Megamix-Plus SSC

Beads for cytometer settings in microparticle analysis

Kit for 50 Tests **Ref. 7803**

BioCytex

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Not for Use in Diagnostic Procedures.

1 INTRODUCTION

Biological microparticles ("MP") are cellular vesicles of heterogeneous size ranging from 0.1 to 1 µm. Due to such small size, MP analysis requires working conditions close to the size-related sensitivity limit of the cytometers. Standardization of MP count requires to master this limit for an optimal compromise between MP analysis and background exclusion. This limit has significantly progressed with the advent of the latest generations of instruments from 0.5 down to 0.3 µm-eq.* (ref.1). Also, experience with Megamix have shown that FCM instruments may not all behave the same in terms of scatter response to sub-micron particles, some being optimized to use Forward Scatter (FS or FSC), e.g. Beckman-Coulter (BC) Gallios/Navios and others making best use of Side Scatter (SS or SSC) parameter, e.g. Becton-Dickinson FACS and LSRs (ref.2).
Megamix-Plus SSC reagent is most appropriate for use with SSC-optimized cytometers (e.g. BD FACS-Canto, -Verse, -Aria and LSR) and allows to standardize a SSC-based MP analysis region comparable in PMP counts to that defined in FSC as 0.3 - 1 µm-eq.

** µm bead-equivalents, a special arbitrary unit used to remind that FCM can not provide absolute measurement of MP size but only reference points to standardize analysis (ref.1).*

2 PRINCIPLE

Megamix-Plus SSC is a mix of fluorescent beads of varied diameters, selected to cover a major part of the theoretical MP size range (0.1 to 1 µm), using SSC as a size-related parameter. Beads acquisition according to the following procedure allows setting the cytometer to study MP within a constant size region and getting reproducible MP counts. Since relative position of biological MP and beads in SSC is different from that in FSC, Megamix-Plus SSC beads feature reference sizes different from those provided in Megamix-Plus FSC. Finally, Megamix-Plus SSC also helps to transfer the protocol and scatter settings from one cytometer to another and to check the stability over-time.

Limitation: flow cytometers using different optical designs for scatter collection may not feature enough SSC resolution to be used for standardized MP counts. This will lead to a bad resolution between 0.16 and 0.20 µm beads (no clear-cut separation in Fig. 2). Some of them may work better using FSC as the prime size-related threshold parameter (e.g. BC Gallios/Navios). For these instruments, standardized MP analysis may rather be done using Megamix-Plus FSC beads (ref. 7802) that are optimized for the use of FSC. Both approaches should provide similar MP counts despite using two different size-related parameters and two different sets of reference beads (ref.2).

Extension: some SSC-optimized flow cytometers may also be capable of using FSC with appropriate resolution (e.g. BD LSR Fortessa equipped with PMT on FSC detector; Stratedigm, …). In such a case, both strategies may be mixed using a mixture of Megamix-Plus FSC (ref.7802) & Megamix-Plus SSC, thus providing a 7 reference beads system, called Gigamix (see ref 2; for technical support please inquire).

3 REAGENT PROVIDED

1 vial of 25 mL of beads.
Megamix-Plus SSC is a mix of beads with following diameters:
0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm.

4 MATERIAL REQUIRED BUT NOT PROVIDED

- Cytometer, SSC-optimized model (e.g. BD FACS-Canto, -Verse, -Aria or LSR). For other models and brands, please inquire.
- Stirring machine type Vortex.
- Cytometry tubes (preferably polypropylene tubes, with low dust and protein adsorption, if compatible with the flow cytometer).
- Adjustable pipette with disposable tips (500 µL).

5 REAGENT PREPARATION AND STORAGE

Unopened vial remains stable until expiration date printed on the label when stored at 2-8°C. Do not freeze.
The reagent is ready for use.

Resuspend this reagent by vortexing for 10 seconds before use.
Stability after opening: until expiration date printed on the label at 2-8°C when free of contamination.

6 PROCEDURE

The reagent must be at room temperature for the procedure.
We recommend to run Megamix-Plus SSC beads before each series of MP analysis.

