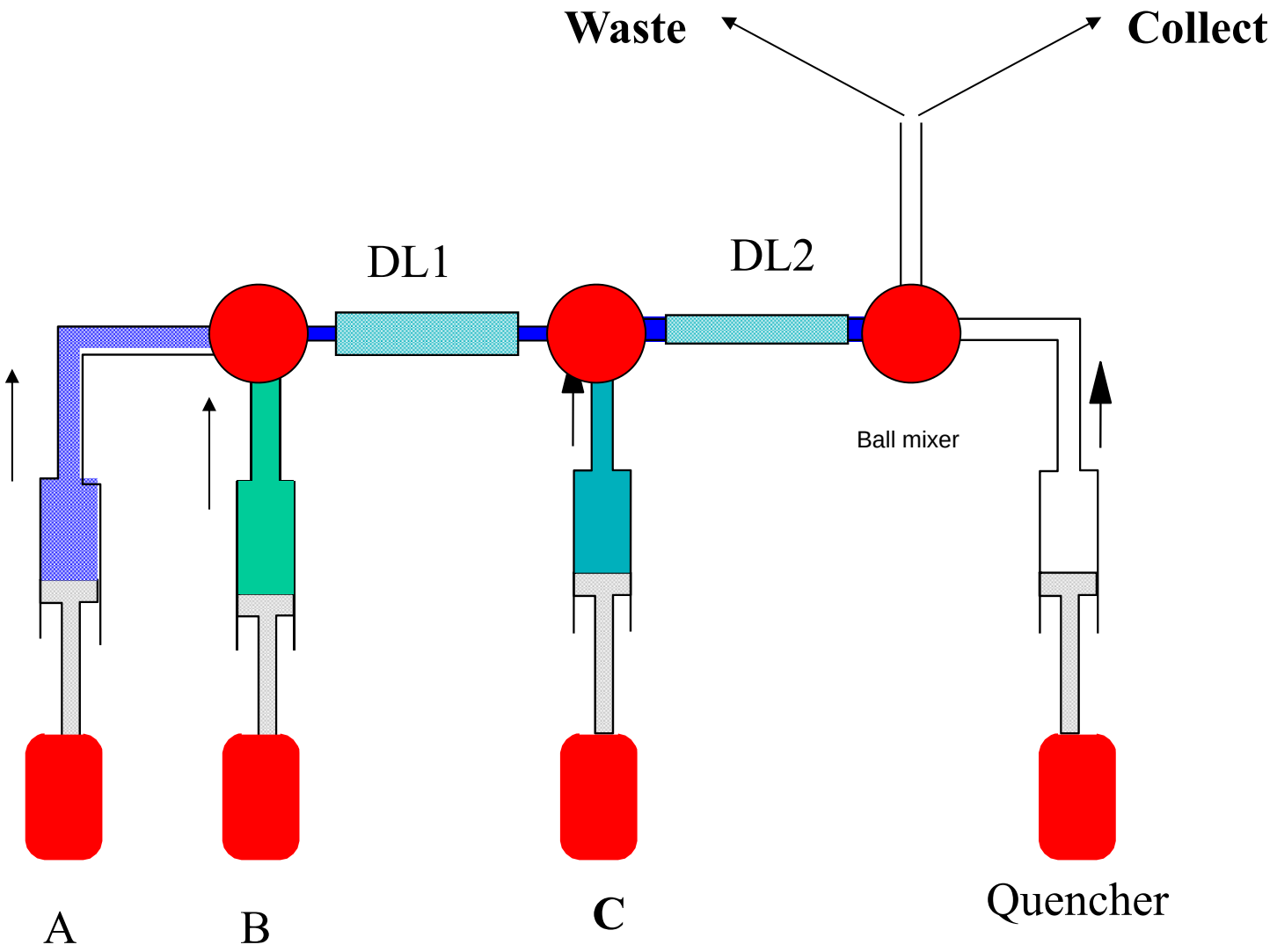
One delay line for all ageing times

10-15 µl of sample only

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

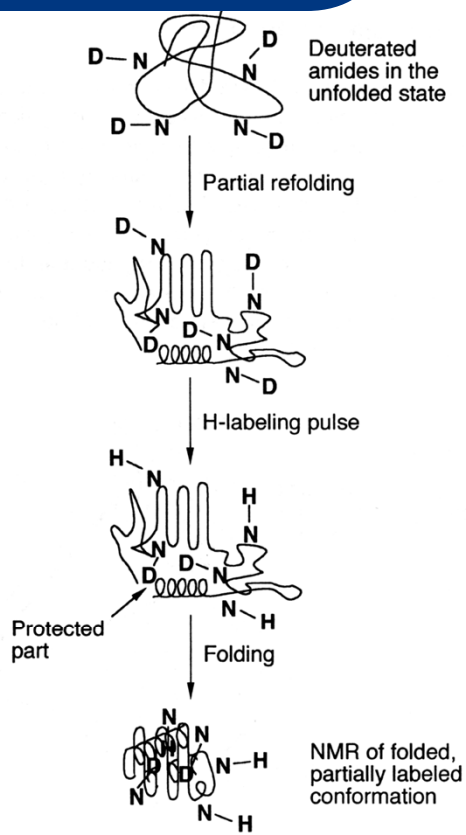


Double mixing quenched-flow instrument

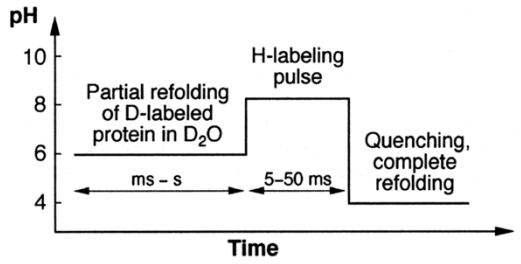