6.1 Bead preparation

Pipet into a cytometric tube **500 µL** of Megamix-Plus SSC reagent after shaking vigorously the reagent vial for at least **10 seconds by vortexing.**

6.2 Generation of cytometric protocol and instrument settings

Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings.
Vortex the tube just prior to analysis.
The following analysis has been performed on BD FACS CantoII cytometer running with DIVA software on 5.5 decades (only 4 are shown in Figs 1-5). The suggested settings below apply for this type of instrument and must be optimized for each individual cytometer.

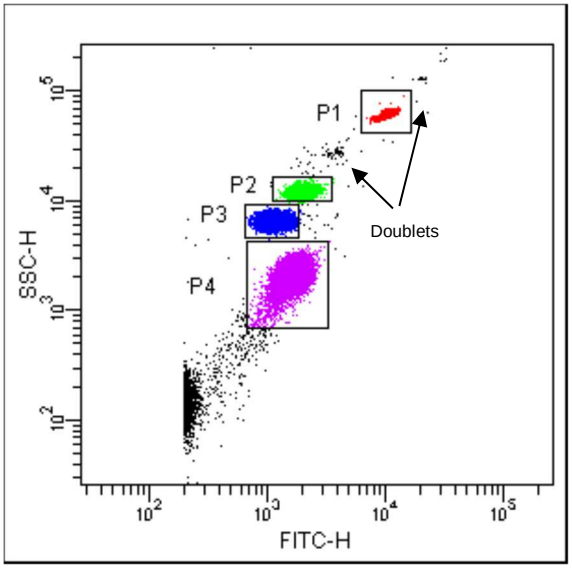
Proceed to the following settings:
- Parameters: select signal height ("XXX-H") for all parameters.
Note: signal height is mandatory for scatter parameters but also recommended (although not mandatory) for fluorescence parameters.
- Time of analysis: 1 to 2 minutes
- Speed of analysis: the lowest available ("LOW", around 10-15µL/mn; in some cases, speed can be increased if SSC resolution is not impaired).
- SSC: Voltage near 600v.
- FSC: Voltage near 800v (optimal value may widely vary depending on individual instrument).
- FITC: Voltage near 580v.
- Logarithmic acquisition for all parameters.
Optional : FL3 (PI or Per-CP) parameter for counting beads and other FLi for MP labelling.

For the acquisition protocol of Megamix-Plus SSC beads, create:
- one cytogram FITC-H x SSC-H (color dot-plot) and four rectangular regions P1 to P4 (Fig.1a).
- one histogram SSC-H x count gated by the Boolean gate "P1 or P2 or P3 or P4" (Fig.2)
- one cytogram FSC-H x SSC-H and one rectangular region "MP" (Figs 3, 4 and 5).
Additional histograms and cytograms are necessary for MP analysis (e.g. Figs 5).

6.2.1 Settings of FITC and SSC voltages (Figs 1)
- Proceed to acquisition of Megamix-Plus SSC beads (Mgx+).
- Select a **threshold on FL1/FITC, threshold = 200 a.u.** to only analyze FL1+ events and to eliminate unfluorescent contaminants (background of the instrument).

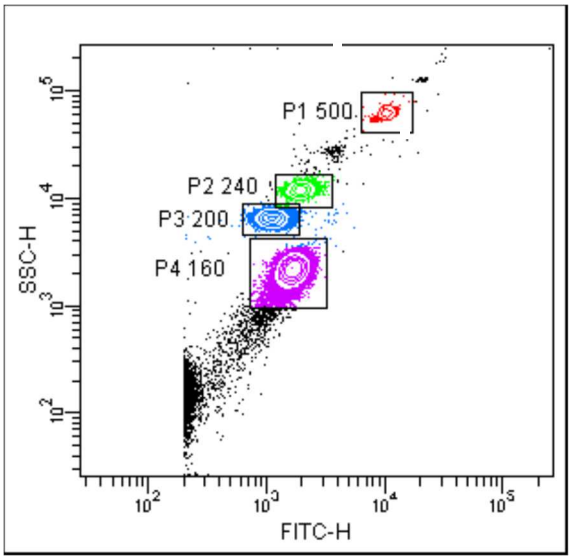
On the FITC-H x SSC-H cytogram:
- Set regions "P1", "P2", "P3" and "P4" around each of the singlet bead populations as shown. Take care to gate each region around the dense cloud of beads corresponding to the singlets and not include doublets. For convenience, you may adjust the "% shown events" to optimize detection of major singlets of beads. Alternatively, an additional contour graph (e.g. Fig.1b) may help to more easily delineate each singlet.
- Adjust the FITC detector voltage in order to set the 0.5 µm bead cloud at the beginning of the 5th decade (~10 to 20,000 a.u.).
- Adjust the SSC voltage in order to set the 0.5 µm bead cloud below the end of the 5th SSC decade (~50 to 80,000 a.u.).