EXEMPLE

H/D exchange experiment

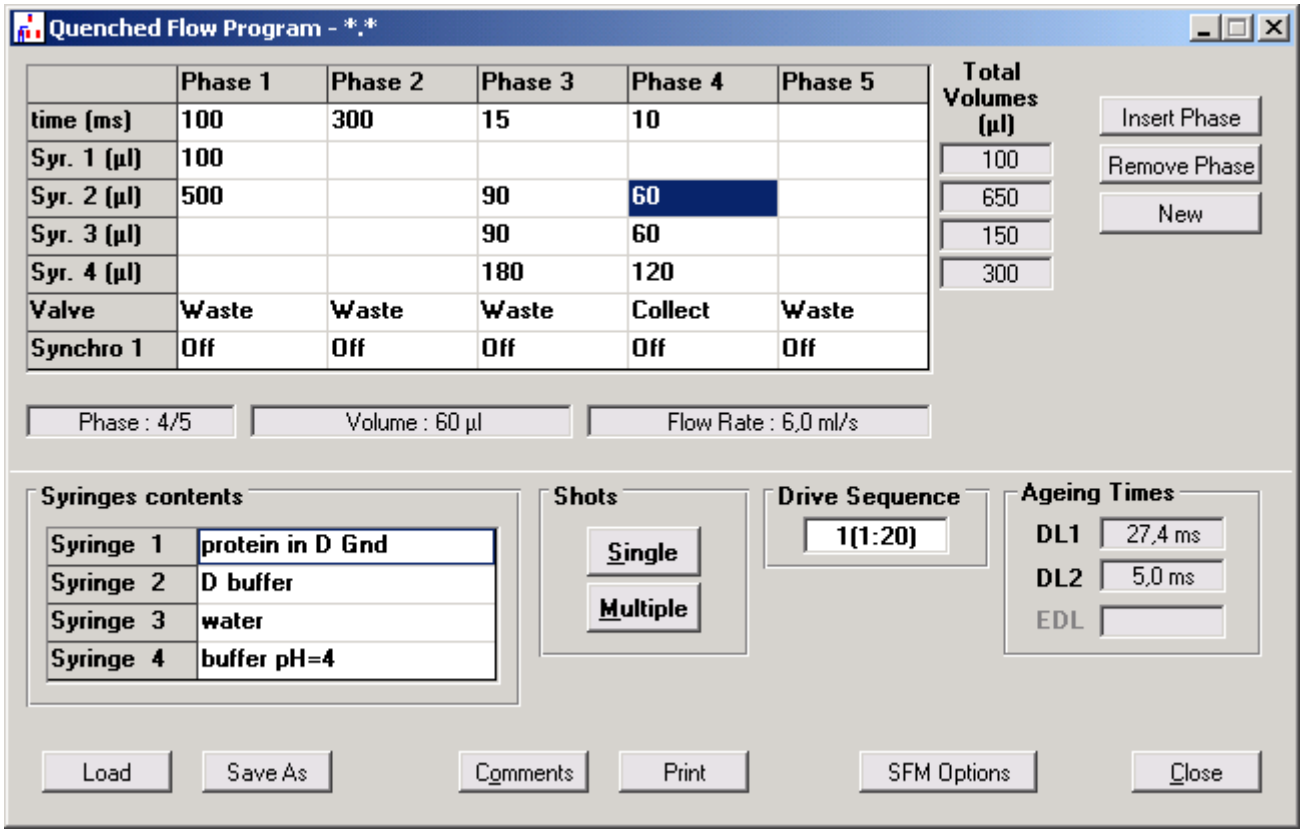


Kinetic H/D exchange experiment. Prior to the refolding reaction, all exchangeable protons were replaced by deuterons in the unfolded state. Refolding is initiated with deuterated