Fig.1a: Settings of FL1/FITC and SSC PMT voltages



Region P1: 0.5 µm beads Region P2: 0.24 µm beads
Region P3: 0.2 µm beads Region P4: 0.16 µm beads

Fig.1b: Settings of FITC and SSC: contour-plot.



Region P1: 500 nm beads Region P2: 240 nm beads
Region P3: 200 nm beads Region P4: 160 nm beads

Option: Settings of other fluorescence PMT

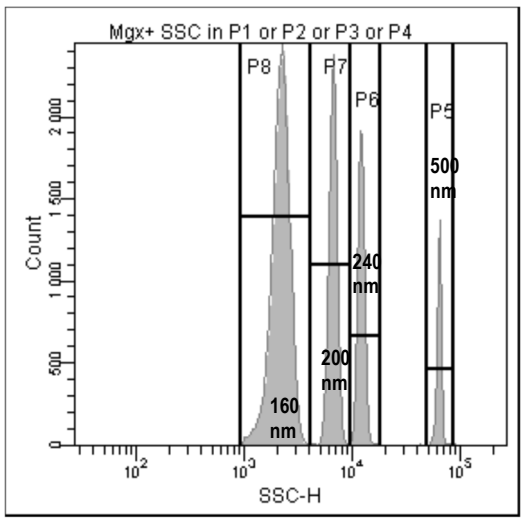
Only 3 detectors (FSC, SSC and FL1/FITC) are specifically needed to run Megamix-Plus SSC beads. Additional detectors (FL2, FL3 … FL9…) may be needed to analyze stained MP and counting beads. These fluorescence detectors will need to be optimized with biological samples (see Fig. 5 and ref.1) as well as counting beads.
Note: Due to the necessary use of very high scatter settings for MP analysis, the diameter of counting beads should not exceed 7 µm (e.g. MP-Count Beads, BioCytex ref. 7804).

6.2.2 Evaluation of SSC resolution and MP regions boundaries (Fig.2)

- On the SSC-H x Count histogram gated on 0.16, 0.20, 0.24, and 0.5 µm bead singlets (gate "P1 or P2 or P3 or P4"), check that 4 individual peaks can be discriminated, as illustrated in Fig.2. Slight overlapping of 0.2 µm with 0.24 µm peaks is common but major overlapping of both peaks and/or slight overlapping of 0.16 µm with 0.20 µm peaks would suggest a too low resolution impeding standardized MP analysis. In such a case, contact technical service for instrument SSC (and fluorescence) optimization.

Option: SSC parameter resolution can be described by a numerical parameter (see § 7).

Fig.2: Beads distribution in SSC parameter



Population	#Events	%Parent	SSC-H Median	SSC-H SD
P5	4 023	7,1	64 145	2 868
P6	9 680	17,0	12 157	852
P7	14 062	24,8	6 604	570
P8	29 018	51,1	2 178	399

- On the same SSC-H x Count histogram, define a region Pi as shown for each of the 4 bead peaks and recover statistics values including #Events, %Parent, Median (optionally SD, see § 7).

To define a standardized "MP gate", calculate the lowest boundary in SSC scale according to formula 1 and take as highest boundary the end (e.g. 99 percentile) of the 0.5 µm SSC-H peak (about $8x10^4$ a.u. in the example of Fig.2*).
* An option is to use the "Snap-to-interval gate" function in BD DIVA software; this will automatically define the end of the 0.5µm peak.