buffer. After partial refolding, the protein solution is mixed with H_2O. Parts of the protein which are protected at this 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



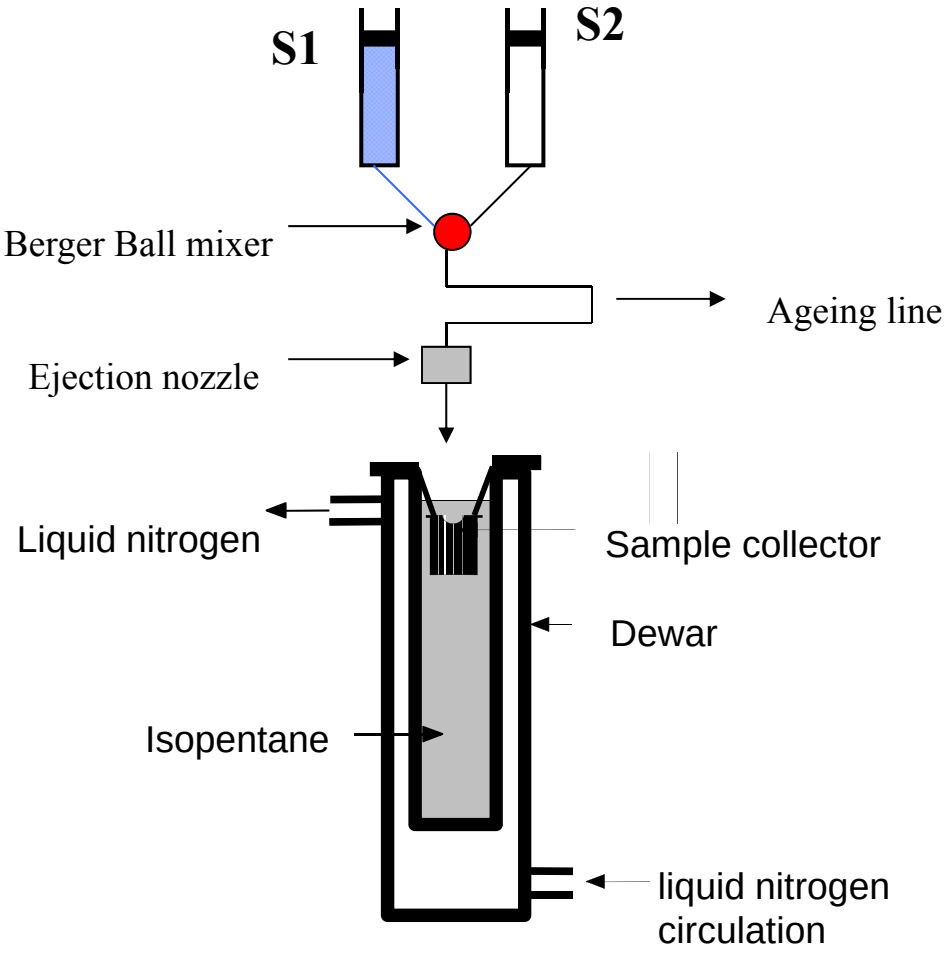
Delay line 1 = 164.3 µl
Delay line 2 = 59.8 µl