Formula 1: Low SSC-H level = Md 0.16 + (0.3 x (Md 0.20 – Md 0.16))
In the present example:
Low SSC-H level = 2,178 + (0.3 x (6,604 - 2,178)) ~ 3,500

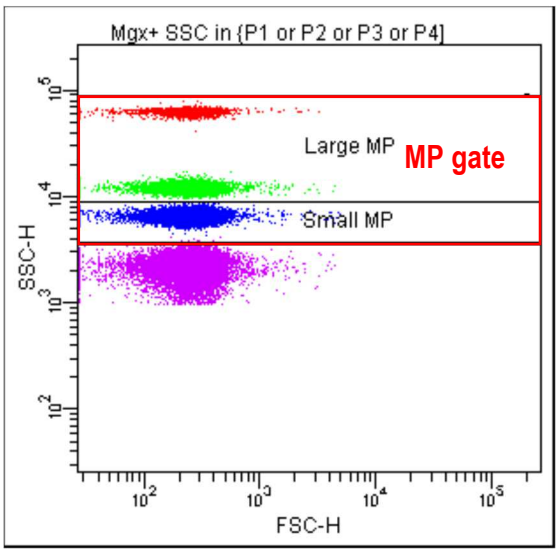
This "MP gate" standardized on SSC will mimick the 0.3 to 1 µm-eq standardized FSC range of the BC Gallios cytometers and thus allow inter-platform comparisons of MP counts (1, 2).

Option: in addition to total MP gate, additional differentiation of "small MP" and "large MP" subsets (as defined in ref.2 mainly for PMP) may be operated by using an additional intermediate boundary, comparable to the historical 0.5 µm-eq FSC limit (ref.1). This is located in the center of the gap between both 0.20 and 0.24 µm peaks (e.g. $9x10^3$ a.u. in Figs 3 and 4).

At that level, it is recommended to store the analysis as an electronic ("fcs 3.0") file (e.g. "Mgx+ SSC sFITC xxx.fcs"). This helps monitoring instrument's behaviour over-time, (see § 7) or requesting technical support if needed.

6.2.3 Setting MP gate(s) in dual scatter (Fig.3)

Fig.3: Setting the MP gate(s) in dual scatter



Once SSC-H MP gate(s) regions' boundaries have been defined/calculated on the SSC-H (log) scale, the corresponding regions can be created on the dual scatter plot that will serve for later MP analysis (illustrated in Fig. 3). The SSC-H boundaries are the ones previously defined.

The FSC-H range may cover a variable part of the whole scale since
a) the variability of FSC resolution is a characteristic feature of this sub-group of cytometers (SSC-optimized instruments)
b) no condition is set on this (actually non-resolutive) parameter for further MP analysis.

Just optimize FSC settings so that the beads are mainly located in the 2nd and/or 3rd decades of the FSC scale (e.g. from 10^2 to 10^3 a.u. as shown in Figs 3 & 4, or from 10^2 to 10^4 a.u., a wide distribution that can be encountered in instruments with very poor FSC resolution or when using elevated sample speed).

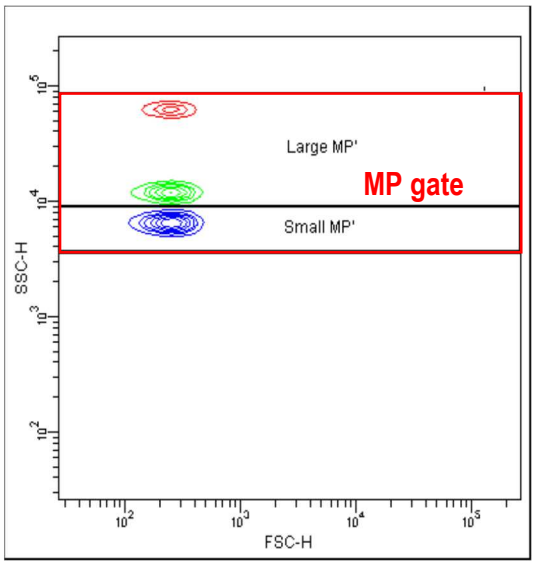
Alternatively, the FSC-H range may be tighter in case of using a dually (FSC- and SSC-) optimized cytometer. Then a clear gap in FSC between 0.5 µm beads and all others can be observed.