Partial refolding = 327.4 msec
H-labelling = 5 msec

Reaction time

=

Ageing time + flying time +freezing time



Applications of chemical quenched-flow

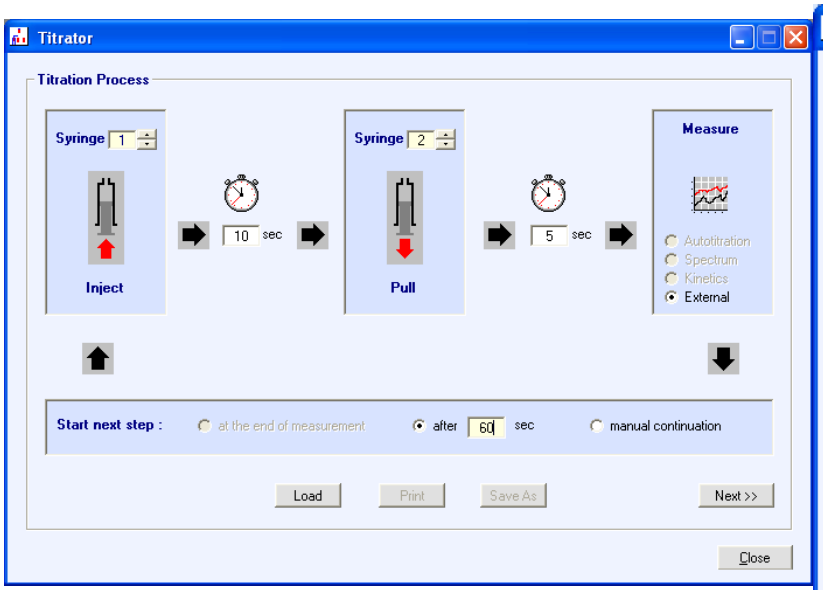
Protein folding Conformational changes Substrate binding Enzyme kinetics Second messenger studies	Radioactive labelling H/D exchange followed by NMR H/D exchange followed by Mass spectrometry Chromatography Gel electrophoresis …