6.2.4 Setting SSC threshold (Fig.4)
- Run Megamix-Plus SSC beads once again.
- Switch threshold to SSC.

Note: Most SSC-optimized cytometers allow the use of several combined threshold parameters. This strategy is difficult to standardize and is not recommended here. Select SSC threshold alone.
In order to set the SSC-H threshold, enter the same a.u. value as previously calculated with formula 1. The entire MP analysis will be restricted to events with SSC values over the threshold, thus excluding all 0.16 µm beads, as illustrated in Fig. 4

Note that the example of Fig.4 does not show significant background in addition to the selected (0.2 to 0.5 µm) beads, due to low optical background of this individual instrument. This is not the general rule, but the calculated lower SSC limit as proposed in § 6.2.2 generally keeps background below an acceptable level, as defined in § 6.3.

Fig.4: Megamix-Plus SSC beads after threshold on SSC (contour-plot)



6.3 Routine analysis of Megamix-Plus SSC

Acquisition protocol being created (cf paragraph 6.2) proceed to the acquisition of Megamix-Plus SSC beads-containing tube.

- Using FL1/FITC threshold alone, check that the beads are located within the pre-existing regions (P1 to P4, cf. Fig.1). If not, adjust FL1 and/or SSC PMT voltages to achieve it.

- Re-actualize the values of MP gate boundaries using formula 1 as described in § 6.2.2 (or simply check that Md values did not change). Option: store electronic file.

- Using SSC threshold set as described, check background noise using ultrapure water (0.1 µm filtered) as the sample: if events/sec exceed 50% of the electronic capacity of the instrument (e.g. > 2,500 events/sec for an instrument capable of treating a maximal rate of ~ 5,000 events/sec.), the instrument is not in appropriate conditions for high-sensitivity MP analysis.

You may either:
a) rule-out any fluidic origin (check the cleanliness of the sheath fluid and tank(s), rinse the sheath line thoroughly, check the absence of micro-bubbles (always use sheath fluid at ambient temperature, not cold fluid) and try again.
b) restrict to "standard sensitivity" MP analysis using a higher SSC threshold value corresponding to the limit between "Large MP" and "Small MP". This limit, described as an option in § 6.2.2, is located in SSC between both 0.20 and 0.24 µm beads and corresponds to a FSC-based cut-off of 0.5 µm-eq.
c) or contact your technical service for optical cleaning in the SSC & fluorescence axis.
Note: high background in the SSC channel may come from problems with the optical coupling gel and/or sub-optimal optical alignment.

After checking these 3 conditions, cytometer settings are optimal and allow a standardized acquisition of MP between ~ 0.17 µm-eq. and 0.5 µm-eq. in SSC (similar range to 0.3 - 1 µm-eq. in FSC).

6.4 Example of platelet MP staining: PMP (Figs 5)

Here is an example of PMP analysis made on a platelet-free plasma sample following AnnexinV-FITC/CD41-PE staining and appropriate fluorescence compensation settings. Figs 5 illustrate the presence of 2 subsets of PMP, so-called "large PMP" and "small PMP". Fig 5a shows the dual fluorescence dot-plot gated on the "MP gate" and colored according to the SSC-based gates noted "Small MP" (cyan) and "Large MP" (pink) in Fig.3.

Apart from a huge number of dual-negative events (mainly background, in Q3 of Fig 5a) and a few AnnV+/CD41- MP (in Q4), the so-called "large PMP" (gate P2) show the highest levels of both Annexin V and CD41 staining and are mostly located in the upper part of the dual scatter plot (red dots in Fig.5b). A so-called "small PMP" subset (gate P3 in Fig.5a → blue dots in Fig.5b) displays the lowest levels of all size-related and fluorescence parameters. The SSC level of the gap between both subsets, as illustrated by the horizontal intermediate line in Figs 3, 4 and 5b, is generally close to 0.22 µm bead-eq. (corresponding to 0.5 µm bead-eq in FSC). Fig. 5c shows color dot-plot obtained after gating on the "MP gate" and coloring according to levels of staining (gates P2 and P3). In Fig. 5d, only PMP subsets gated on P2 or P3 (and colored accordingly) are displayed in a dual scatter contour-plot, illustrating the discrimination of small and large PMP.