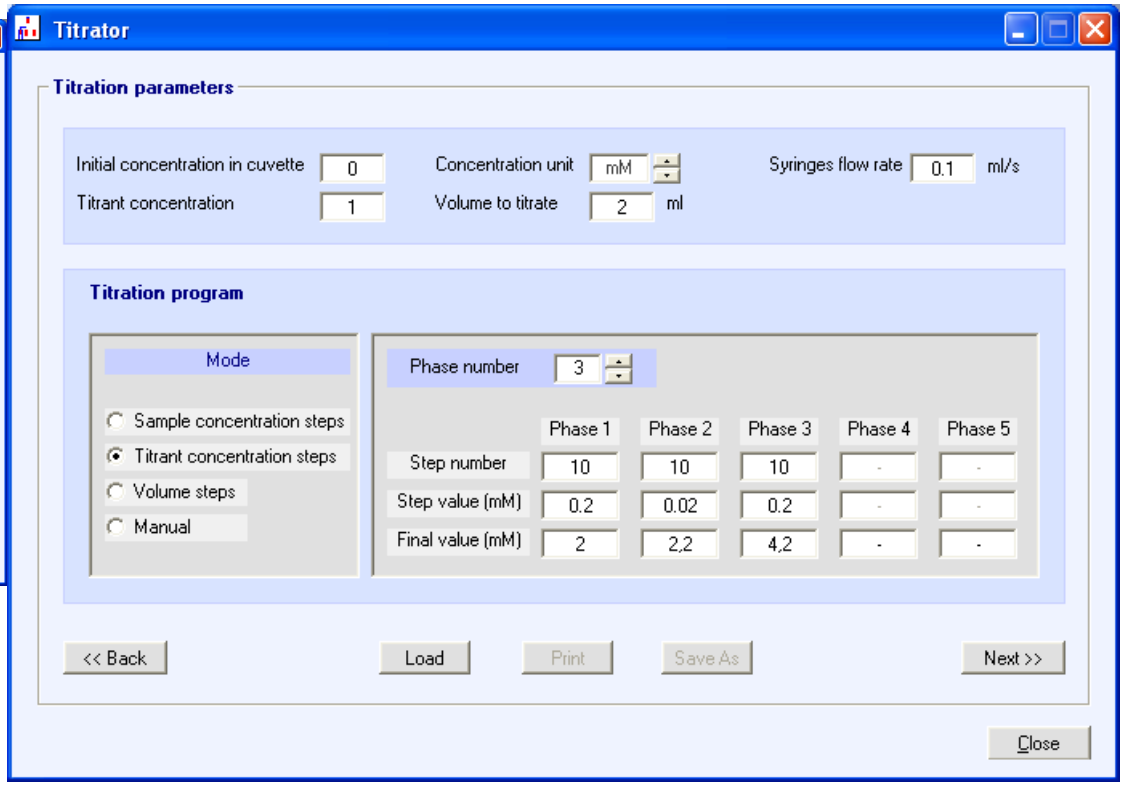
Applications of freeze quench

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

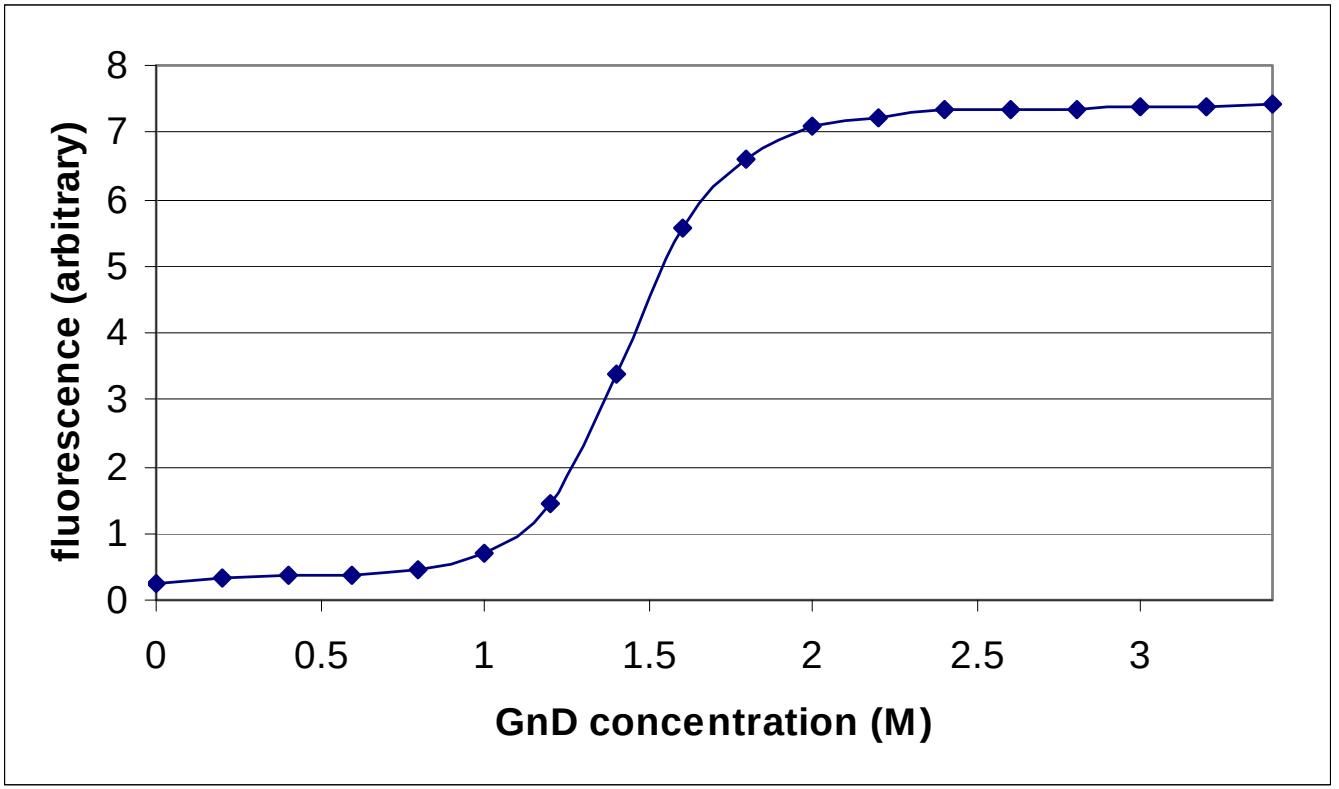




Exemple : Titration of cytochrome c from horse heart

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