Fig. 5a: Color dot-plot of all events gated on the "MP gate".
Cyan: "Small MP" Pink: "Large MP"

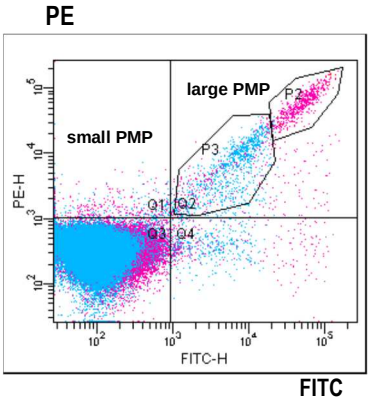


Fig. 5b: Color dot-plot of all events gated on the "MP gate"
Red: P2 Blue: P3
Grey: background & non PMP events

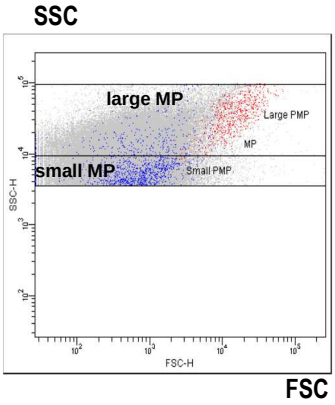


Fig. 5c: Color dot-plot of all events gated on the "MP gate".
Red: P2 Blue: P3
Grey: background & non PMP events

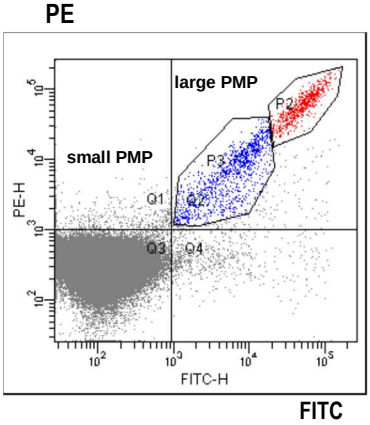
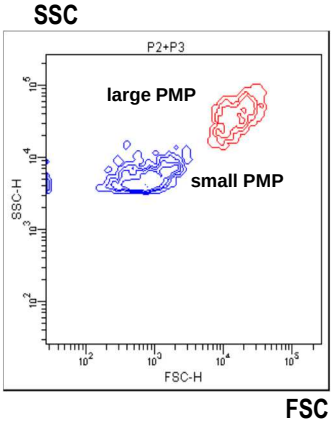


Fig. 5d: Colored contour-plot of PMP subsets size distribution.
Red: P2 Blue: P3



Note: the instrument used in this PMP analysis also had a favorable FSC resolution, in addition to appropriate resolution in SSC. Thus, the discrimination of PMP subsets in FSC may not be always as good as illustrated here, depending on FSC resolution of individual instrument. On the contrary, similar discrimination in SSC scale may be expected.

7 Monitoring of SSC resolution

SSC parameter resolution can be described by a numerical parameter called Separation Index or S.I. (adapted from ref.3). This provides opportunity for instrument monitoring (e.g. Levy-Jennings plots) or inter-instrument comparisons in terms of SSC resolution.

$S.I._{200\text{-}160} = [Md_{200} - Md_{160}] / [SD_{160} + SD_{200}]$ where:
Md_{200} = Median SSC of the 200 nm (resp. 160 nm) beads
SD_{200} = Standard Deviation SSC of the 200 nm (resp. 160 nm) beads.
In the present example, $S.I._{200\text{-}160}$ = (6,604-2,178)/(570+399) ~ 4.6
$S.I._{200\text{-}160}$ is currently in the range of 3 to 8 using BD FACS and LSRs.
A value below 3 is sign of an unappropriate SSC resolution for high-sensitivity FCM analysis of MP. This may suggest:
a) keeping to standard FCM MP analysis or
b) requesting a microparticle-dedicated technical service from the manufacturer, with optics cleaning and realignment.

8 Troubleshooting

If you are using polystyrene tubes (e.g. Falcon 4 mL FACS tubes) and the bead suspension has spent more than 1 hour waiting in this tube, the relative amounts of each bead subset may be different from those illustrated here (risk for differential disappearance of beads sticking to the tube wall). Check that the amounts of all beads remain appropriate to properly see all peaks shown in Figs 1 and 2 and thus apply the remaining procedure. In case of doubt, take a new sample of Megamix-Plus SSC beads.

REFERENCES

1) Robert S et al, Artherioscler Thromb Vasc Biol, 2012; 32: 1054-58.
2) Poncelet P et al, Transf Apher Sci 2015,53(2):110-26.
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